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EDITORIAL NOTES

Note from the Editor-in-Chief

Since the Journal's inception, it has been printed in a 6×9-in. format. In 2002, the cover appearance was modified, but this issue (January, 2008) is the first substantive change. The new 8.5×11-in. format with two-column text will allow us to better accommodate large figures and tables. Additionally, each cover will now have a color photograph that relates to an article contained in that issue. We invite authors to submit color photographs for consideration *after* an article has been accepted. These should be sent directly to the Journal e-mail jce@chuma1.cas.usf.edu.

The process of electronic submission of articles through our Editorial Manager system https://www.editorialmanager.com/joce/default.asp, and the practice of "Online First" publication, followed by electronic and hard copy issues will continue without change.

We believe that all of these are positive changes that will be welcomed by authors and readers.

John T. Romeo, Editor-in-Chief

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The Defensive Role of Volatile Emission and Extrafloral Nectar Secretion for Lima Bean in Nature

Christian Kost · Martin Heil

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Abstract Lima bean (*Phaseolus lunatus*) features two indirect anti-herbivore defenses—emission of volatile organic compounds (VOCs) and secretion of extrafloral nectar (EFN)—which are both inducible upon herbivore damage. In a previous field study, Lima bean benefited from the simultaneous induction of the two defenses, yet it remained unclear whether both had contributed to plant protection. Our experimental approach aimed at studying the defensive role of both indirect defenses simultaneously. Tendrils were sprayed with jasmonic acid (JA) to induce both defenses, and performance was compared to that of others that were treated with a synthetic blend of either EFN or VOCs. Confirming earlier results, JA treatment and application of the VOC mixture induced EFN secretion in treated tendrils in quantitatively similar amounts. The composition of the applied synthetic blend of EFN was adjusted to match the concentration of EFN secreted from JA- and VOC-treated tendrils. Repeated application of either enhanced the performance of several fitness-relevant plant parameters such as growth rate and flower production. Tendrils treated with JA showed a similar trend, yet some fitness-related parameters responded less to this treatment. This suggests a minor importance of any putative JA-dependent direct defense traits or higher costs of JA-elicited responses as compared to VOCS and EFN, as otherwise JA-treated tendrils should have outperformed VOC- and EFN-treated tendrils. Moreover, the beneficial effect of applying synthetic EFN alone equaled or exceeded that of VOCs and JA. Ants were by far the dominant group among the arthropods that was attracted to JA-, VOC-, or EFN-treated tendrils. The results suggest that EFN plays a more important role as an indirect defense of lima bean than VOCs or any other JA-responsive trait.

Keywords Ants · Extrafloral nectar · Indirect defense · *Phaseolus lunatus* · Plant–plant communication · Volatile organic compounds

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C. Kost · M. Heil
Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology, Beutenberg Campus, Hans-Knöll-Str. 8, 07745 Jena, Germany

Present address:
C. Kost (✉)
Evolutionary Genetics and Microbial Ecology Laboratory, New Zealand Institute for Advanced Study, Massey University, Private Bag 102 904, North Shore Mail Centre, Auckland, New Zealand
e-mail: christiankost@web.de

Present address:
M. Heil
Departamento de Ingeniería Genética, CINVESTAV–Irapuato, Km. 9.6 Libramiento Norte, Irapuato, Guanajuato CP 36821, Mexico

Introduction

Plants respond to herbivore attack with an array of changes in plant chemistry, morphology, and physiology, which frequently result in the increased resistance of plants to further attack (Karban and Baldwin 1997). Induced resistance may be caused by direct effects on the herbivore through plant-derived toxic metabolites or anti-digestive and anti-nutritive compounds. Furthermore, plants may utilize indirect defenses that facilitate top-down control of

herbivore populations by the herbivore's predators and parasitoids (Price et al. 1980).

One way a plant may attract beneficial arthropods is by providing suitable food sources such as extrafloral nectar (EFN; Koptur 1992). EFN is an aqueous solution, with sugars and amino acids being the most abundant solutes (Ruffner and Clark 1986; Galetto and Bernardello 1992; Heil et al. 2000), which is secreted from specialized organs, the extrafloral nectaries. In addition, plants may increase the emission rate of volatile organic compounds (VOCs) in response to herbivore attack. VOCs can serve as a cue that guides foraging parasitoids and predators to the feeding herbivore (Turlings et al. 1990; Paré and Tumlinson 1997). The composition of the herbivore-induced volatile blend depends not only on the plant species or cultivar but also varies with the species and even the larval stage of the herbivore (for review, see Arimura et al. 2005), thus providing highly reliable signals to the members of the third trophic level.

In many cases, plants do not rely on a single defense strategy but employ a complex array of different defensive mechanisms. For example, several plant species feature both VOC emission and EFN secretion (Arimura et al. 2005). The presence of two indirect defenses within one individual plant gives rise to the questions whether both defenses contribute to plant fitness and how they interact in attracting beneficial arthropods (Price et al. 1980). Studies on the benefit of indirect defense traits in nature are generally rare (but see Thaler 1999 and Kessler and Baldwin 2001). No study to date has tried to study the defensive roles of two indirect defenses within one plant species simultaneously.

The lima bean (*Phaseolus lunatus*) is a common model system in studies on induced indirect defenses. Heil (2004b) showed that plants growing in the wild, which had been induced by exogenous application of the phytohormone jasmonic acid (JA), responded by increasing both their VOC emission and EFN secretion. The application of JA repeatedly (i.e., every 3 days) resulted in benefits for the treated plants as reflected by a decreased herbivory rate and an increased seed set. However, JA also affects fitness-relevant processes such as fruit development and ripening (Creelman and Mullet 1997). Thus, the multiple effects constrain the interpretation of the observed morphological changes as being exclusively attributable to induced defenses. Furthermore, Kost and Heil (2005) demonstrated that an artificial increase of the amount of available EFN benefits lima bean in nature by attracting predacious and parasitoid arthropods. These two pilot studies represented a starting point for unraveling whether both induced defense traits contribute to plant defense or whether EFN secretion solely is responsible for lima bean protection.

We chose an experimental approach similar to the one used in Heil (2004b): Groups of five tendrils were used as experimental units (Fig. 1; Table 1). Within each unit, two tendrils were either treated with JA or left untreated. The inclusion of two additional bean tendrils with artificially increased amounts of either VOCs or EFN enabled us to study the protective effect of the two defenses. A fifth group served as a treatment control.

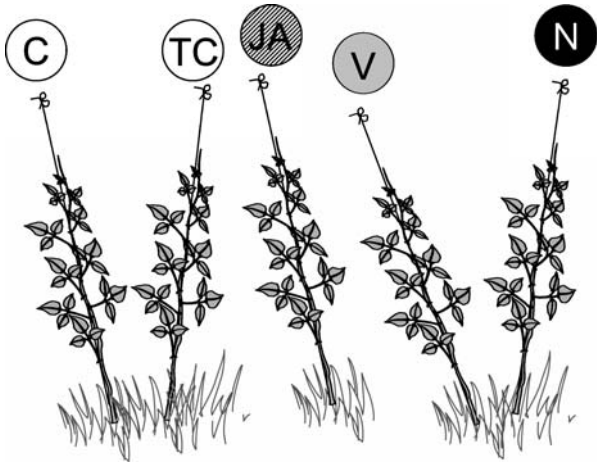


Fig. 1 Experimental design. Five groups of lima bean tendrils (*P. lunatus*) served as experimental unit with *C* no treatment, *TC* application of lanolin paste, *JA* spraying with jasmonic acid, *V* application of a synthetic volatile blend dissolved in lanolin paste, and *N* application of a synthetic mixture of EFN. See Table 1 for details

We repeatedly applied these five treatments to address the following questions: (1) What is the relative contribution of the two indirect defenses—volatile emission and extrafloral nectar secretion —to the overall herbivore defense of the lima bean? (2) Is there an additional influence of a putative JA-dependent direct defense? And (3) what kind of arthropods (including putative defenders) are attracted to the treated tendrils?

Methods and Materials

Study Site and Species This study was conducted in the coastal area near Puerto Escondido in the state of Oaxaca, Mexico. The climate in the study area is characterized by one main rainy season from June to October, which follows a bimodal distribution peaking in July and September. Annual rainfall averages between 1 and 1.4 m, and the mean annual temperature is 28°C (Strässne 1999). The two sites were used in previous studies (Heil 2004b; Kost and Heil 2005) and located 15 km northwest of Puerto Escondido and about 3 km apart (15°55.596N/097° 09.118W and 15°55.357N/97°08.336W). In this study, lima bean grows naturally along dirt roads that lead to extensively used pastures or plantations. All experiments were performed on this native population of lima bean plants in 2003 and 2004, during the transition from wet to dry season (October to December).

Table 1 Five chemical treatments and their effect on treated tendrils

Group Name (Abbreviation)	Treatment	Defensive Traits (*i*=Induced, *e*=Experimentally Increased)	Effect
Control (C)	Untreated	None	Untreated control
Treatment control (TC)	Lanolin paste	None	Lanolin paste only
JA-treatment (JA)	JA	VOCs (i) EFN (i) putative direct resistance (i)	Induction of VOCs, EFN, and putative JA-dependent direct defenses (Heil 2004b)
Volatile treatment (V)	Volatile mixture dissolved in lanolin paste	VOCs (e) EFN (i)	Induction of EFN, attraction of arthropods to VOCs and EFN (Kost and Heil 2006)
Nectar treatment (N)	Synthetic mixture of EFN	EFN (e)	Attraction of arthropods to EFN (Kost and Heil 2005)

JA Jasmonic acid, *EFN* extrafloral nectar, *VOC* volatile organic compound

Design of the Long-Term Experiment To reduce environmental and genotypic variability, 23 groups of five lima bean tendrils were selected as experimental units. Within a unit, the maximum distance between the two outer tendrils was less than 3 m, and tendrils forming one group usually were part of the same plant individual, although this could not always be ensured because of the tangled growth of the lima bean. Eleven groups were located at site 1 and 12 at site 2. All selected tendrils were trained along supporting ropes, and the tendrils of each group assigned randomly to one of five treatments (for details, see the next section): Tendrils were either left untreated (control group) or treated every 3–4 days with lanolin paste only (treatment control group), sprayed with an aqueous solution of JA (JA group), or supplied with a synthetic blend of VOCs (volatile group) or EFN (EFN group; Fig. 1; Table 1). Application of pure lanolin served as a procedural control to test for any effect caused by lanolin alone. See Table 1 for a summary of the expected treatment effects. Starting 27th October 2003, the experiment lasted 25 days and comprised six applications. During this time, the initial number of tendril groups was reduced to a final sample size of 17 because of cattle and human impact.

Treatment of Tendril Groups Tendrils of the JA group were sprayed with an aqueous solution of 1 mmol JA until runoff (i.e., approximately 10 µl per cm^2 leaf area). Levels of JA were adjusted to those of previous experiments in which similar concentrations were applied without phytotoxic effects becoming apparent (Heil 2004b). The synthetic volatile blend and the synthetic mixture of EFN were adjusted to mimic their natural models within 3 days post-induction according to previous experiments (Kost and Heil 2005, 2006). However, the amount of VOCs and EFN cumulatively applied ranged largely below the physiological limit of a plant for producing the two indirect defenses within this period (Kost and Heil 2005, 2006).

The synthetic volatile blend consisted of 0.12 µg (*R*)-(−)-linalool, 0.13 µg β-caryophyllene, 0.19 µg methyl salicylate, 0.26 µg (*Z*)-jasmone (all purchased from Sigma-Aldrich), 0.02 µg (3*Z*)-hexen-1-yl acetate (Avocado Research Chemicals Ltd., Leysham, Lancaster, UK), 0.85 µg (*E*,*Z*)-β-ocimene (mixture of *E*/*Z*-isomers approximately 70:30; kindly provided by Roger Snowden, Firmenich, Geneva, Switzerland), 0.63 µg (3*E*)-4,8-dimethyl-nona-1,3,7-triene (DMNT), and 0.9 µg (3*E*,7*E*)-4,8,12-trimethyltrideca-1,3,7,11-tetraene (TMTT; synthesized by standard methods; Pattenden and Weedon 1968) per microliter lanolin. The purity of all compounds was >98%. Pure lanolin and lanolin paste that contained volatiles were spotted on green plastic strips attached to tendrils to prevent any diffusion of compounds into the treated plant. A total of 120 µl of paste were applied per five leaves of either the volatile- or the treatment-control group.

The synthetic EFN consisted of an aqueous solution of 4.01 g l^{-1} sucrose and 24.24 g l^{-1} of each fructose and glucose. Of this blend, 40 µl were applied with an Eppendorf pipette directly to the extrafloral nectaries of every trifoliate leaf of the EFN group.

Comparison of Treatments To ensure comparability between experimental groups of the long-term experiment, we quantified VOC and EFN levels of differently treated tendrils in preceding experiments.

The ability of our VOC mixture to mimic the volatile emission of herbivore-damaged and JA-induced tendrils was examined by quantifying the VOCs present in the headspace of tendrils that had received one of five treatments. Tendrils were either left untreated or treated with lanolin paste, infested for 48 h with a representative mixture of natural herbivores of lima bean (six Ensifera or Caelifera and ten beetles of various species, i.e., *Cerotoma ruficornis*, *Gynandrobrotica guerreroensis*, *Epilachna var-*

ivestis, and a curculionid species; for details, see Kost and Heil 2006), treated with a blend of synthetic VOCs, or sprayed with a 1-mmol aqueous solution of JA. Each treatment was replicated six to eight times. Groups of five tendrils that had received the different treatments originated from plants that were spaced apart at a maximum distance of approximately 3 m, had five leaves, matched each other in terms of leaf age and visual appearance, and were collected at the same time for simultaneous volatile collection. Treated tendrils were detached, immediately supplied with a water reservoir, and bagged in a PET foil ('Bratenschlauch', Toppits, Minden, Germany) that does not emit detectable amounts of volatiles by itself. Emitted VOCs were collected continuously on charcoal traps (1.5 mg charcoal, CLSA Filters, Le Ruisseau de Montbrun, France) by using air circulation, as described by Donath and Boland (1995). After 24 h, volatiles were eluted from the carbon trap with dichloromethane (40 µl) that contained 1-bromodecane (200 ng μl^{-1}) as an internal standard. Samples were transferred to glass capillaries, sealed by melting the open end, and stored at <5°C for transport. Samples were analyzed on a gas chromatography–trace mass spectrometer (Thermo Finnigan, http://www.thermofinnigan.com) according to Koch et al. (1999). Individual compounds (peak areas) were quantified with respect to the peak area of the internal standard.

To compare quantitatively the amount of EFN secreted from tendrils after exposure to airborne VOCs or induction with JA, 11 groups of three tendrils spread across the two sites were selected; these groups were located <1 m apart. Each tendril was basifixed and had five leaves. The first tendril within each group was treated with lanolin paste, the second with lanolin paste that contained volatiles, and the third was sprayed with JA. All treatments were similar to those described above. The three tendrils were then placed in gauze bags (mesh size, 0.5 mm), and a ring of sticky resin (Tangletrap®, Tanglefoot Company, Grand Rapids, MI, USA) was applied at the base as protection against flying and crawling nectar consumers. After 24 h, the amount of newly produced EFN was measured as the amount of secreted soluble solids (i.e., sugars, amino acids; see Heil et al. 2000) by quantifying the nectar volume with micro-capillaries and the nectar concentration with a portable, temperature-compensated refractometer (Heil et al. 2000, 2001). Both the amount of VOCs emitted and EFN secreted were referenced to the dry weight of the measured tendrils.

Fitness-Relevant Plant Parameters To assess the effect of treatments on the fitness of the study tendrils, the following fitness-relevant plant parameters were considered: number of leaves, inflorescences, living and dead shoot tips, and herbivory rate. Herbivory rate was estimated as percent leaf loss, as described in Kost and Heil (2005), and the remaining parameters were quantified by counting. All five parameters were assessed at the beginning of the experiment and after 25 days. Differences between these two values were calculated to determine the development of the respective parameter in the course of the study period.

Insect Counts and Sticky Traps The insect community that visited the treated bean tendrils was assessed by counting and with sticky traps. Two series of insect countings were performed, in which 20 tendril groups of the long-term experiment located at both sites were visited repeatedly. The first series of countings started on day 7 and the second on day 18 after the onset of the experiment. Ten groups of tendrils were selected at each study site, and the number of ants, wasps, or flies present on the plants was recorded, as these insects represented the most abundant groups. The first census was performed before tendril treatment at 8:00 A.M. Thereafter, all tendrils were treated, and insects on all experimental tendrils were counted repeatedly every 2 or 3 h until midnight. Two additional censuses were performed at 9:00 and 10:00 A.M. on the following 2 days, resulting in a total of 14 monitorings. The number of all insects counted per tendril was summed up to test for an effect of the treatment on the total number of insects observed.

To assess the functional groups of insects attracted to the experimental tendrils, two sticky traps were attached with plastic strings to each tendril of 14 groups that were equally distributed between the study sites. The sticky traps consisted of 100 cm^2 pieces of green plastic foil that had been coated with a thin layer of a trapping adhesive (Tangletrap®). After 24 h of exposure, traps were collected and the insects transferred to 75% ethanol. Insects were identified to order or family level with keys and information provided by Arnett (2000), Schaefer et al. (1994), and Noyes (2003). On the basis of the natural history information provided by Kelsey (1969, 1981), Honomichl et al. (1996), Matile (1997), and Daly et al. (1998), the collected arthropods were assigned to the following guilds according to nutritional or functional aspects: Predator/entomophaga (R), parasitoid (P), utilization of plant-derived resources including floral or extrafloral nectar, pollen and honeydew (S), frugivore (F), herbivore and flower feeder (H), detrivore including phytosaprophage and zoosaprophage (D), blood-sucking and ectoparasitic (B), and fungivore (M).

Statistical Analysis Amounts of volatiles emitted from differentially treated tendrils were compared with a Kruskal–Wallis rank sum test and a subsequent nonparametrical multiple test procedure of the Behrens–Fisher type (Munzel

and Hothorn 2001) by using the open source software R 2.3.1 (http://www.r-project.org).

Our randomized complete block design allowed us to analyze the data of the EFN-induction experiment, the fitness-relevant plant parameters, and the cumulative insect numbers with a mixed-effect model (univariate GLM procedure) with 'treatment' as a fixed and 'tendril group' as a random factor. The following variables were transformed to meet the assumption of homogeneity of variances (transformation in brackets): number of living shot tips and number of wasps (square root), number of inflorescences and dead shoot tips (log), number of leaves, cumulative number of ants and flies (ln). Post hoc comparisons (least significant difference, LSD) were performed to test for statistically significant differences among treatments. These statistical analyses were done using Statistical Package for the Social Sciences 13.0 (SPSS Inc., Chicago, USA).

Results

Comparison of Treatments The headspace of lima bean tendrils, which had been treated with either a blend of synthetic VOCs or with JA, largely resembled the volatile blend typically emitted from herbivore-damaged bean tendrils in terms of quantity and quality (Table 2). Only (3*Z*)-hexen-1-yl acetate was emitted in significantly lower amounts from the synthetic VOC mixture than from herbivore-induced tendrils. Also, JA- and herbivore-treated tendrils showed a marked quantitative similarity in the composition of their emitted volatile bouquets. However, JA-treated tendrils emitted significantly lower amounts of (*R*)-(−)-linalool and higher amounts of DMNT compared to herbivore-damaged tendrils. In general, volatile blends emitted from herbivore-, volatile-, and JA-treated plants were characterized by high quantitative variability (Table 2).

A similar trend became obvious when EFN secretion rates were compared among treatments. Both the application of the synthetic volatile mixture and the JA treatment increased the EFN secretion rate within 24 h compared to controls treated with lanolin only (univariate analysis of variance, ANOVA, $P < 0.01$, $N=11$). The EFN secretion rate in both treatments was twice that of the treatment control (Fig. 2). However, no significant difference was detected between the volatile- and the JA-treated group (LSD post hoc test, $P>0.05$).

Effect of the Treatments on Fitness-Relevant Plant Parameters The three treatments, JA, volatiles, and EFN, significantly affected vegetative and reproductive plant traits after 25 days as compared to the controls. Tendril groups treated with volatiles or EFN showed a significant increase in the number of newly produced leaves, shoot tips, and inflorescences, bore fewer dead shoot tips, and had suffered less herbivore damage than the controls (Fig. 3). JA treatment also significantly decreased the number of dead shoot tips and the herbivory rate, whereas no difference could be detected for the number of living shoot tips, leaves, and inflorescences. According to a LSD post hoc test, the tendrils of the JA group ranked between the volatile and EFN group on one hand and the two controls on the other (Fig. 3).

Table 2 Quantitative comparison of five-leaved lima bean tendrils, which were left untreated (C); treated with lanolin paste (Tc); exposed to a mixture of herbivores, which are characteristic for the lima bean for 48 h (H); treated with the artificial volatile blend dissolved in lanolin paste (V); or sprayed with JA

Compound	Emission ($A_{VOC}\ A_{IS}^{-1}\ g^{-1}\ 24\ h^{-1}$)					
	C (N=8)	TC (N=7)	H (N=6)	VOCs (N=7)	JA (N=7)	KW test
(3*Z*)-Hexen-1-yl acetate	0.03 ± 0.03^{a}	0.01 ± 0.01^{a}	0.15 ± 0.21^{a}	0.03 ± 0.06^{b}	0.34 ± 0.35^{a}	=0.07
(*Z*)-β-Ocimene	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.03 ± 0.05^{ac}	0.13 ± 0.10^{bc}	0.07 ± 0.12^{ac}	<0.001
(*E*)-β-Ocimene	0.01 ± 0.01^{a}	0.02 ± 0.04^{a}	0.42 ± 0.64^{bc}	0.23 ± 0.19^{bc}	0.86 ± 1.60^{ac}	<0.01
(R)-(-)-Linalool	0.05 ± 0.07^{a}	0.06 ± 0.06^{a}	0.64 ± 0.55^{b}	0.39 ± 0.41^{b}	0.06 ± 0.09^{a}	<0.01
DMNT	0.04 ± 0.05^{a}	0.07 ± 0.07^{a}	0.76 ± 0.91^{b}	1.18 ± 0.86^{bc}	0.96 ± 1.56^{ac}	<0.001
$C_{10}H_{14}$	0.03 ± 0.06^{a}	0.18 ± 0.22^{a}	1.43 ± 0.81^{bc}	1.16 ± 0.97^{bc}	0.32 ± 0.42^{ac}	<0.001
Methyl salicylate	0.02 ± 0.02^{a}	0.13 ± 0.10^{ac}	0.34 ± 0.31^{ad}	0.78 ± 0.58^{bcd}	0.07 ± 0.12^{a}	<0.01
$C_{10}H_{16}O$	0.08 ± 0.11^{a}	0.15 ± 0.28^{a}	0.45 ± 1.01^{a}	0.45 ± 0.67^{a}	0.68 ± 1.03^{a}	=0.64
(*Z*)-Jasmone	0.01 ± 0.02^{ac}	0.00 ± 0.00^{a}	0.21 ± 0.22^{bd}	1.21 ± 1.21^{be}	0.35 ± 0.44^{cde}	<0.01
β-Caryophyllene	0.01 ± 0.01^{a}	0.03 ± 0.05^{a}	1.23 ± 2.10^{b}	0.75 ± 0.66^{b}	0.26 ± 0.28^{b}	<0.001
TMTT	0.02 ± 0.02^{a}	0.09 ± 0.08^{ac}	0.43 ± 0.34^{bd}	0.85 ± 0.71^{bce}	0.69 ± 1.21^{ade}	<0.01

The dominant emitted volatiles are given as mean peak area (±95% confidence interval) relative to the peak area of an internal standard per 24 h and per gram dry weight. The last column shows *P* values of a Kruskal–Wallis (KW) test. Significant treatment effects are indicated by different superscript letters (nonparametrical multiple test procedure of the Behrens–Fisher type, $P<0.05$).

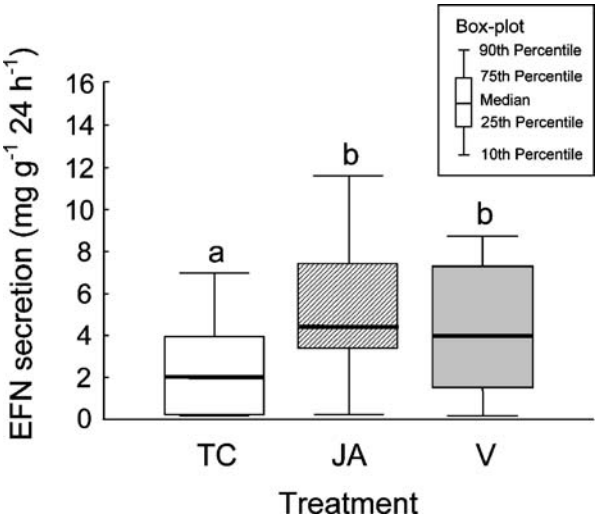


Fig. 2 Effect of different treatments on the secretion rate of extrafloral nectar (*EFN*) given in milligrams soluble solids per gram leaf dry mass per 24 h. Comparisons between tendrils treated with lanolin paste (*TC*), the synthetic volatile blend dissolved in lanolin (*V*), and jasmonic acid (*JA*) are shown. *Different letters* indicate significant differences among treatments (univariate ANOVA, $P<0.05$ according to LSD post hoc test). Sample size was eleven groups of tendrils

Effect of the Treatments on Insect Abundance Ants, wasps, and flies were the most abundant groups observed visiting the study tendrils, with ants being by far the dominant group. Their numeric abundance on the experimental tendrils exceeded that of flies 5- to 15-fold and that of wasps 20- to 30-fold (Fig. 4).

The five treatments significantly affected the insect visitation rate of the experimental tendrils. The mean cumulative number of ants was increased significantly on tendrils treated with JA, volatiles, and EFN relative to the two control tendrils (Fig. 4, LSD post hoc test after univariate ANOVA, $P < 0.05$). Volatile treatment doubled the median cumulative number of ants on the experimental tendrils, whereas the JA and nectar treatment led to a threefold increase over the number on the untreated state. Correspondingly, the median wasp number ranged between 1.5 (JA group), 2 (volatile group), and 2.5 (EFN group), whereas these insects were significantly less frequently encountered on control tendrils (Fig. 4; LSD post hoc test after univariate ANOVA, $P < 0.05$). Flies responded less to the five treatments. Despite the significantly increased visitation rate of flies to the JA-treated tendrils as compared to the controls (Fig. 4, LSD post hoc test after univariate ANOVA, $P < 0.05$), no such effect was observed for VOC- and EFN-treated tendrils (Fig. 4). The two latter groups were statistically indistinguishable from the JA-treated tendrils and both control groups (Fig. 4, LSD post hoc test after univariate ANOVA, $P>0.05$).

Community Composition of Arthropods Visiting Lima Bean In total, 899 arthropods were caught on sticky traps, and >94% were identified to the order or family level. Among them, Diptera (55%) and Hymenoptera (26%) were the most abundant (Table 1; Fig. 4). Other groups trapped included Coleoptera (6%), Araneida (5%), and Thysanoptera (3%).

Based on information derived from the literature, the trapped arthropods were assigned to feeding guilds, whereas multiple affiliations per taxon were allowed. This analysis indicated that 73% of all trapped arthropods were characterized by parasitoid or predacious life habits and may, thus, be classified as potentially beneficial to the lima bean (Fig. 5). On the other hand, only 16% of the trapped arthropods were assigned to herbivorous or flower-feeding groups, which potentially could have had detrimental effects on the plant. Other plant-derived food sources, such as EFN, pollen, or honeydew, are known to be used by 67% of all trapped arthropod groups, and 19% of all trapped taxa additionally rely on other food sources such as fungi, fruits, or detritus. The two latter groups may be classified as 'tourists' and are likely to have a neutral effect on the lima bean (Fig. 5; Table S1).

A closer look at the trapped arthropod taxa revealed that Dolichopodidae (34% of all trapped Diptera), Phoridae (24%), and Chloropidae (7%) were the most abundantly trapped Dipterans (Table S1). All three groups share parasitoid and predacious life habits and are occasionally known to feed on EFN (Table S1). Members of the Chalcidoidea contributed preponderantly to the captured Hymenoptera (64%). The individuals trapped by this superfamily of parasitoid wasps belonged to 16 different families, with Eulopidae (13% of all trapped Hymenoptera), Encyrtidae (12%), and Pteromalidae (8%) being most frequently trapped. Among the hymenopterans, Formicidae (12%) and Braconidae (6%) were the most often captured non-chalcid families.

Discussion

Comparison of Treatments The aim of the present study was to study simultaneously the protective effect of the two indirect defenses, EFN secretion and VOC emission, on the lima bean under field conditions. The performance of plants that were induced by spraying with JA and, thus, had increased amounts of VOCs and EFN, was monitored and compared to plants of which the amount of either VOCs or EFN was increased experimentally.

An important prerequisite for any conclusion that can be drawn from such an experimental design is knowledge of the performance of the two indirect defenses under

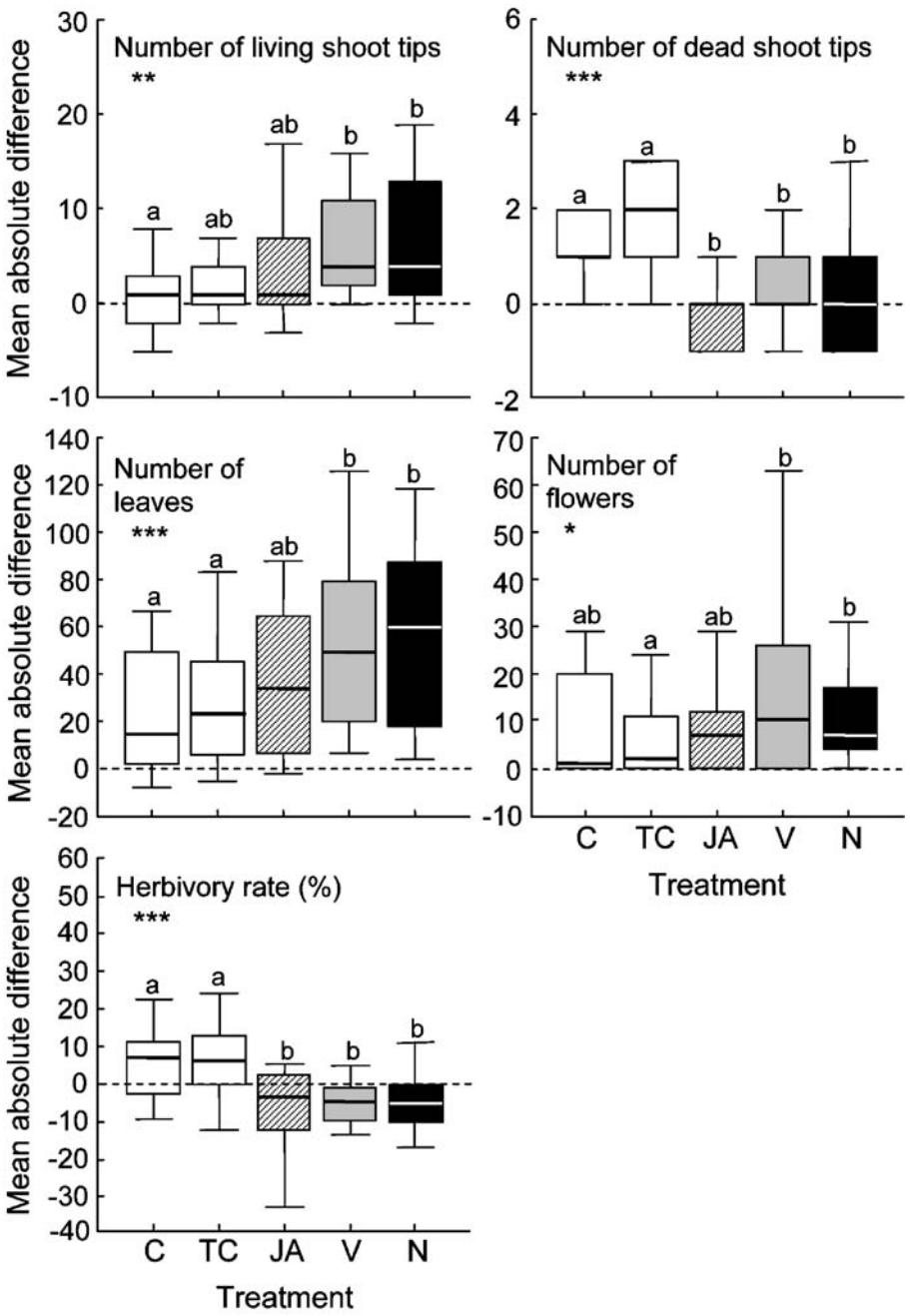
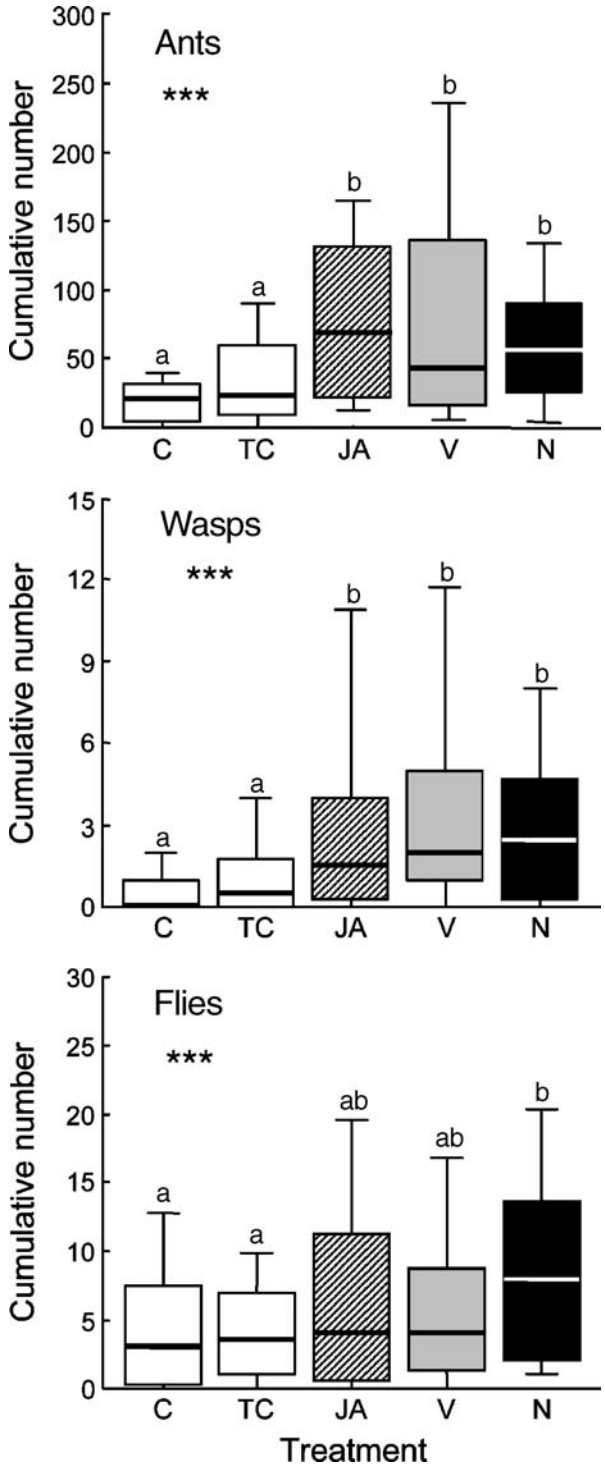


Fig. 3 Effect of five treatments on fitness-relevant plant parameters. Each tendril within groups of five lima bean tendrils received one of five treatments: no treatment (*C*), treatment with lanolin paste (*TC*), spraying with jasmonic acid (*JA*), application of a synthetic volatile blend dissolved in lanolin paste (*V*), and application of a synthetic mixture of EFN (*N*). See Table 1 for details. Mean absolute differences between days 0 and 25 of the experiment are displayed. Values above zero (*dashed line*) indicate an increase and values below a decrease in comparison to the starting situation. *Asterisks* indicate significant treatment effects (univariate ANOVA; $*P<0.05$, $**P<0.01$, $***P<0.001$), and *different letters* indicate significant differences among treatments (LSD post hoc test, $P<0.05$). Sample size was 20 groups of tendrils

investigation. A quantitative and qualitative comparison of the VOC blends emitted from herbivore-damaged, volatile-treated, and JA-sprayed lima bean tendrils revealed—besides subtle differences—a pronounced similarity among these three groups with respect to the 13 dominant emitted compounds (Table 2). However, the volatile blends emitted from these three groups were characterized by a high degree of quantitative variation, an observation well known from literature (Kessler and Baldwin 2001; Fritsche-Hoballah et al. 2002; Röse and Tumlinson 2004). The question whether such differences hamper the ability of predators and parasitoids to locate the VOC-emitting plant has been studied intensively under laboratory conditions, where it has been shown that carnivorous arthropods can discriminate even minor differences in volatile blends offered (e.g., changes in the enantiomer ratios; Dicke et al. 1990). A species of curculionid beetles, for example, which also was observed feeding on lima bean in this study (C. Kost, personal observation), in laboratory experiments, used VOCs of slightly induced plants as a host-location cue, yet avoided plants with high induction levels (Heil 2004a). In the field, plants generally show a higher quantitative and qualitative variability of their emitted VOCs than under constant laboratory conditions (Gouinguene et al. 2001; Gouinguene



◀ **Fig. 4** Effect of the five treatments on insect numbers visiting the lima bean. Insects perching on lima bean tendrils that received one of five treatments were counted. Treatments were no treatment (*C*), treatment with lanolin paste (*TC*), spraying with jasmonic acid (*JA*), application of a synthetic volatile blend dissolved in lanolin paste (*V*), and application of a synthetic mixture of EFN (*N*). See Table 1 for details. Fourteen censuses were performed within 3 days at two sites (site 1 on day 7 and site 2 on day 18 after the beginning of the experiment). Insect numbers were pooled per counted tendril. *Asterisks* indicate significant treatment effects (univariate ANOVA; $*P<0.05$, $**P<0.01$, $***P<0.001$), and *different letters* indicate significant differences among treatments (LSD post hoc test, $P<0.05$). Sample size was 20 tendril groups

and Turlings 2002; Vallat et al. 2005). Foraging predators should respond to this situation by relaxing the specificity of their search patterns (Dicke et al. 2003). Indeed, field studies on the attractiveness of VOCs suggest that predatory and predacious insects are also attracted to jasmonate-induced plants (Thaler 1999; Kessler and Baldwin 2001), even if the emitted volatiles differ substantially from blends emitted by herbivore-damaged plants (Farag and Paré 2002). Moreover, field experiments indicate that the presence of single compounds can be sufficient to attract carnivorous insects (James 2003, 2005; James and Price 2004), which then act as indirect plant defenders (Kessler and Baldwin 2001). Consequently, under our experimental conditions, the VOCs of both JA- and volatile-treated tendrils likely have attracted plant defenders.

Our volatile treatment induced the secretion of EFN (Fig. 2), thus, confirming recent results obtained for the same plant species under laboratory (Choh et al. 2006) and field conditions (Heil and Kost 2006; Kost and Heil 2006). Hence, the tendrils of the volatile group experienced the combined defensive effect of both VOCs and EFN to an extent comparable to the tendrils of the JA group. These two defenses are not only connected by the shared signaling molecule JA (Heil 2004b) but are also airborne VOCs implicated in the induction of EFN secretion within one (Heil and Silva Bueno 2007) or between two conspecific plant individuals (Kost and Heil 2006). Consequently, this physiological linkage precludes an experimental separation of the two indirect defenses.

Treatment Effects on the Plant Tendrils of the JA, the volatile, and the EFN group generally benefited from the respective treatments, as these tendrils suffered less herbivory by leaf-chewing herbivores and bore fewer dead shoot tips than the two control groups (Fig. 3). The picture, however, changes when other fitness-relevant parameters are considered: The numbers of living shoot tips, leaves, and inflorescences differed significantly from the controls only in tendrils treated with VOCs and EFN (Fig. 3). A possible explanation for the weaker effect experienced by the JA-treated tendrils could be that induction with JA incurred allocation costs to the treated tendrils, which were greater than costs imposed by the VOC-induced EFN secretion and absent in EFN-treated tendrils (Heil 2002; Strauss et al. 2002). Beyond eliciting stress-related responses, such as insect and disease resistance, JA is known to be involved in various physiological or morphological changes not necessarily related to resistance

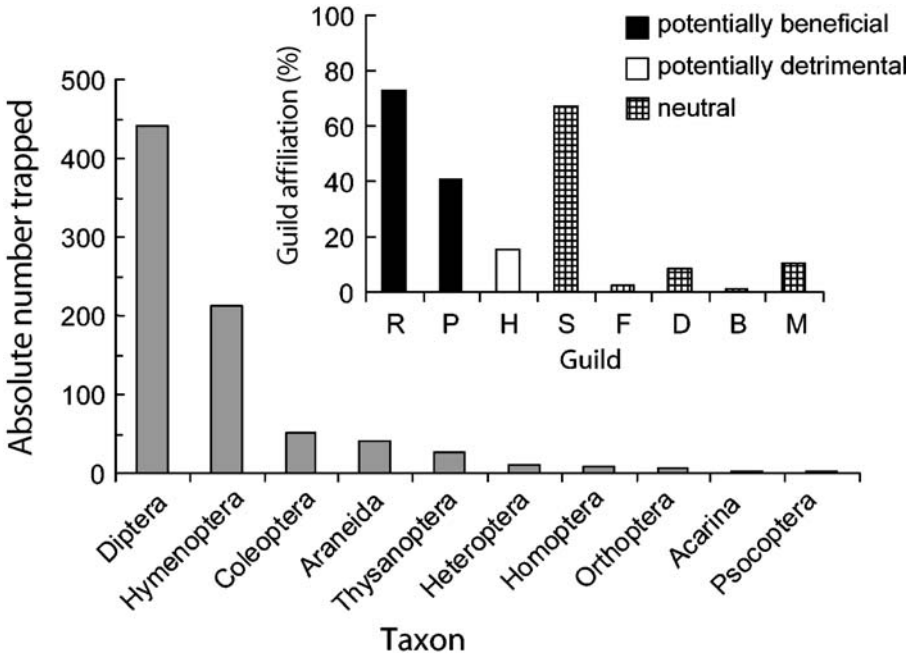


Fig. 5 Arthropods taxa trapped on sticky traps. *Insert* Affiliation of trapped taxa to different guilds: predator/entomophaga (R); parasitoid (P); utilization of plant-derived resources including floral or extrafloral nectar, pollen and honeydew (*S*); frugivore (*F*); herbivore and flower feeder (*H*); detrivore including phytosaprophage and zoosaprophage (*D*); blood-sucking and ectoparasitic (*B*); and fungivore (*M*). Arthropod groups were assigned to guilds according to nutritional or functional aspects whenever larval or adult stages feature the respective trait. Multiple affiliations per taxon were allowed. Sample size was two traps per 14 tendril groups

(Creelman and Mullet 1997). Given that these are costly in terms of metabolic resources, the induction of such processes may have affected the measured fitness parameters and could explain the weaker growth and reproductive status of JA-treated tendrils (Agrawal et al. 1999). JA may also directly affect processes such as fruit development and ripening (Creelman and Mullet 1997). An increased seed set of JA-treated plants, therefore, does not necessarily reflect an enhancement of the plant's defense status. Furthermore, external application of JA in elevated concentrations can, in principle, have detrimental effects on a plant and cause chlorosis, necrosis, or abscission of leaves (Husain et al. 1993; Oka et al. 1999; Pilotti et al. 2004). However, no such symptoms of phytotoxicity were observed. In contrast, the leaves of JA-treated tendrils looked even healthier than those of both control tendrils.

Additionally, JA application may have induced putative direct defenses (Halitschke and Baldwin 2004). However, no such alternative defense strategy has yet been described for lima bean. In its close relative, *P. vulgaris*, English-Loeb and Karban (1991) did not find evidence for induced direct resistance to spider mites. However, the protective effect of a direct defense, which in our long-term experiment could have been induced after JA application, cannot be excluded. In this case, the direct defense did not significantly contribute to plant protection because tendrils with increased amounts of EFN only (EFN group) or volatiles and EFN combined (volatile group) performed better than tendrils with volatiles, EFN, and the putative direct defense (JA group; Fig. 3).

The development of the fitness-relevant plant parameters measured in the JA-treated group and the two controls confirmed a preceding study (Heil 2004b). In both studies, JA treatment increased the number of newly produced leaves and decreased both the number of dead shoot tips, as well as the herbivory rate. The induction of these two indirect defenses was shown to benefit lima bean plants in two independent studies performed in two consecutive years. By applying synthetic VOCs and EFN externally, we could trace the observed effects back to these two kinds of defensive metabolites and corroborate the importance of EFN secretion and VOC emission for lima bean defense in nature.

Treatment Effects on the Insect Community Ants were by far the dominant insect group observed on experimental tendrils, and they were significantly attracted to tendrils that experienced the JA, volatile, and EFN treatment (Fig. 4). Although chemical cues are generally important for ants (Vander Meer et al. 1998; Keeling et al. 2004), little is known about whether plant-derived volatiles also influence ant behavior. Some reports are available on the role of yet unidentified plant chemicals that orient ants to their host plant (Fiala and Maschwitz 1990; Agrawal and Dubin-Thaler 1999; Djieto-Lordon and Dejean 1999a,b) or facilitate within-host plant patrolling behavior (Brouat et al. 2000). In lima bean, herbivory induces both VOC emission and EFN secretion (Heil 2004b). Ants could use herbivore-induced volatiles as long-distance cues to detect patches of increased availability of EFN, which simultaneously are characterized by the increased presence of herbivores (i.e., potential prey). However, preliminary

experiments with *Camponotus novogranadensis*, one of the three dominant ant species that visit lima bean at the two study sites (Kost and Heil 2005) did not support this hypothesis. Given the choice in a Y-olfactometer, workers of *C. novogranadensis* neither preferred lanolin paste that contained volatiles over pure lanolin paste. They also did not discriminate between JA-induced vs water-sprayed control plants. However, in previous trials they significantly chose an arm with mashed banana (C. Kost, unpublished data). More experiments of this kind will clarify the role of plant volatiles in ant foraging behavior.

In contrast to VOCs, EFN is a known attractant to ants (Koptur 1992) that has been reported to translate into enhanced plant protection in the vast majority of studies (for review, see Bentley 1977; Koptur 1992; Heil and McKey 2003; Oliveira and Freitas 2004), although some studies did not detect a defensive effect (O'Dowd and Catchpole 1983; Boecklen 1984; Becerra and Venable 1989; Rashbrook et al. 1992).

Also, wasps were more attracted to the tendrils of the JA, volatile, and EFN groups than to the control tendrils, yet in much smaller numbers than ants. The majority of wasps trapped on experimental tendrils belonged to predacious or parasitoid families (Table S1). These observations suggest that not only ants but also wasps likely contributed to the protection of the treated tendrils. Because of the intrinsic physiological linkage between volatiles and EFN, however, it remains unclear which indirect defense was mainly responsible for wasp attraction. As the emission of VOCs from a lima bean plant is correlated with its amount of EFN secreted, it appears reasonable to assume that wasps may have used VOCs as long-distance cues to detect patches with an increased herbivore density and/or EFN availability.

Comprehensive evidence for the volatile-mediated attraction of wasp is available from many laboratory-based studies (e.g., Turlings et al. 1990; Takabayashi et al. 1995; Du et al. 1996), yet relatively few field studies. Among them, James (2005) identified methyl salicylate as an attractant for parasitic wasps such as Encyrtidae and Mymaridae. Furthermore, braconid wasps were attracted to (3*Z*)-hexen-1-yl acetate and (*Z*)-jasmone (James 2005). These three compounds were also constituents of the synthetic volatile blend used in our study, and the above-mentioned parasitic wasps were also trapped on our experimental tendrils. In another study, DMNT (another constituent of the lima bean's induced VOC blend) emitted from molasses grass (*Melinis minutiflora*) significantly attracted parasitoid wasps in the Y-tube olfactometer and was likely involved in increasing the parasitation rate of stem-borer larvae that were feeding on nearby growing maize plants (Khan et al. 1997). Wasps feeding on EFN have been reported for several different plant species (for review, see Koptur 1992), yet so far, only one study has also demonstrated a fitness-benefit for the EFN-secreting plant (Cuautle and Rico-Gray 2003). A more detailed analysis of the attractive effects of VOCs and EFN on wasps is required, in which single blend constituents should be tested under field conditions.

Flies responded differently to tendril treatment than ants and wasps. They were only significantly attracted to tendrils of the nectar group (Fig. 4), suggesting that flies were more attracted to synthetic EFN than to airborne VOCs. The community of trapped Diptera covered a diverse spectrum of feeding habits ranging from predacious or parasitoid over herbivorous to detritus- or fungi-feeding (Table S1). This heterogeneous composition complicates a clear functional assignment of the caught flies. In most cases, the offered EFN seems to have been exploited by the flies as an additional food source rather than having contributed to a large extent to plant protection. In this case, the consumption of EFN without providing plant protection would cause ecological costs because the EFN-producing plant would be less protected against herbivores (Heil 2002; Heil et al. 2004).

In summary, ecological studies on the benefit of indirect defenses have generally focused on the protective role of a single defensive trait. While this simplification is easy to understand from the viewpoint of experimental feasibility, such univariate approaches may be inappropriate because they do not appreciate the complex interplay of several plant defenses that can co-occur within one plant species (Duffey and Stout 1996). This study takes a first step in this direction.

The mere application of synthetic EFN (nectar group) resulted in a fitness benefit that was always stronger or quantitatively similar to that experienced by tendrils of the JA and VOC treatments (Fig. 3). Moreover, the number of ants observed on the experimental tendrils (i.e., typical EFN feeders but less known to respond to VOCs) overwhelmingly exceeded the number of all other arthropod groups (Fig. 4). These observations suggest that, under our experimental conditions, the presence of EFN was more important for plant defense than was the VOC-mediated attraction of arthropods.

However, the inducing effect of VOCs on EFN impeded an experimental separation of VOCs and EFN and, thus, the exclusion of an attractive effect of airborne VOCs on flying or crawling arthropods. This finding underlines the necessity of studying different plant traits such as indirect defenses simultaneously in their ecological context. Studying them separately may provide a distorted picture of their true function and likely cause an under- or overestimation of their true effect.

This issue needs to be addressed in future studies, which should focus especially on the role of volatiles and EFN for

short- and long-distance attraction of herbivores and plant defenders. Several of the arthropod taxa that were identified in this study could serve as possible targets for such analyses. Further laboratory and field-based experimentation is needed to study whether inductive situations exist, in which either the volatile emission or the EFN secretion is differentially up- or down-regulated or if both defenses always respond similarly to herbivore attack.

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References

AGRAWAL, A. A., and DUBIN-THALER, B. J. 1999. Induced responses to herbivory in the Neotropical ant-plant association between *Azteca* ants and *Cecropia* trees: response of ants to potential inducing cues. *Behav. Ecol. Sociobiol* 45:47–54.

AGRAWAL, A. A., STRAUSS, S. Y., and STOUT, M. J. 1999. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53:1093–1104.

ARIMURA, G. I., KOST, C., and BOLAND, W. 2005. Herbivore-induced, indirect plant defenses. *Biochim. Biophys. Acta, Lipid Lipid Metab*. 1734:91–111.

ARNETT, R. H. 2000. American Insects—A Handbook of the Insects of America North of Mexico. CRC Press, Boca Raton.

BECERRA, J. X. I., and VENABLE, D. L. 1989. Extrafloral nectaries: a defense against ant–Homoptera mutualisms? *Oikos* 55:276–280.

BENTLEY, B. L. 1977. Extrafloral nectaries and protection by pugnacious bodyguards. *Annu. Rev. Ecol. Syst* 8:407–427.

BOECKLEN, W. J. 1984. The role of extrafloral nectaries in the herbivore defense of *Cassia fasciculata*. *Ecol. Entomol* 9:243–249.

BROUAT, C., MCKEY, D., BESSIERE, J. M., PASCAL, L., and HOSSAERT-MCKEY, M. 2000. Leaf volatile compounds and the distribution of ant patrolling in an ant–plant protection mutualism: preliminary results on *Leonardoxa* (Fabaceae: Caesalpinioideae) and *Petalomyrmex* (Formicidae: Formicinae). *Acta Oecol* 21:349–357.

CHOH, Y., KUGIMIYA, S., and TAKABAYASHI, J. 2006. Induced production of extrafloral nectar in intact lima bean plants in response to volatiles from spider mite-infested conspecific plants as a possible indirect defense against spider mites. *Oecologia* 147:455–460.

CREELMAN, R. A., and MULLET, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant. Mol. Biol* 48:355–381.

CUAUTLE, M., and ICO-GRAY, V. 2003. The effect of wasps and ants on the reproductive success of the extrafloral nectaried plant *Turnera ulmifolia* (Turneraceae). *Funct. Ecol* 17:417–423.

DALY, H. V., DOYEN, J. T., and PURCELL, A. H. 1998. Introduction to Insect Biology and Diversity. Oxford University Press, Oxford.

DICKE, M., DE BOER, J. G., HOFTE, M., and OCHA-GRANADOS, M. C. 2003. Mixed blends of herbivore-induced plant volatiles and foraging success of carnivorous arthropods. *Oikos* 101:38–48.

DICKE, M., VANBEEK, T. A., POSTHUMUS, M. A., BENDOM, N., VANBOKHOVEN, H., and DEGROOT, A. E. 1990. Isolation and identification of volatile kairomone that affects acarine predator-prey interactions—involvement of host plant in its production. *J. Chem. Ecol* 16:381–396.

DJIETO-LORDON, C., and DEJEAN, A. 1999a. Innate attraction supplants experience during host selection in an obligate plant-ant. *Behav. Process* 46:181–187.

DJIETO-LORDON, C., and DEJEAN, A. 1999b. Tropical arboreal ant mosaics: innate attraction and imprinting determine nest site selection in dominant ants. *Behav. Ecol. Sociobiol* 45:219–225.

DONATH, J., and BOLAND, W. 1995. Biosynthesis of acyclic homoterpenes: enzyme selectivity and absolute configuration of the nerolidol precursor. *Phytochemistry* 39:785–790.

DU, Y. J., POPPY, G. M., and POWELL, W. 1996. Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of *Aphidius ervi*. *J. Chem. Ecol* 22:1591–1605.

DUFFEY, S. S., and STOUT, M. J. 1996. Antinutritive and toxic components of plant defense against insects. *Arch. Insect Biochem. Physiol* 32:3–37.

ENGLISH-LOEB, G. M., and KARBAN, R. 1991. Consequences of mite feeding injury to beans on the fecundity and survivorship of the two-spotted spider mite (Acari: Tetranychidae). *Exp. Appl. Acarol* 11:125–136.

FARAG, M. A., and PARÉ, P. W. 2002. C_6-Green leaf volatiles trigger local and systemic VOC emissions in tomato. *Phytochemistry* 61:545–554.

FIALA, B., and MASCHWITZ, U. 1990. Studies on the Southeast Asian ant–plant association *Crematogaster borneensis*/*Macaranga*: adaptations of the ant partner. *Insectes Soc* 37:212–231.

FRITSCHE-HOBALLAH, M. E., TAMO, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important? *J. Chem. Ecol* 28:951–968.

GALETTO, L., and BERNARDELLO, L. M. 1992. Extrafloral nectaries that attract ants in Bromeliaceae: structure and nectar composition. *Can. J. Bot* 70:1101–1106.

GOUINGUENE, S., DEGEN, T., and TURLINGS, T. C. J. 2001. Variability in herbivore-induced odour emissions among maize cultivars and their wild ancestors (teosinte). *Chemoecology* 11:9–16.

GOUINGUENE, S. P., and TURLINGS, T. C. J. 2002. The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Phys* 129:1296–1307.

HALITSCHKE, R., and BALDWIN, I. T. 2004. Jasmonates and related compounds in plant–insect interactions. *J. Plant Growth Regul* 23:238–245.

HEIL, M. 2002. Ecological costs of induced resistance. *Curr. Opin. Plant Biol* 5:345–350.

HEIL, M. 2004a. Direct defense or ecological costs: responses of herbivorous beetles to volatiles released by wild lima bean. *J. Chem. Ecol* 30:1289–1295.

HEIL, M. 2004b. Induction of two indirect defenses benefits lima bean (*Phaseolus lunatus*, Fabaceae) in nature. *J. Ecol* 92:527–536.

HEIL, M., FIALA, B., BAUMANN, B., and LINSENMAIR, K. E. 2000. Temporal, spatial and biotic variations in extrafloral nectar secretion by *Macaranga tanarius*. *Funct. Ecol* 14:749–757.

HEIL, M., HILPERT, A., KRÜGER, R., and LINSENMAIR, K. E. 2004. Competition among visitors to extrafloral nectaries as a source of ecological costs of an indirect defense. *J. Trop. Ecol* 20:201–208.

HEIL, M., KOCH, T., HILPERT, A., FIALA, B., BOLAND, W., and LINSENMAIR, K. E. 2001. Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius,* is an induced, indirect, defensive response elicited by jasmonic acid. *Proc. Natl. Acad. Sci. USA* 98:1083–1088.

HEIL, M., and KOST, C. 2006. Priming of indirect defenses. *Ecol. Lett* 9:813–817.

HEIL, M., and MCKEY, D. 2003. Protective ant–plant interactions as model systems in ecological and evolutionary research. *Annu. Rev. Ecol. Evol. Syst* 34:425–453.

HEIL, M., and SILVA BUENO, J. C. 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proc. Natl. Acad. Sci. USA* 104:5467–5472.

HONOMICHL, K., BELLMANN, H., JACOBS, W., and ENNER, M. 1996. Biologie und Ökologie der Insekten. Gustav Fischer Verlag, Stuttgart.

HUSAIN, A., AHMAD, A., and AGRAWAL, P. K. 1993. (−)-Jasmonic acid, a phytotoxic substance from *Botryodiplodia theobromae*—characterization by NMR spectroscopic methods. *J. Nat. Prod* 56:2008–2011.

JAMES, D. G. 2003. Field evaluation of herbivore-induced plant volatiles as attractants for beneficial insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. *J. Chem. Ecol* 29:1601–1609.

JAMES, D. G. 2005. Further field evaluation of synthetic herbivore-induced plant volatiles as attractants for beneficial insects. *J. Chem. Ecol* 31:481–495.

JAMES, D. G., and PRICE, T. S. 2004. Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. *J. Chem. Ecol* 30:1613–1628.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

KEELING, C. I., PLETTNER, E., and SLESSOR, K. N. 2004. Hymenopteran semiochemicals. *Top. Curr. Chem* 239:133–177.

KELSEY, L. P. 1969. A revision of the Scenopinidae (Diptera) of the world. *Bull. US Natl. Mus* 277:1–336.

KELSEY, L. P. 1981. New Scenopinidae (Diptera) from the Palearctic. *Folia Entomol. Hung* 2:85–93.

KESSLER, A., and BALDWIN, I. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141–2144.

KHAN, Z. R., AMPONG-NYARKO, K., CHILISWA, P., HASSANALI, A., KIMANI, S., LWANDE, W., OVERHOLT, W. A., PICKETT, J. A., SMART, L. E., WADHAMS, L. J., and WOODCOCK, C. M. 1997. Intercropping increases parasitism of pests. *Nature* 388:631–632.

KOCH, T., KRUMM, T., JUNG, V., ENGELBERTH, J., and BOLAND, W. 1999. Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol* 121:153–162.

KOPTUR, S. 1992. Extrafloral nectary-mediated interactions between insects and plants, pp. 81–129, in E. Bernays (ed.). Insect–Plant InteractionsCRC Press, Boca Raton.

KOST, C., and HEIL, M. 2005. Increased availability of extrafloral nectar reduces herbivory in lima beans (*Phaseolus lunatus*, Fabaceae). *Basic Appl. Ecol* 6:237–248.

KOST, C., and HEIL, M. 2006. Herbivore-induced plant volatiles induce an indirect defense in neighbouring plants. *J. Ecol* 94:619–628.

MATILE, L. 1997. Phylogeny and evolution of the larval diet in the Sciaroidea (Diptera, Bibionomorpha) since the Mesozoic. *Mem. Mus. Natl. Hist. Nat* 173:273–303.

MUNZEL, U., and HOTHORN, L. A. 2001. A unified approach to simultaneous rank test procedures in the unbalanced one-way layout. *Biometrical J* 43:553–569.

NOYES, J. S. 2003. Universal Chalcidoidea Database. World Wide Web Electronic Publication. http://www.nhm.ac.uk/entomology/chalcidoids/index.html. Cited 05 Sep. 2003. The Natural History Museum, London, UK.

O'DOWD, D. J., and CATCHPOLE, E. A. 1983. Ants and extrafloral nectaries: no evidence for plant protection in *Helichrysum* spp–ant interactions. *Oecologia* 59:191–200.

OKA, Y., COHEN, Y., and SPIEGEL, Y. 1999. Local and systemic induced resistance to the root-knot nematode in tomato by DL-beta-amino-*n*-butyric acid. *Phytopathology* 89:1138–1143.

OLIVEIRA, P. S., and FREITAS, A. V. L. 2004. Ant–plant–herbivore interactions in the neotropical cerrado savanna. *Naturwissenschaften* 91:557–570.

PARÉ, P. W., and TUMLINSON, J. H. 1997. *De novo* biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol* 114:1161–1167.

PATTENDEN, G., and WEEDON, B. L. L. 1968. Carotenoids and related compounds. Part XVIII. Synthesis of *cis*-and di-*cis*-polyenes by reactions of the Wittig type. *J. Chem. Soc. C* 16:1984–1997.

PILOTTI, M., FESTA, S., GERVASI, E., and COPPOLA, F. 2004. Biological assays to study induction of resistance in *Platanus* × *acerifolia* to *Ceratocystis fimbriata*. *J. Plant Pathol* 86:71–83.

PRICE, P. W., BOUTON, C. E., GROSS, P., MCPHERON, B. A., THOMPSON, J. N., and WEIS, A. E. 1980. Interactions among 3 trophic levels—influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst* 11:41–65.

RASHBROOK, V. K., COMPTON, S. G., and LAWTON, J. H. 1992. Ant–herbivore interactions: reasons for the absence of benefits to a fern with foliar nectaries. *Ecology* 73:2167–2174.

RÖSE, U. S. R., and TUMLINSON, J. H. 2004. Volatiles released from cotton plants in response to *Helicoverpa zea* feeding damage on cotton flower buds. *Planta* 218:824–832.

RUFFNER, G. A., and CLARK, W. D. 1986. Extrafloral nectar of *Ferocactus acanthodes* (Cactaceae): composition and its importance to ants. *Am. J. Bot* 73:185–189.

SCHAEFER, M., BOHLKEN, H., EICHSTEIN, H., SATTLER, K., SCHMIDT, E., SICK, F., and TISCHLER, W. 1994. Brohmer–Fauna von Deutschland. Quelle und Meyer Bestimmungsbücher, Heidelberg.

STRÄSSNE, M. 1999. Klimadiagramme der Erde–Teil 2: Asien, Lateinamerika, Afrika, Australien und Ozeanien, Polarländer. Dortmunder Vertrieb für Bau-und Planungsliteratur, Dortmund.

STRAUSS, S. Y., UDGERS, J. A., LAU, J. A., and IRWIN, R. E. 2002. Direct and ecological costs of resistance to herbivory. *Trends Ecol. Evol* 17:278–285.

TAKABAYASHI, J., TAKAHASHI, S., DICKE, M., and POSTHUMUS, M. A. 1995. Developmental stage of herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol* 21:273–287.

THALER, J. S. 1999. Jasmonate-inducible plant defenses cause increased parasitism of herbivores. *Nature* 399:686–688.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

VALLAT, A., GU, H. N., and DORN, S. 2005. How rainfall, relative humidity and temperature influence volatile emissions from apple trees *in situ*. *Phytochemistry* 66:1540–1550.

VANDER MEER, R., BREED, M., ESPELIE, K., and WINSTON, M. 1998. Pheromone Communication in Social Insects: Ants, Wasps, Bees, and Termites. Westview Press, Boulder.

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Floral Odors of *Silene otites*: Their Variability and Attractiveness to Mosquitoes

Umma Salma Jhumur · Stefan Dötterl · Andreas Jürgens

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Abstract Inflorescence scent samples from nine populations of dioecious *Silene otites*, a plant pollinated by moths and mosquitoes, were collected by dynamic headspace extraction. Sixty-three scent samples were analyzed by gas chromatography–mass spectrometry. Out of 38 found, 35 compounds were identified, most of which were monoterpenoids, fatty acid derivatives, and benzenoids. Phenyl acetaldehyde was the most dominant compound in the majority of samples. The variability in scent composition was high, and population and sex differences were found. Nevertheless, wind tunnel experiments proved similar attraction of *Culex pipiens pipiens* biotype *molestus* mosquitoes to the inflorescence odor of *S. otites* of different populations, indicating that different blends are similarly attractive to mosquitoes. The electrophysiological responses of mosquitoes to the 12 most common and abundant odor compounds of *S. otites* differed. Linalool oxide (furanoid) and linalool evoked the strongest responses in male and female mosquitoes, and (*Z*)-3-hexenyl acetate was strongly active in females. Medium responses were evoked in males by (*Z*)-3-hexenyl acetate, in females by benzaldehyde and methyl salicylate, and in both sexes by lilac aldehyde, lilac alcohol, and linalool oxide (pyranoid).

Keywords *Silene otites* · Flower odor variability · Wind tunnel bioassays · *Culex pipiens pipiens* biotype *molestus* · Electroantennography · Attraction · Nectar host plant

U. S. Jhumur (✉) · S. Dötterl
Department of Plant Systematics, University of Bayreuth,
95440 Bayreuth, Germany
e-mail: jhumurjb@yahoo.com

A. Jürgens
School of Botany and Zoology, University of Natal,
P. Bag XO I, Scottsville,
Pietermaritzburg 3209, South Africa

Introduction

Carbohydrates are vital resources for adult male and female mosquitoes. Uptake of sugar plays a critical role in longevity, fecundity, flight capacity, and host-seeking behavior (Harada et al. 1971; Nayar and Saurman 1971, 1975; Magnarelli 1978; Klowden 1986). The primary sugar source for mosquitoes is nectar (Haeger 1955; Sandholm and Price 1962; Grimstad and DeFoliart 1974), and mosquitoes prefer some plants to others as nectar sources (Grimstad and DeFoliart 1974; Magnarelli 1978; Gadawaski and Smith 1992). However, the specific cues that mosquitoes use to find and to select nectar sources are not well understood. Many flower visitors, mosquitoes included, are known to be attracted to floral scents (Vargo and Foster 1982; Dudareva and Pichersky 2000).

For finding effective nectar-related attractants for biological control of mosquitoes, it is important to determine which plant species produce the most attractive floral compounds and to identify these compounds. Plant species adapted to mosquitoes as pollinators are expected to emit more mosquito-attracting compounds than plants pollinated primarily by other pollen vectors.

Worldwide, effective pollination by mosquitoes has been described only in the orchid *Habenaria (Platanthera) obtusata* (Banks ex Pursh) Richardson (Stoutamire 1968) and in *Silene otites* L. Wibel (Caryophyllaceae) (Brantjes and Leemans 1976), which is usually a perennial and dioecious species widely distributed in Middle, East, and South Europe and in Central Asia. The small and white-greenish flowers are arranged in terminal cymes. Jürgens et al. (2002) described the floral scent composition of *S. otites*. The scent of a few plants of a single *S. otites* population was analyzed. Therefore, nothing is known about the variability in the scent of this plant among populations or between males and females.

Compounds with low variability may be more important for the attraction of pollinators than compounds with high variability (Ayasse at al. 2000), as pollinators may exert selective pressure on scent composition, resulting in regular emission of attractive compounds, whereas nonattractive compounds may be more variable. So far, only a single major volatile component of *S. otites*, phenyl acetaldehyde, has been shown to attract mosquitoes (Jhumur et al. 2006), whereas the importance of the total floral scent emitted by *S. otites* is unknown for attraction of its flower-visiting mosquitoes (e.g., *Culex pipiens* L. and *Culiseta annulata* Schrank; Brantjes and Leemans 1976).

The aim of this study was to analyze the geographic variability of the floral scent composition of *S. otites* (L.) Wibel (Caryophyllaceae), and to assess the attractiveness of floral bouquets of different *S. otites* populations to *Culex pipiens pipiens* biotype *molestus* Forskal 1775. Furthermore, the antennal electrophysiological responses of *C. p. molestus* to the most common and abundant odor compounds in *S. otites* were measured.

Methods and Materials

Plant Material Inflorescence scent samples were collected from 63 individuals of 9 different populations. The geographic origin of eight populations and the number of females and males sampled are shown in Fig. 1. For one population (h), from which three males and four females were sampled, the geographic origin is unknown. Seed of the different populations were provided by several botanical gardens. To reduce environmental variation among populations, plants were grown under the same conditions (e.g., soil, temperature) in pots in the greenhouse until they built up a rosette, and thereafter the pots were placed in flower beds in the field.

Odor Collection S. otites is a nocturnal plant. Its floral scent emission is strongest in the early night hours (Jürgens et al. 2002). Male flowers remain functional for two nights, whereas female flowers emit scent over several days until they are pollinated (Brantjes and Leemans 1976). For studying the variability of floral scents, floral odors of *S. otites* were collected from one to four inflorescences of each individual plant 2–3 d after the onset of floral bloom when most of the flowers in an inflorescence had opened for the first time. Thus, the inflorescences used were of the same age; however, the flowers of these inflorescences were in different developmental stages. It is unclear whether there is variation in scent of *S. otites* among flowers of different ages on the same plant and whether this possible variation contributed to the observed variability among populations. However, as scent was collected from inflorescences of the same age, the possible variation in scent among flowers of different ages is not expected to have influenced our measurements. Furthermore, in a closely related species, *S. latifolia*, no differences in scent composition of flowers of different stages were found (Dötterl et al. 2005b).

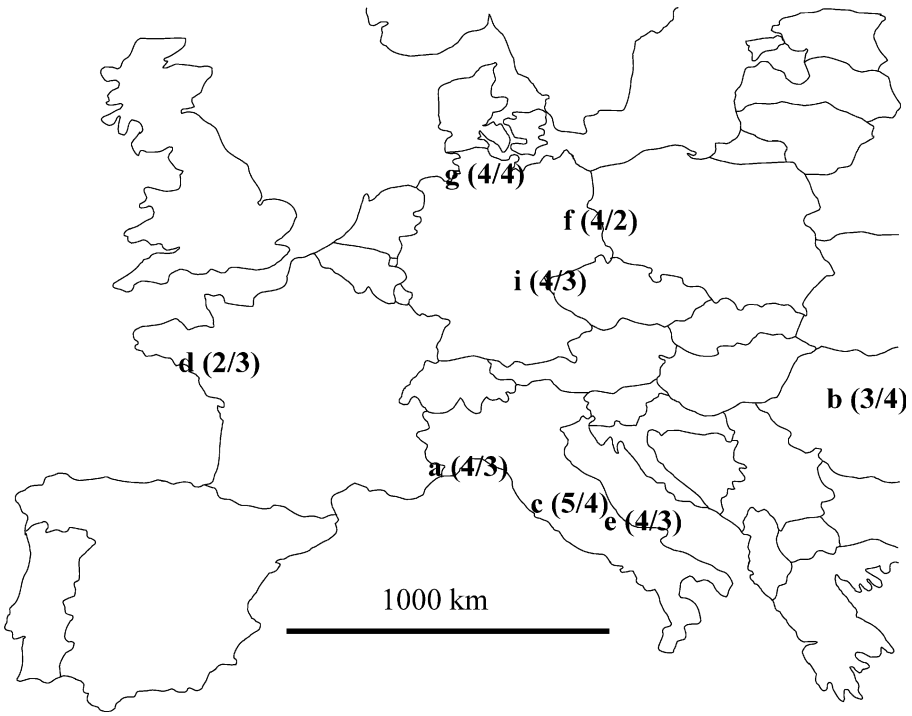

Fig. 1 Geographic origin of eight out of nine *S. otites* populations analyzed (a–f, i; the geographic origin of population 'h' is unknown). The number of sampled male and female individuals of each population is given in parenthesis

To collect odors, potted plants were placed under the extractor hood in the laboratory. Volatiles were collected by using the dynamic headspace method described by Dötterl et al. (2005b). Inflorescences were enclosed in a polyester oven bag (20×8 cm; Toppits®, Germany) 1–1 1/2 hr after sunset, and volatiles were trapped in an adsorbent tube for 2 min by using a membrane pump (ASF Thomas) with a flow rate of 200 ml/min. The adsorbent tubes were filled with a mixture (1:1) of 3 mg Tenax-TA (mesh 60–80) and Carbotrap (mesh 20–40). To distinguish between plant volatiles and ambient contaminants, surrounding air was collected for comparison. Furthermore, to discriminate odor emitted by flowers from odor derived from vegetative parts, scent was also collected from nonflowering shoots. However, as insects attracted to plants may detect green leaf volatiles and floral odors, we also included vegetative odors in subsequent analyses (see below).

Preparation of Plant Material for Bioassays To facilitate the work with the night-active plant-flower visitor system, plants were shifted from flower beds to a climatic chamber with an inverted day and night rhythm shortly before onset of flowering. Maintenance of the climatic chamber was dark (9 hr: from 9 A.M. to 6 P.M.) and light (15 hr: from 6 P.M. to 9 A.M.) with 20.5°C and 24.5°C, respectively. One or 2 d after moving, when flower opening had adjusted to the changed day and night rhythm, inflorescences were used for bioassays. Flower odors were collected before and after each bioassay, and are expressed as the mean total amount of emitted odors during bioassays. Flowering inflorescences (three to five) of males or females of a population were cut and placed together in small glass bottles filled with water. Within 5 min, inflorescences were bagged, and thereafter volatiles were collected for 2 min as described above. With the exception of higher amounts of green leaf odors in cut plants, the scent compositions of clock-shifted plants were the same as those of *in situ* plants (Jhumur, unpublished data).

Preparation of Insects for Bioassays We used flower-naïve individuals of the autogenous *Culex pipiens pipiens* biotype *molestus* Forskal 1775 (European strain) for experiments. Mosquitoes were reared according to Jhumur et al. (2006) with an inverted day and night rhythm in accordance with the designed bioassays. For bioassays, the sugar supply was removed 61–63 hr before the experiment. For electrophysiological measurements, regularly fed mosquitoes were used.

Chemical Scent Analysis Scent samples were analyzed on a Varian Saturn 2000 mass spectrometer coupled to a Varian 3800 gas chromatograph equipped with a 1079 injector that had been fitted with the ChromatoProbe kit. The adsorbent tube containing sample was placed in the Chromatoprobe and then inserted into the modified GC injector. The injector split vent was opened (1/20), and the injector was heated to 40°C to flush any air from the system. The split vent was closed after 2 min, and the injector was heated to 200°C (200° C/min); this temperature was held for 4.2 min. Then, the split vent was opened again (1/10) while the injector was cooled. For analyses, a ZB-5 column (5% phenyl polysiloxane; 60 m long, i.d. 0.25 mm, film thickness 0.25 μm, Phenomenex) was used. A constant flow of carrier gas (helium, 1.8 ml/min) was maintained by electronic flow control. The GC oven temperature was held for 7 min at 40°C, then increased by 6°C/min to 250°C, and held for 1 min. The MS interface was 260°C, and the ion trap worked at 175°C. The mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1 scan sec^{-1} from *m/z* 30 to 350. The GC–MS data were processed by using the Saturn Software package 5.2.1. Component identification was carried out with the NIST 02 mass spectral data base or MassFinder 2.3, and confirmed by comparison of retention times with published data (Adams 1995). Identification of individual components was confirmed by comparison of both mass spectra and GC retention data with those of authentic standards.

For quantification of odors emitted from inflorescences, known amounts of lilac aldehydes (>99%, synthesized according to Dötterl et al. 2006b), (Z/E)-β-ocimene (>99%, provided by Jette T. Knudsen, Lund University, Sweden), (*Z*)-3-hexenyl acetate, benzaldehyde, phenyl acetaldehyde, and veratrole (all purchased from Sigma-Aldrich with highest purity available) were injected into the GC for calibration.

Bioassays A 160×75×75-cm wind tunnel (Dötterl et al. 2006b; Jhumur et al. 2006) was used for bioassays. A Fischbach speed controller fan (D340/E1, FDR32, Neunkirchen, Germany) continuously circulated air through the tunnel with an air speed of 0.35 m/sec. Incoming air was cleaned through four charcoal filters (145×457 mm, carbon thickness 16 mm, Camfil Farr). To allow mosquitoes to adapt to the wind tunnel environment, they were kept in the wind tunnel room for about 12 hr before the experiment started. To avoid contamination, all equipment was cleaned with ethanol, burned in flame, and then sterilized at 200°C, and surgical gloves were worn during mosquito handling and bioassays.

At the conditions described above, *S. otites* emitted the highest amounts of floral odors in the 2nd and 3rd hr after onset of darkness (Jhumur, unpublished data). Therefore, bioassays were conducted within this time frame. The inflorescences, the cut ends of which were already inserted in water, were placed at the upwind end of the tunnel

behind gauze and different aluminum screens. They were invisible to the mosquitoes.

A group of 10–15 randomly chosen male and/or female mosquitoes (the behaviors of mosquitoes were not influenced by the opposite sex, see also Jhumur et al. 2006) were released from a chamber (16×8 cm) at the downwind end of the tunnel. Mosquitoes were observed for 1 hr. Landing on the gauze (20×10 cm) in front of the odor source was considered as attraction to the source. In addition, the latency time before landing was measured. After landing, the behavior was classified into two types: "sitting" and "searching". "Sitting" was characterized simply as sitting without moving or doing anything on the gauze for 15 sec after landing, and "searching" was characterized by excited movement of mosquitoes on the gauze and repeated penetration of gauze with their proboscis, presumably in search for a food source. To avoid recording the behavior of any responding mosquito twice, landing mosquitoes were removed from the wind tunnel after 15 sec with an aspirator.

From other tests with mosquitoes in the same wind tunnel, we know that almost no mosquitoes land just by chance in front of the odor source (Jhumur et al. 2006). Therefore, we did not test the mosquitoes' response to clean air or room air. Furthermore, given that a small number of mosquitoes would land just by chance on the gauze in front of the odor source, this number should be similar for odor from all *S. otites* populations, and thus, not affect the comparison of attractiveness of *S. otites* odor from different populations.

Dependent upon the availability of flowers, 25 bioassays were conducted with *S. otites* plants of six populations. Male and female inflorescences were tested separately. However, female inflorescences were not available for the 'a' and 'c' populations (Table 2). Nine bioassays were conducted with population 'i', six with 'f', four with 'g', and two each with 'c', 'b', and 'a'. Most of the inflorescences of one plant (one bioassay) were tested with two groups of mosquitoes, and the behavioral responses (percentage of individuals landing) of these 20–30 mosquitoes were used for subsequent statistical analyses (see below). However, for population 'a', only one group of mosquitoes was used for each of the two inflorescence samples. In total, 113 male and 531 female mosquitoes were tested. Male mosquitoes were not available during the bioassays with male inflorescences of populations 'a', 'c', and 'g', and female inflorescences of population 'b'.

Electrophysiology

Authentic Standard Compounds The most frequently found 12 floral scent compounds of *S. otites* were used for electrophysiological measurements. Among these, lilac aldehyde (purity >99%) was synthesized as described by Dötterl et al. (2006b); lilac alcohol and linalool were provided by Karlheinz Seifert (University Bayreuth, Germany; purity >99%); and the other compounds were purchased from Sigma-Aldrich (hexanol, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexenyl acetate >98%; benzaldehyde 99%; phenylethyl alcohol 99%, acetophenone 98%; linalool oxide [furanoid] 97%; phenyl acetaldehyde 90%; methyl salicylate 98%) or Wako (linalool oxide [pyranoid] 98%). Among these 12 compounds, all monoterpenoids were used as stereoisomeric mixtures. To obtain dose–response curves and to compare the sensitivity of mosquitoes to different compounds, electroantennographic (EAG) recordings were performed with a dilution series of standard compounds (Schütz et al. 1999). Dilutions were prepared in paraffin oil (Uvasol, MERCK, Darmstadt, Germany).

Preparation Four- to 5-d-old *C. p. molestus* were used for EAG. For measurements, an excised antenna was mounted between glass micropipette electrodes filled with insect ringer (8.0 g/l NaCl, 0.4 g/l KCl, 0.4 g/l $CaCl_2$). The electrodes were connected to silver wires. Signals were interfaced with a two-channel USB acquisition controller (provided by Syntech, Hilversum, The Netherlands) to a PC as described by Dötterl et al. (2005a). Twenty microliters of a test compound was placed onto a piece of filter paper (2.5×1.5 cm^2) inside a 5-ml plastic syringe (Omnifix, B/Braun, Melsungen). Separate syringes were used for each stimulus. Stimuli were released into a continuous flow of humidified air that passed over the antenna with a pulse duration of 0.5 sec, and a flow of 10 ml/sec regulated by a CS-01 Stimulus Controller (Syntech). Each compound and each dilution was tested on four to six mosquitoes. In all EAG tests, antennae were stimulated at 30–40 sec intervals. To discriminate between the antennal response elicited by the air flow or by paraffin and by the tested scent compound, a filter paper that contained only paraffin was tested as the first and last measurement on each antenna. To counterbalance for the loss of antennal sensitivity during measurements, the antennal response to a syringe containing (*Z*)-3-hexen-1-ol (10^{-1} in paraffin) was recorded as the second measurement from the beginning to the end. (*Z*)-3-Hexen-1-ol is a compound frequently found in sampling of *S. otites*. As this was used as the standard for EAG recordings, it was not used to obtain dose–response curves.

Statistical Analysis We used the Primer 6 program (Clarke and Warwick 2001; Clarke and Gorley 2006) to assess the variability in scent of *S. otites* individuals of different populations. Semiquantitative data of compounds (percentages=relative amounts with respect to total peak areas) were used because the total amount of emitted volatiles varied greatly among different individuals (see also Dötterl

et al. 2005b). We used multidimensional scaling (MDS) based on Bray–Curtis similarities to detect similarities among samples. To evaluate how well or poorly the particular configuration produces the observed distance matrix, the stress value is given. The smaller the stress value, the better the fit of the reproduced ordination to the observed distance matrix (Clarke 1993). We used ANOSIM (two-way crossed design, factors: sex, population) in Primer to test for the differences in scent between male and female flowers and among populations. SIMPER (two-way crossed design, factors: sex, population) was used in Primer to identify the compounds responsible for dissimilarities between sexes and among populations. RELATE was used in Primer to correlate the scent matrix with the distance matrix (in km) of the populations. To obtain the scent matrix, mean relative amounts of compounds were calculated for the different populations, and these values were used to calculate the Bray–Curtis similarities finally used for the analysis.

Chi-square tests were used to assess the differences in attractiveness between male and female mosquitoes (number of males responding–males not responding vs. number of females responding–females not responding) to male and female inflorescences of different populations of *S. otites*. No differences in responses between males and females were found (Jhumur, unpublished data). Therefore, the responses of males and females were pooled for further analyses.

In individual bioassays with specific inflorescences, the number of landing (attractive) mosquitoes (%) was determined at first, and among the landed mosquitoes thereafter the proportion of searching mosquitoes (%) was calculated. Kruskal–Wallis–ANOVA followed by the Tukey–Kramer post hoc test for nonparametric data in STATISTICA (StatSoft 2004) was used to compare these behavioral responses to the flower odors of different populations. ANOVA was used to compare the latency time of individual mosquitoes to different populations. Normality was tested with the Kolmogorov–Smirnov test; homogeneity of variances was tested by using the Hartley test.

For analyzing the EAG recordings, at first, the responses from the blank syringes were measured and subtracted from the recordings in between. Then, the response to (*Z*)-3-hexen-1-ol as the second measurement from the beginning of each measurement was set to 100%. As the sensitivity of antennae decreased during measurements, the response to (*Z*)-3-hexen-1-ol was also measured as the second measurement from the end, to determine the loss of sensitivity and to compensate for this. The responses to different compounds and dilutions are given as proportions of the responses to (*Z*)-3-hexen-1-ol (10^{-1} in paraffin). These data were directly used without transformation for further analyses. A general linear model (GLM) in STATISTICA was used to compare the differences in the responses of males and females to different dilutions and different compounds. The α-level for all statistical analyses was 0.05.

Results

Variability in Floral Scents of S. otites Thirty-eight compounds were detected in the inflorescence odor samples of *S. otites* of 9 geographical locations, 35 of which were tentatively identified by comparing mass spectra and retention index with literature data (Adams 1995). In addition, the identity of 27 of these compounds was confirmed by authentic standards (see Table 1). Among these, six compounds were also emitted from leaves. The identified compounds belong to 5 classes: fatty acid derivatives (8), benzenoids (6), nitrogen-containing compounds (1), monoterpenoids (18), and sesquiterpenoids (2). The benzenoid phenyl acetaldehyde (PAA) was the dominant odor compound in most of the individuals. However, one specimen emitted no PAA but instead high relative amounts of lilac aldehyde. Out of the 38 compounds, 19 were common to the scent samples of all populations.

Semiquantitative differences in the odor samples based on Bray–Curtis similarities are shown in Fig. 2. Variation among samples was high with significant differences among the samples from different populations (within sexes; two-way ANOSIM: $R=0.454$; $P<0.001$). SIMPER analyses revealed the compounds responsible for the differences among populations. Most populations and samples were dominated by phenyl acetaldehyde, but in some samples, high relative amounts of lilac aldehyde (e.g., samples of population ‘g’) or (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate (e.g., samples of population ‘h’) were present. One sample of population ‘i’ was characterized by a high percentage (33%) of linalool. There was no correlation between scent and the distance matrix of the populations (RELATE: $\rho=-0.02$, $P=0.52$), indicating that populations close to each other were not more similar in their scents than distant populations.

Within populations, we found significant differences in scent between male and female plants (two-way ANOSIM: $R=0.129$; $P=0.038$). However, differences between males and females were less pronounced than the observed differences among populations. Within populations, both males and females emitted the same compounds, but the proportions of some differed between males and females. According to the SIMPER analysis, phenyl acetaldehyde and (*Z*)-3-hexenyl acetate were the main compounds responsible for the differences between males and females

Table 1 Relative amounts of compounds (mean±SE) in inflorescence odors of 63 *S. otites* plants from different populations

Compounds	a (7)	b (7)	c (9)	d (5)	e (7)	f (6)	g (8)	h (7)	i (7)
Fatty acid derivatives									
Hexanol[a]	0.36±0.16	0.14±0.03	0.13±0.05	0.15±0.06	0.12±0.04	0.19±0.06	0.26±0.22	0.04±0.02	0.28±0.08
(*Z*)-3-Hexen-1-ol[a,b]	3.64±1.92	6.32±1.75	2.33±1.25	1.83±0.47	3.39±2.22	7.21±2.09	3.2±1.69	15.01±6.01	8.74±1.71
(*E*)-3-Hexen-1-ol	0.53±0.16	0.14±0.06	0.4±0.19	0.41±0.22	0.36±0.35	0.19±0.08	0.01±0.01	0.23±0.08	0.45±0.14
(*Z*)-3-Hexenyl acetate[a,b]	4.27±1.84	10.33±2.73	1.85±0.81	5.36±1.89	8.12±4.1	6.95±2.65	5.31±2.68	15.18±5.39	7.26±1.64
(*E*)-2-Hexenyl acetate[a]	0.11±0.05	0.04±0.04	0.08±0.06	0.06±0.04	0.73±0.55	0.02±0.02	–	–	0.03±0.02
Hexyl acetate[a]	–	0.09±0.08	0.03±0.02	0.01±0.01	2.61±1.71	0.17±0.1	–	–	0.14±0.08
(*Z*)–3-Hexenyl butyrate[b]	–	0.23±0.08	0.01±0.01	0.08±0.07	–	0.35±0.17	0.01±0.01	0.05±0.03	0.33±0.14
(*E*)-4,8-Dimethyl 1,3,7 nonatriene[b]	–	0.01±0.01	0.15±0.08	–	–	–	–	–	–
Benzenoids									
Benzaldehyde [a,b]	4.62±0.67	7.26±1.37	2.08±0.37	7.1±1.02	4.05±1.18	8.26±1.74	6.67±1.26	2.59±0.73	5.12±1.08
Benzyl alcohol[b]	0.02±0.02	0.17±0.06	0.01±0.01	0.04±0.03	0.01±0.01	0.84±0.42	0.14±0.06	0.48±0.26	4.68±1.43
Phenyl acetaldehyde[b]	47.71±4.43	38.97±4.78	35.94±1.54	42.27±7.64	40.89±11.69	41.02±4.42	31.66±3.29	14.85±4.64	26.05±4.29
Acetophenone[b]	0.36±0.22	0.31±0.17	0.48±0.32	0.58±0.36	0.32±0.29	1.38±0.56	3.01±2.01	0.07±0.07	1.08±0.5
Phenylethyl alcohol[b]	0.82±0.25	5.18±0.74	1.82±0.47	2.02±0.72	1.87±0.83	5.31±0.68	1.25±0.38	1.44±0.65	6.98±0.96
Methyl salicylate[b]	0.07±0.03	3.27±1.16	0.16±0.06	0.03±0.03	–	0.41±0.17	0.03±0.03	2.36±1.32	0.07±0.04
N- bearing compounds									
3-Methyl-butyl-aldoxime (syn/anti)[b]	–	–	0.01±0.01	–	–	–	0.01±0	0.01±0.01	–
Monoterpenoids									
α-Pinene[b,c]	–	0.02±0.02	–	0.15±0.09	0.08±0.04	0.13±0.06	0.12±0.05	0.35±0.12	0.14±0.1
β-Pinene[b,c]	0.05±0.04	0.01±0.01	–	0.04±0.02	0.05±0.03	0.12±0.05	0.01±0.01	0.23±0.13	0.25±0.08
D-Limonene[b,c]	–	–	–	–	0.04±0.02	–	–	–	–
(*E*)-β-Ocimene[b]	–	–	tr	–	–	–	0.06±0.06	0.31±0.2	0.54±0.54
(*Z*)-Linalool oxide furanoid[b,c]	0.28±0.15	tr	0.87±0.21	–	0.78±0.52	0.02±0.02	0.01±0.01	–	0.16±0.04
(*E*)-Linalool oxide furanoid[b,c]	–	0.6±0.11	0.01±0.01	4.45±2.35	0.12±0.07	1.46±0.39	0.03±0.03	7.17±1.16	3.04±0.39
Linalool[b,d]	–	3.13±1.09	0.84±0.22	0.49±0.19	0.06±0.04	4.26±1.63	0.52±0.12	5.64±1.72	8.11±4.24
Hotrienol[c]	–	2.16±0.48	0.25±0.2	1.52±0.88	0.1±0.04	1.63±0.5	0.66±0.16	0.6±0.39	2.57±0.49
2,2,6-Trimethyl-2-vinyl-5-ketotetrahydropyran[c]	0.4±0.13	0.05±0.01	0.71±0.16	0.53±0.21	0.42±0.16	0.16±0.05	0.06±0.04	0.73±0.33	0.73±0.11
Lilac aldehyde A[b,d]	12.37±2.01	6.94±0.86	14.42±1.43	10.46±1.43	8.14±1.91	6.27±0.65	20.16±2.23	12.03±2.45	5.96±1.58
Lilac aldehyde B+C[b,d]	13.72±1.9	8.32±1.13	16.53±1.11	11.31±1.36	10.43±2.35	7.48±0.59	19.42±1.26	10.19±1.78	6.47±1.52
Lilac aldehyde D[b,d]	2.9±0.6	2.29±0.52	4.22±0.47	3.02±0.41	2.73±0.5	1.94±0.29	4.67±0.61	2.29±0.49	1.12±0.31
(*Z*)-Linalool oxide pyranoid[b,c]	5.99±2.97	0.33±0.13	10.11±4.99	0.23±0.22	8.71±4.95	0.76±0.23	1.47±0.95	0.43±0.17	0.84±0.19
(*E*)-Linalool oxide pyranoid[b,c]	0.66±0.3	0.59±0.21	4.36±1.21	6.74±2.08	3.83±1.58	2.45±0.66	0.1±0.05	5.28±2.3	5.76±1.02
Lilac alcohol A[b,d]	0.32±0.07	0.39±0.18	0.96±0.19	0.4±0.12	0.71±0.49	0.19±0.07	0.37±0.11	1.3±0.41	0.54±0.12
Lilac alcohol B+C[b,d]	0.36±0.08	0.49±0.13	1.03±0.18	0.51±0.15	0.94±0.68	0.36±0.1	0.59±0.15	0.81±0.27	0.7±0.16
Lilac alcohol D[b,d]	0.03±0.02	0.13±0.04	0.2±0.04	0.04±0.02	0.22±0.17	0.05±0.03	0.11±0.03	0.05±0.03	0.07±0.02
1-Hydroxy linalool[c]	–	1.55±0.49	–	0.04±0.02	–	0.29±0.28	0.05±0.05	–	1.79±0.88
Monoterpenoid 43, 67, 79, 91, 105, 121	–	0.01±0.01	–	0.08±0.06	0.09±0.06	0.05±0.03	0.01±0.01	0.2±0.06	0.01±0.01
Monoterpene oxide 39, 65, 79,91,105, 121, 135, 150	–	0.01±0.01	–	0.03±0.03	0.02±0.02	0.06±0.06	–	–	0.01±0.01
Sesquiterpenoids									
(*E*)-β-Caryophyllene[b]	–	0.1±0.1	–	0.02±0.01	0.04±0.02	0.03±0.02	–	0.06±0.06	–
Geranyl isovalerate	0.38±0.25	0.38±0.38	0.02±0.02	–	–	–	–	–	–
Unknown									
43, 67, 93, 109, 123, 151	0.04±0.02	0.04±0.02	–	–	–	–	–	–	–

The number of individuals sampled in each population (a–i) is given in parenthesis.
[a] Compounds were also found in samples collected from leaves.
[b] Compounds were identified by comparing mass spectra and retention times with authentic standards.
[c] Enantiomeric composition was not determined
[c] Enantiomeric composition was determined by Dötterl et al. (2006a).

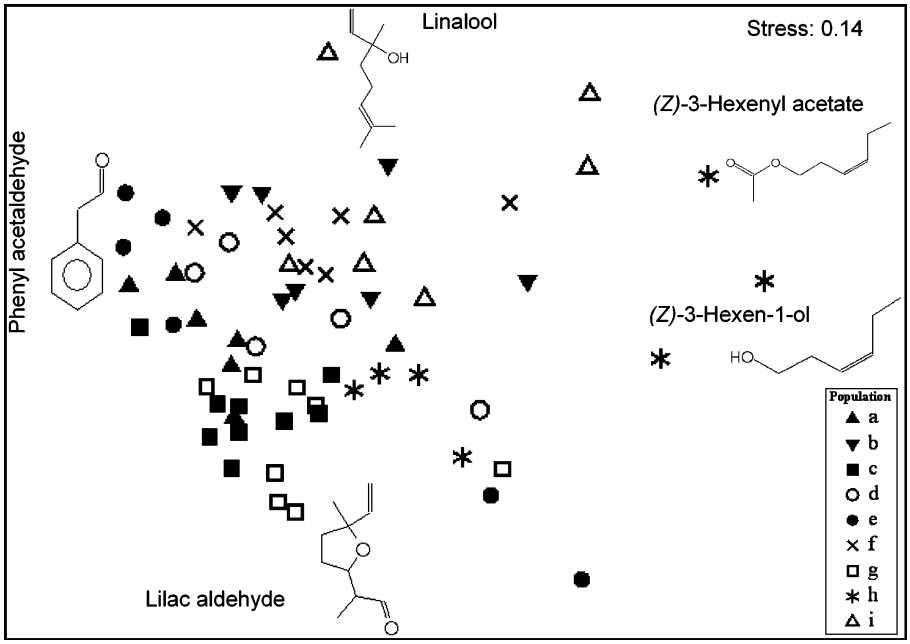

Fig. 2 MDS based on Bray–Curtis similarities of the odor composition of 63 inflorescences from 9 populations of *S. otites*. Most of the samples were dominated by high relative amounts of phenyl acetaldehyde; however, in some samples, high relative amounts of other compounds, such as lilac aldehyde, were found

(PAA=38% in males, 31% in females; (*Z*)-3-hexenyl acetate=8% in females, 6% in males).

Behavioral Responses of Mosquitoes to Odors of S. otites Inflorescences The wind tunnel bioassays revealed that about 50% of tested mosquitoes were attracted to scents emitted from inflorescences of *S. otites* of different populations. Male and female inflorescences were equally attractive to mosquitoes ('b': *Chi-square test*: $\phi^2_{df=1}=0.25$, $P=0.62$; 'f': $\phi^2_{df=1}=0.03$, $P=0.87$; 'g': $\phi^2_{df=1}=1.26$, $P=0.26$; 'i': $\phi^2_{df=1}=1.74$, $P=0.19$). Therefore, the responses to female and male inflorescences were pooled for further analyses. No differences in attractiveness among populations were found (Kruskal–Wallis–ANOVA: H (5, 25)=4.3; $P=0.5$). There was high variability in attraction within populations, which could not be explained by the different total amounts of scent emitted (Table 2, Fig. 3). As an example, most inflorescences of populations 'i' emitted similar total amounts of floral scent, but their attractiveness differed strongly (34–73%).

The latency time of mosquitoes did not differ among populations (ANOVA: F (5, 314)=0.33; $P=0.89$), and was on average 30 min. However, overall significant differences were found in the post choice behavior (Kruskal–Wallis–ANOVA: H (5, 25)=11.139; $P=0.049$). The 'searching' behavior was recorded most often when inflorescences of population 'a' were offered to the mosquitoes, and less often when they were offered inflorescences of population 'c'. Nevertheless, there were no significant differences in post hoc tests.

Electrophysiological Recordings EAG responses of male and female *C. p. molestus* to several odor components of *S. otites* are shown in Fig. 4. All tested compounds elicited EAG responses, and the effect of dilution was evident for each compound. EAG responses generally increased with increasing dose of tested compounds. However, mosquitoes responded differently to compounds tested, and we also recorded differences in the responses of males and females to different compounds (Table 3). The strongest responses (110–151%) were elicited by linalool oxide (furanoid) and linalool. Furthermore, females responded strongly to (*Z*)-3-hexenyl acetate. Weak responses (<80%) were obtained from both sexes to phenyl acetaldehyde, phenylethyl alcohol, acetophenone, and hexanol.

Discussion

Most compounds found in this study have been reported earlier as part of the floral odor bouquet in other angiosperms (Knudsen et al. 2006), but only nine of the compounds identified in this study were also found in the *S. otites* samples analyzed by Jürgens et al. (2002). In total, we found 22 new compounds in the floral scent of *S. otites* that have not been reported previously in that species. On the other hand, Jürgens et al. (2002) identified nine compounds that were not detectable in our samples. Furthermore, only small amounts of phenyl acetaldehyde were found in that study, but we found that this was the dominant compound in nine populations. Some of these differences might be ascribed to different scent collection

Table 2 Attraction, post choice behavior, and latency time of mosquitoes with respect to the emitted scent from *S. otites* inflorescences (three to five) of different populations (a–i)

Population (Numbers of Female (F) and Male (M) Inflorescence Samples Tested in Bioassays)	Number of Mosquitoes Tested	Odor Emission of Inflorescence Samples (ng/2 min) Median (Min–Max)	Number of Landed (Attracted) Mosquitoes (%) Median (Min–Max)[a]	Number of Landed Mosquitoes Showing Searching Behavior (%) Median (Min–Max)[b]	Latency Time of Mosquitoes Until Landing (Min) Median (Min–Max)[c]
a (2 M)	30	357 (337–378)	49 (36–63)	75 (50–100)	25 (5–53)
b (1 M, 1 F)	46	240 (223–378)	50 (44–55)	55 (50–60)	20 (1–59)
c (2 M)	40	219.3 (217–221)	60 (47–64)	18 (18–18)	31 (1–59)
f (2 M, 4 F)	170	146 (9–370)	51 (35–72)	40 (12–80)	25 (1–60)
g (1 M, 3 F)	98	463 (329–1387)	38 (30–52)	32 (0–33)	23 (3–59)
i (5 M, 4 F)	260	115 (82–234)	53 (34–73)	20 (0–35)	25 (1–59)

[a] Kruskal–Wallis–ANOVA: H (5, 25)=4.3; P=0.5
[b] Kruskal–Wallis–ANOVA: H (5, 25)=11.13; P=0.05
[c] ANOVA: F (5; 314) = 0.33; P=0.89

methods, but probably such differences are also due to sampling of plants of different geographical origin. Different populations of *S. otites* emit population-specific scent profiles with only 19 out of 38 inflorescence volatiles being common to plants of the 9 populations studied here.

Although intraspecific variation in floral scent has been observed for many angiosperms, comprehensive screening for population/geographic variation in floral scent composition has been investigated only in few species, e.g., *Yucca filamentosa* L (Agavaceae; Svensson et al. 2005), *Magnolia kobus* DC (Magnoliaceae; Azuma et al. 2001), *Geonoma macrostachys* Mart. (Arecaceae; Knudsen 2002), *Silene latifolia* L. (Caryophyllaceae; Dötterl et al. 2005b), and *Ophrys* species (Orchidaceae; Mant et al. 2005). The intraspecific variability found in our dataset was comparable to variability found in other studied taxa. Such variability may be the result of genetic drift or natural selection (Tollsten and Bergström 1993). Furthermore, different chemotypes may be adapted to different pollinators (Whitten and Williams 1992; Tollsten and Bergström 1993).

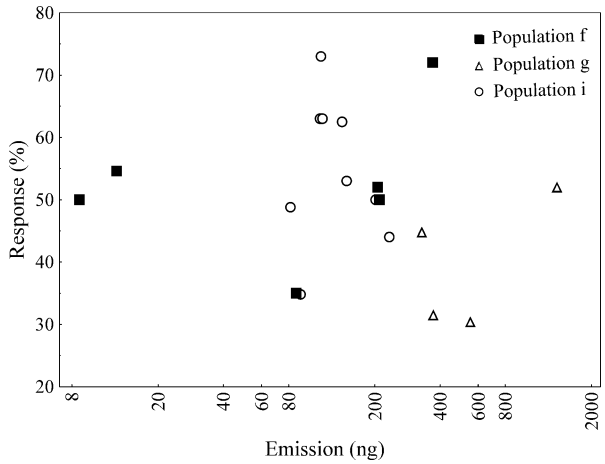

Fig. 3 Total amount of scent emission from *S. otites* inflorescences and % mosquitoes attracted in the wind tunnel (N=100%=170, 98, 260 for 'f', 'g', and 'i' population, respectively)

So far, we do not know the evolutionary factors that trigger the observed odor variability among *S. otites* populations. Different pollinators associated with the different populations might exert different selective pressures on the odor. Only a few species of nocturnal Lepidoptera and mosquitoes have been recorded as pollinators in this species (Brantjes and Leemans 1976); among them, *Autographa gamma* L. and *Culex pipiens*. Whereas *A. gamma* is known to be strongly attracted by lilac aldehyde (Plepys et al. 2002a, b), *C. pipiens* is known to respond strongly to phenyl acetaldehyde (Jhumur et al. 2006).

In this study, phenyl acetaldehyde was the dominant and abundant odor compound, followed by lilac aldehyde, (*Z*)-3-hexenyl acetate, linalool oxide (pyranoid), (*Z*)-3-hexen-1-ol, benzaldehyde, phenylethyl alcohol, linalool, linalool oxide (furanoid), lilac alcohol, acetophenone, methyl salicylate, and hexanol. Most of these compounds are known to elicit strong antennal responses and/or to be attractive to moths such as *Hadena bicruris* Hufn. (Lepidoptera: Noctuidae, Dötterl et al. 2006b), *Sphinx perelegans* Edwards (Lepidoptera: Sphingidae, Raguso and Light 1998), *Hyles lineata* L. (Lepidoptera: Sphingidae, Raguso et al. 1996), *Argyresthia conjugella* Zeller (Lepidoptera: Argyresthiidae, Bengtsson et al. 2007), *Cydia pomenella* L. (Lepidoptera: Tortricidae, Bengtsson et al. 2007), and *Mamestra brassicae* L. (Lepidoptera: Noctuidae, Rojas 1999), whereas only phenyl acetaldehyde has been reported as being attractive to mosquitoes (Howse 2003; Jhumur et al. 2006). It is interesting to note that 19 out of 35 identified compounds in *S. otites* were also found in other closely related *Silene* species, which have been described as moth-pollinated flowers (Jürgens et al. 2002). Thus, it is not surprising that besides mosquitoes, moths

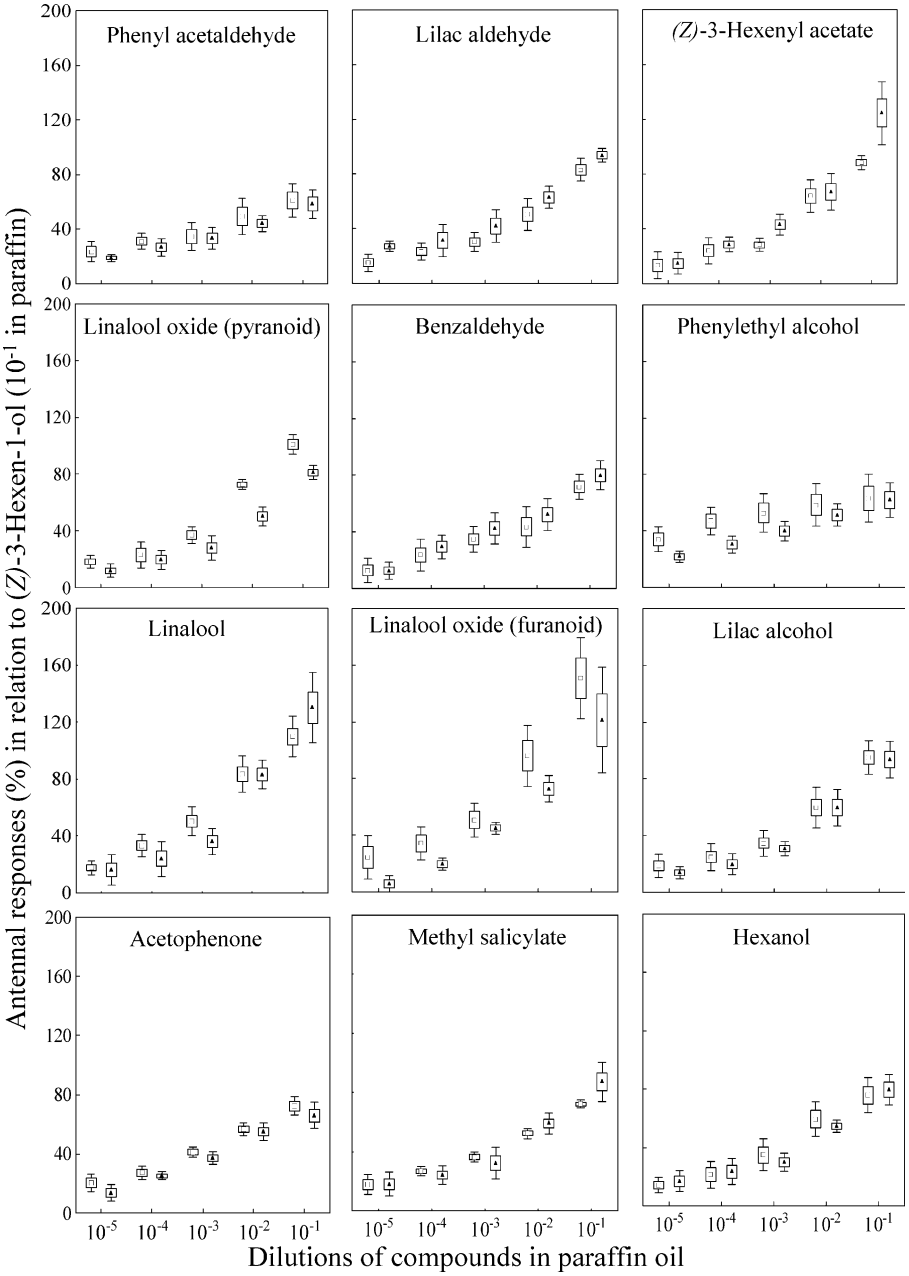

Fig. 4 EAG responses of male (*rectangular*) and female (*triangular*) *Culex pipiens pipiens* biotype *molestus* to different dilutions (in paraffin) of common floral scent compounds of *S. otites* of different populations. Twenty microliters of each dilution of 12 scent compounds were tested on 4–6 mosquitoes. The antennal responses are given in relation to a standard stimulus (Z-3-Hexen-1-ol). Odor compounds have been sorted according to their mean percentage amounts in *S. otites*. All monoterpenoids were used as stereoisomeric mixtures

have also been reported as pollinators of *S. otites* (Brantjes and Leemans 1976).

Our study showed that in the absence of visual stimuli, mosquitoes were attracted to male and female inflorescences of *S. otites* by scent only. The attractiveness of both sexes of this dioecious plant was similarly strong in bioassays, although female and male inflorescences differed with respect to the relative amounts of scent compounds. We found no significant differences in intensity or latency time of response to the inflorescence scents of six different populations. Therefore, different compound mixtures seem to have the same attractiveness.

Table 3 Multiple comparisons based on a GLM of antennal responses of male and female mosquitoes to different compounds and dilutions

Effect	*df*	MS	*F*	*P*
Intercept	1	1,181,681	11,128.31	<0.001
Sex	1	569	5.36	0.02
Dilution	4	92,933	875.18	<0.001
Compound	12	2,603	24.51	<0.001
Sex × dilution	4	87	0.82	0.511
Sex × compound	12	841	7.92	<0.001
Dilution × compound	48	960	9.05	<0.001
Sex × dilution × compound	48	135	1.27	0.116
Error	455	106		

Even within *S. otites* populations that showed low qualitative and semiquantitative scent variation, no positive relation between the total amount of scent emitted and the number of mosquitoes attracted was found. This finding is in contrast to the results of Bowen (1992) who found that behavioral response increased with stimulus concentration. Microclimatic conditions in the wind tunnel, such as temperature (which ranged from 20°C to 25°C), humidity, and atmospheric pressure, might have influenced the results obtained in this study (Grimstad and DeFoliart 1975). Furthermore, inflorescences might have emitted not only attractive compounds, but also compounds repellent to mosquitoes (Kessler and Baldwin 2007). The effect of repellency could increase with increasing concentration of these repellent compounds. Jhumur et al. (2006) found that the dominant odor compound of *S. otites*, phenyl acetaldehyde, attracted about 65% of *C. p. molestus*, whereas only about 50% of the mosquitoes were attracted to the entire *S. otites* inflorescence odor in our study. This finding supports the hypothesis that *S. otites* emits not only attractive, but also repellent compounds.

EAG studies were conducted to examine whether mosquitoes are able to detect components of the *S. otites* odor profile other than phenyl acetaldehyde. Mosquitoes responded to all tested compounds, and all may be involved in host-plant finding by mosquitoes. Bioassays are needed to test the behavioral response of *C. pipiens* to these compounds. Compounds tested in the EAG studies were representative of the floral scent composition of *S. otites*, accounting for 97% on average of all samples of this species. Both male and female mosquitoes detected all 13 floral scent compounds (including (*Z*)-3-hexen-1-ol) till 10^{-5} dilutions. Therefore, if some of these compounds also prove to be attractive, then they might be used as reliable cues for finding *S. otites*, and as long-range attractants by *C. pipiens*.

From this and previous studies, mosquitoes have been proven to detect or be attracted to 15 floral volatiles (Mauer and Rowley 1999; Howse 2003; Kline et al. 2003; Jhumur et al. 2006). It is interesting to note that the ranking of the EAG responses does not correlate with the dominance of the volatiles in floral scent profiles. For example, phenyl acetaldehyde elicited only weak responses in EAGs although it is the main compound (35% mean percentage amount) in the scent of *S. otites*. Furthermore, this compound was attractive to mosquitoes (Jhumur et al. 2006). On the other hand, the mean percentage amounts of linalool and linalool oxide (furanoid) were only 3% and 2%, respectively, but elicited the strongest EAG responses.

Several studies provide evidence that release of linalool oxide (furanoid) and linalool may reflect adaptations by plants to attract lepidopteran pollinators (Raguso et al. 1996; Raguso and Light 1998; Andersson et al. 2002; Andersson and Dobson 2003). Linalool also occurs in plants pollinated by bats, bees, flies, beetles, and wasps (Borg-Karlson et al. 1996; Raguso and Pichersky 1999). These monoterpenoids may also be important for attraction of mosquitoes and could explain the mixed pollinator guild found in *S. otites*, mainly moths and mosquitoes. Indeed, the attractiveness of linalool for mosquitoes was confirmed by Kline et al. (2003). In a dual-port olfactometer, more *Aedes aegypti* (L.) individuals were attracted by linalool than by a control. Although these two oxygenated monoterpenes are generally assumed to be pollinator attractants, Ômura et al. (2000) reported that linalool oxide (furanoid) acted as a weak deterrent in proboscis extension responses and a weak repellent in flower alighting tests with the cabbage butterfly *Pieris rapae* L., indicating that this compound can be repellent and attractive to insects. Bioassays are needed to determine the behavioral response to linalool oxide in mosquitoes.

Similarly to phenyl acetaldehyde, phenylethyl alcohol (3% mean percentage) elicited only weak EAG responses, although this compound may also be attractive to mosquitoes. Mauer and Rowley (1999) found that *C. pipiens* was attracted to the scent of the common milkweed *Asclepias syriaca* L., which is dominated by phenylethyl alcohol and benzyl alcohol. The authors assumed that these two benzenoids were responsible for the attraction of mosquitoes to *A. syriaca*, but they failed to attract mosquitoes in a dual-port olfactometer to a synthetic mixture of these two compounds.

It is interesting to note that in our study, *C. pipiens* also responded to the typical green leaf odors of *S. otites*, such as (*Z*)-3-hexenyl acetate. These compounds are not only released from several plant species in response to herbivory, but also serve as attractants for a variety of predatory and parasitic insects (see Röse et al. 1998; James 2005). In the natural environment, green leaf compounds are widespread and would not necessarily guide insects directly to flowers (Honda et al. 1998), although being directed to vegetation would certainly increase the probability of finding flowers.

In summary, floral scent compositions of *S. otites* populations from different geographical origin are highly variable, but nevertheless similarly attractive to *Culex pipiens molestus* mosquitoes. Mosquitoes can detect the most common and abundant scent compounds of *S. otites* inflorescences, but knowledge of the biological significance of most of the compounds is still lacking (e.g., attractant or repellent). By means of bioassays, we are presently evaluating the role of these compounds in the plant–pollinator interactions of *S. otites* and mosquitoes, which might lead to the development of new means of pest control and mosquito attractants and repellents.

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References

ADAMS, R. P. 1995. Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. Allured Publishing Corporation, Carol Stream, Illinois.

ANDERSSON, S., and DOBSON, H. E. M. 2003. Antennal responses to floral scents in the butterfly *Heliconius melpomene*. *J. Chem. Ecol* 29:2319–2330.

ANDERSSON, S., NILSSON, L. A., GROTH, I., and BERGSTRÖM, G. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Bot. J. Linn. Soc* 140:129–153.

AYASSE, M., SCHIESTL, F. P., Paulus, H. F., LÖFSTEDT, C., HANSSON, B., IBARRA, F., and FRANCKE, W. 2000. Evolution of reproductive strategies in the sexually deceptive orchid *Ophrys sphegodes*: How does flower-specific variation of odor signals influence reproductive success. *Evolution* 54:1995–2006.

AZUMA, H., TOYOTA, M., and ASAKAWA, Y. 2001. Intraspecific variation of floral scent chemistry in *Magnolia kobus* DC. (Magnoliaceae). *J. Plant Res* 114:411–422.

BENGTSSON, M. C., WITZGALL, P., KOBRO, S., JAASTAD, G., LOFQVIST, J. and LINDHE, C. 2007. Attractant for apple fruit moth and other insect pests of apple. United States Patent: 20070004686 A1.

BORG-KARLSON, A. K., UNELIUS, C. R., VALTEROVÁ, I., and NILSSON, L. A. 1996. Floral fragrance chemistry in the early flowering shrub *Daphne mezereum*. *Phytochemistry* 41:1477–1483.

BOWEN, M. F. 1992. Terpene sensitive receptors in female *Culex pipiens* mosquitoes: electrophysiology and behavior. *J. Insect Physiol* 38:759–764.

BRANTJES, N. B. M., and LEEMANS, J. A. A. M. 1976. *Silene otites* (Caryophyllaceae) pollinated by nocturnal Lepidoptera and mosquitoes. *Acta Bot. Neerl* 25:281–295.

CLARKE, K. R. 1993. Non-parametric multivariate analyses of changes in community structure. *Aust. J. Ecol* 18:117–143.

CLARKE, K. R., and WARWICK, R. M. 2001. Change in Marine Communities: An Approach to Statistical Analysis and Interpretation, 2nd edition. Primer-E, Plymouth.

CLARKE, K. R. and GORLEY, R. N. 2006. Primer v6: User Manual/Tutorial. Primer-E, Plymouth.1–91.

DÖTTERL, S., FÜSSEL, U., JÜRGENS, A., and AAS, G. 2005a. 1,4-Dimethoxybenzene, a floral scent compound in willows that attracts an oligolectic bee. *J. Chem. Ecol* 31:2993–2998.

DÖTTERL, S., WOLFE, L. M., and JÜRGENS, A. 2005b. Qualitative and quantitative analyses of flower scent in *Silene latifolia*. *Phytochemistry* 66:203–213.

DÖTTERL, S., BURKHARDT, D., WEIßBECKER, B., JÜRGENS, A., SCHÜTZ, S., and MOSANDL, A. 2006a. Linalool and lilac aldehyde/alcohol in flower scents. Electrophysiological detection of lilac aldehyde stereoisomers by a moth. *J. Chromatograph. A* 1113:231–238.

DÖTTERL, S., JÜRGENS, A., SEIFERT, K., LAUBE, T., WEIßBECKER, B., and SCHÜTZ, S. 2006b. Nursery pollination by a moth in *Silene latifolia*: the role of odours in eliciting antennal and behavioural responses. *New Phytol* 169:707–718.

DUDAREVA, N., and PICHERSKY, E. 2000. Biochemical and molecular genetic aspects of floral scents. *Plant Physiol* 122:627–633.

GADAWASKI, R., and SMITH, S. M. 1992. Nectar sources and age structure in a population of *Aedes provocans* (Diptera: Culicidae). *Entomol. Soc. Am* 29:876–886.

GRIMSTAD, P. R., and DEFOLIART, G. R. 1974. Nectar sources of Wisconsin mosquitoes. *J. Med. Entomol* 11:331–341.

GRIMSTAD, P. R., and DEFOLIART, G. R. 1975. Mosquito nectar feeding in Wisconsin in relation to twilight and microclimate. *J. Med. Entomol* 11:691–698.

HAEGER, J. S. 1955. The non-blood feeding habits of *Aedes taeniorhyndus* (Diptera, Culicidae) on Sanibel Island, Florida. *Mosq. News* 15:21–26.

HARADA, F., MORIYA, K., and YABE, T. 1971. Observations on the survival and longevity of the adult *Culex* mosquitoes fed on the flowers of some nectar plants. *Jpn. J. Sanit. Zool* 22:18–23.

HONDA, K., ÔMURA, H., and HAYASHI, N. 1998. Identification of floral volatiles from *Ligustrum japonicum* that stimulate flower visiting by cabbage butterfly, *Pieris rapae*. *J. Chem. Ecol* 24:2167–2180.

HOWSE, E. P. 2003. Insect attractant. European Patent Specification EP 0 838998 B1.

JAMES, D. G. 2005. Further field evaluation of synthetic herbivore-induced plant volatiles as attractants for beneficial insects. *J. Chem. Ecol* 31:481–495.

JHUMUR, U. S., DÖTTERL, S., and JÜRGENS, A. 2006. Naïve and conditioned responses of *Culex pipiens pipiens* biotype *molestus* (Diptera: Culicidae) to flower odors. *J. Med. Entomol* 43:1164–1170.

JÜRGENS, A., WITT, T., and GOTTSBERGER, G. 2002. Flower scent composition in night-flowering *Silene* species (Caryophyllaceae). *Biochem. Syst. Ecol* 30:383–397.

KESSLER, D., and BALDWIN, I. T. 2007. Making sense of nectar scents: the effects of nectar secondary metabolites on floral visitors of *Nicotiana attenuata*. *Plant J* 49:840–854.

KLINE, D. L., BERNIER, U. R., POSEY, K. H., and BARNARD, D. R. 2003. Olfactometric evaluation of spatial repellents for Aedes aegypti. *J. Med. Entomol* 40:463–467.

KLOWDEN, M. J. 1986. Effects of sugar deprivation on the host-seeking behavior of gravid Aedes aegypti mosquitoes. *J. Insect Physiol* 32:479–483.

KNUDSEN, J. T. 2002. Variation in floral scent composition within and between populations of *Geonoma macrostachys* (Arecaceae) in the western Amazon. *Am. J. Bot* 89:1772–1778.

KNUDSEN, J. T., ERIKSSON, R., GERSHENZON, J., and STÅHL, B. 2006. Diversity and distribution of floral scent. *Bot. Rev* 72:1–120.

MAGNARELLI, L. A. 1978. Nectar-feeding by female mosquitoes and its relation to follicular development and parity. *J. Med. Entomol* 14:527–530.

MANT, J., PEAKALL, R., and SCHIESTL, F. P. 2005. Does selection on floral odor promote differentiation among populations and species of the sexually deceptive orchid genus Ophrys. *Evolution* 59:1449–1463.

MAUER, D. J., and ROWLEY, W. A. 1999. Attraction of *Culex pipiens pipiens* (Diptera: Culicidae) to flower volatiles. *J. Med. Entomol* 36:503–507.

NAYAR, J. K., and SAURMAN, D. M. 1971. The effect of diet on life-span, fecundity and flight potential of female *Aedes taeniorhynchus* adults. *J. Med. Entomol* 8:506–513.

NAYAR, J. K., and SAURMAN, D. M. 1975. The effects of nutrition on survival and fecundity in Florida mosquitoes. Part 1. Utilization of sugar for survival. *J. Med. Entomol* 12:92–98.

ÔMURA, H., HONDA, K., and HAYASHI, N. 2000. Floral scent of *Osmanthus fragrans* discourages foraging behavior of cabbage butterfly, *Pieris rapae*. *J. Chem. Ecol* 26:655–666.

PLEPYS, D., IBARRA, F., FRANCKE, W., and LÖFSTEDT, C. 2002a. Odour-mediated nectar foraging in the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae): behavioural and electrophysiological responses to floral volatiles. *Oikos* 99:75–82.

PLEPYS, D., IBARRA, F., and LÖFSTEDT, C. 2002b. Volatiles from flowers of *Platanthera bifolia* (Orchidaceae) attractive to the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae). *Oikos* 99:69–74.

RAGUSO, R. A., and LIGHT, D. M. 1998. Electroantennogram responses of male *Sphinx perelegans* hawkmoths to floral and 'green-leaf volatiles. *Entomol. Exp. Appl* 86:287–293.

RAGUSO, R. A., and PICHERSKY, E. 1999. A day in the life of a linalool molecule: Chemical communication in a plant-pollinator system. Part 1: Linalool biosynthesis in flowering plants. *Plant Species Biol* 14:95–120.

RAGUSO, R. A., LIGHT, D. M., and PICKERSKY, E. 1996. Electroantennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *J. Chem. Ecol* 22:1735–1766.

ROJAS, J. C. 1999. Electrophysiological and behavioural responses of the cabbage moth to plant volatiles. *J. Chem. Ecol* 25:1867–1883.

RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1998. Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. *J. Chem. Ecol* 24:303–319.

SANDHOLM, H. A., and PRICE, R. D. 1962. Field observations on the nectar feeding habits of some Minnesota mosquitoes. *Mosq. News* 22:346–349.

SCHÜTZ, S., WEIßBECKER, B., KOCH, U. T., and HUMMEL, H. E. 1999. Detection of volatiles released by diseased potato tubers using a biosensor on the basis of intact insect antennae. *Biosens. Bioelectron* 14:221–228.

Statsoft, Inc. 2004. STATISTICA for Windows Version 7. www.statsoft.com.

STOUTAMIRE, W. P. 1968. Mosquito pollination of *Habenaria obtusata* (Orchidaceae). *Mich. Bot* 7:203–212.

SVENSSON, G. P., HICKMAN, M. O., BARTRAM, S., BOLAND, W., PELLMYR, O., and RAGUSO, R. A. 2005. Chemistry and geographic variation of floral scent in *Yucca filamentosa* (Agavaceae). *Am. J. Bot* 92:1624–1631.

TOLLSTEN, L., and BERGSTRÖM, G. 1993. Fragrance chemotypes of *Platanthera* (Orchidaceae): The result of adaptation to pollinating moths. *Nord. J. Bot* 13:607–613.

VARGO, A. M., and FOSTER, W. A. 1982. Responsiveness of female *Aedes aegypti* (Diptera, Culicidae) to flower extracts. *J. Med. Entomol* 19:710–718.

WHITTEN, W. M., and WILLIAMS, N. H. 1992. Floral fragrances of *Stanhopea* (Orchidaceae). *Lindleyana* 7:130–153.

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Effect of Long-Term Forest Fertilization on Scots Pine Xylem Quality and Wood Borer Performance

Juha Heijari · Anne-Marja Nerg · Pirjo Kainulainen · Uwe Noldt · Teuvo Levula · Hannu Raitio · Jarmo K. Holopainen

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Abstract We tested whether changes in long-term nutrient availability would affect the xylem quality and characteristics of Scots pine trees as a food source for the larvae of the xylophagous wood borer *Hylotrupes bajulus* L. (Cerambycidae). We looked for an effect of host plant growth and xylem structural traits on *H. bajulus* larval performance, and looked for delayed effects of long-term forest fertilization on xylem chemical quality. In general, larval performance was dependent on larval developmental stage. However, the growth of larvae also varied with host plant quality (increases in the concentration of nitrogen and carbon-based secondary compounds of xylem were correlated with a decrease in the larval growth rate). The greater annual growth of trees reduced tracheid length and correlated positively with second-instar *H. bajulus* growth rate. This is consistent with the hypothesis that intrinsic growth patterns of host plants influence the development of the xylophagous wood borer *H. bajulus.*

J. Heijari (✉) · A.-M. Nerg · P. Kainulainen · J. K. Holopainen
Department of Environmental Science, University of Kuopio,
P.O. Box 1627, 70211 Kuopio, Finland
e-mail: Juha.Heijari@uku.fi

H. Raitio
The Finnish Forest Research Institute, Helsinki Unit,
Unioninkatu 40A,
00170 Helsinki, Finland

U. Noldt
Bundesforschungsanstalt für Forst- und Holzwirtschaft,
Institut für Holzbiologie und Holzschutz,
Postfach 800209,
21002 Hamburg, Germany

T. Levula
Parkano Research Station, The Finnish Forest Research Institute,
39700 Parkano, Finland

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Introduction

Wood-boring beetles (Coleoptera: Cerambycidae) are predominantly xylophagous insects and are commonly referred to as long-horned beetles when they are adults and as round-headed borers when they are larvae (Linsley 1959). They attack the xylem of host plants in a variety of conditions ranging from living trees to dead decaying wood (Linsley 1959; Hanks 1999). Since they have adapted to such highly variable hosts, wood borer species have tremendous diversity, and many of these species are serious pests of forest trees. The larvae of the old house borer, *Hylotrupes bajulus* L. (Coleoptera: Cerambycidae) feed on the sapwood of dying or dead host species such as pine, spruce, or fir in both natural and managed forests as well as construction timber used in buildings. *H. bajulus* larvae may consume heartwood only if the sapwood has been destroyed by the larvae (Robinson and Cannon 1979). The larvae spend most of their lifetime (2–12 years) inside the trunk consuming the xylem tissue. Although the taxonomy, species distributions, and larval host ranges of long-horned beetles have been extensively studied, little is known about their chemical ecology in relation to growth and survival (Ljungkvist 1983; Allison et al. 2004).

Nitrogen is a vital component of the diet of insects, and the levels of nitrogenous compounds can influence the interactions between herbivorous insects and their host plants (Mattson 1980; Holopainen et al. 1995). In forestry, nitrogen fertilizers are used to enhance timber production,

but much less attention has been paid to the effects of fertilizers on the characteristics of xylem and on the performance of the xylophagous insects that attack woody plants (Kytö et al. 1996). The modification of plant resource balances by fertilization treatments might change the structure of the wood, e.g., tracheid properties (Mäkinen et al. 2002), as well as have effects on mechanical properties of xylem (Lucas et al. 2000). This, in turn, might have an impact on the performance of herbivores. These are all topics that need clarification. It is known that the carbon-based secondary compounds (e.g., terpenes and resin acids) in conifers deter insect pests and fungal pathogens (Gershenzon and Croteau 1991; Nerg et al. 2004). Xylem resin reduces the feeding activity of *H. bajulus* larvae (Holm and Ekbom 1958). This study examined the effects of a long-term increase in nutrient availability on tree growth, tracheid properties, the nutritional value of xylem tissue, and the growth of wood-boring *H. bajulus* L. larvae.

Methods and Materials

Site Description and Treatments The Scots pine (*Pinus sylvestris* L.) trees used in this study were part of an earlier experiment established by the Finnish Forest Research Institute in which several experimental sites in Finland were set up in the 1950s to investigate the effects of forest fertilization on tree growth. The experimental sites used were SITE1—Padasjoki (61°23′N, 25°3′E), SITE2—Vilppula (62°4′N, 24°29′E), and SITE3—Punkaharju (61°40′N, 29°18′E) (Turtola et al. 2002). Three fertilization treatments were applied to the plots at each site: 1) plots with no fertilization (control), 2) plots fertilized with nitrogen (N), and 3) plots fertilized simultaneously with calcium, nitrogen, and phosphorus (CaNP). There was one plot per treatment at each site, as individual trees were considered to act as replicates in these forestry experiments designed in the 1950s. The size of each plot was 40×40 m, with a 30×30 m area delimited for tree felling centered in each plot. Fertilization treatments (year of fertilization) were initiated in SITE1 when the trees were 12 yr old: N (1958, 1963, 1968, 1973, 1978, 1983, 1988, 1993, 1998); Ca (1959, 1978); P (1958, 1978, 1993); SITE2 when trees were 12 yr old: N (1959, 1964, 1969, 1974, 1979, 1984, 1989, 1994); Ca (1959, 1979); P (1959, 1979, 1994); SITE3 when trees were 14 yr old: N (1959, 1969, 1979, 1989); Ca (1959, 1979); P (1959, 1979). Fertilization treatments: N=82 kg ha^{-1} of nitrogen applied as ammonium sulfate in 1958–1959, 92 kg ha^{-1} as urea in 1963–1969, and 150–180 kg ha^{-1} as ammonium nitrate in 1973–1998; Ca=calcium applied as limestone, first at 2000 kg ha^{-1}, and later at 4000 kg ha^{-1}; P=phosphorus, initially applied at 29 kg ha^{-1} as finely ground rock phosphate, and subsequently at 40 kg ha^{-1} as superphosphate.

Sampling All trees from the 30×30 m area were divided into three groups according to their basal area. From each of these groups, two trees were randomly selected for felling. In April 2000, 18 trees (six per treatment) from each site were harvested. For chemical and xylem property analysis, consecutive xylem disks (about 2 cm thick) with bark from breast height (H1; 130 cm from ground level) and canopy height (H2; 0.7 × tree height) were sawed. The xylem disks were frozen and stored at −20°C before analysis. All xylem used in the following analysis was taken from the sapwood. The analyses of nutrient and tracheid properties were performed on xylem disks from both H1 and H2, while for secondary compound analysis, only xylem disks from H1 were used. To examine the growth performance tests of *H. bajulus* L. larvae, an additional 20-cm-long xylem disk was sawed from each tree, just above the disks taken from H1 and H2 for chemical and xylem property analysis. The sapwood of these xylem disks was sawed into blocks 80×40×20 mm and air-dried at room temperature before use in the insect performance tests. The sapwood diameter from the latest 20 annual rings was measured.

Tracheid Properties At H1, tracheid length was measured from annual ring numbers 12, 30, and 40 from the pith outwards. The annual ring number 12 was at the border of the sapwood and heartwood. At H2, tracheid length was measured from annual ring numbers 5, 10, and 12 from the pith outwards. Early- and latewood samples were separated from these annual rings and macerated with glacial acetic acid/hydrogen peroxide (1:1, *v*/*v*) at 60°C overnight. The suspension was washed with distilled water and stained with safranin (1%, 10 sec). Tracheids were placed on glass slides, and 50 unbroken tracheids were measured with a light microscope.

Chemical Analysis A 0.125-g (dry weight) powdered xylem sample was weighed for the nitrogen (N) analysis. Nitrogen was analyzed with a LECO CHN 2000 analyzer (USA). The analyzer was calibrated with EDTA (containing 40.97 % carbon, 9.57 % nitrogen, and 5.48 % hydrogen) as a standard with every eighth sample being a pine control sample. The performance of the apparatus was monitored once a day with certified reference samples (BRC 101 and NIST 1547). The total nitrogen content was calculated as mg g^{-1} dry weight. Other xylem characteristics, such as, cellulose, hemicellulose, lignin, and starch were measured (Heijari et al. 2005), but these parameters did not have any significant effect on *H. bajulus* performance (data not

Table 1 Analysis of variance of the main effects of site, fertilization treatments and sampling height and their interactions on the relative growth rate (RGR) of neonatal (i1l), second- (i2l), and third instar *hylotrupes bajulus* larvaE (I3L)

Between-subject effects	RGR		
	I1L	I2L	I3L
Site	$F_{2,\ 44}$=0.005 P=0.995	$F_{2,\ 101}$=5.131 P=0.008	$F_{2,\ 79}$=0.977 P=0.382
Fertilization	$F_{2,\ 44}$=2.000 P=0.151	$F_{2,\ 101}$=0.583 P=0.561	$F_{2,\ 79}$=0.308 P=0.736
Height	$F_{2,\ 44}$=3.394 P=0.074	$F_{2,\ 101}$=35.923 P<0.001	$F_{2,\ 79}$=9.131 P=0.004
Site × Fertilization	$F_{2,\ 44}$=0.991 P=0.408	$F_{2,\ 101}$=2.594 P=0.042	$F_{2,\ 79}$=0.778 P=0.544
Site × Height	$F_{2,\ 44}$=0.263 P=0.611	$F_{2,\ 101}$=1.363 P=0.261	$F_{2,\ 79}$=1.875 P=0.162
Fertilization × Height	$F_{2,\ 44}$=0.571 P=0.570	$F_{2,\ 101}$=0.372 P=0.690	$F_{2,\ 79}$=0.403 P=0.607
Site × Fertilization × Height	n.c.	$F_{2,\ 101}$=1.262 P=0.291	$F_{2,\ 79}$=0.675 P=0.612

[a] n.c.=not calculable

shown). In the monoterpene analysis, sapwood samples (two replicate wood pieces) were extracted with *n*-hexane as described earlier by Manninen et al. (2002). Wood resin acids were extracted from freeze-dried and powdered xylem samples (two replicate wood pieces) with petroleum ether-diethyl ether. Monoterpene and resin acid extracts were analyzed by gas chromatography-mass spectrometry (Hewlett Packard GC type 6890, MSD 5973) using a 30-m-long HP-5MS (0.25 mm ID, 0.25-µm film thickness, Hewlett Packard) capillary column (Manninen et al. 2002). Helium was used as the carrier gas. The temperature program for monoterpenes rose from 50°C to 250°C, and for resin acids from 50°C to 270°C. The heating rate was 5°C min^{-1}. The SCAN technique (mass numbers from *m*/*z* 30 to 350 were recorded; signal ions in monitoring; 93, 133, 136, 161, 204 *m*/*z*) was used for monoterpene samples, and the technique of selected ion monitoring (SIM) 299, 301, 314, 316 *m*/*z* for resin acid samples. For quantification of resin acids and terpenes, calibrations were made from known amounts of available pure compounds relative to known amounts of the internal standard (1-chloro-octane for monoterpenes and heptadecanoic acid for resin acids).

Larval Performance Laboratory grown *Hylotrupes bajulus* L. larvae were pre-grown on protein-yeast enriched pine sapwood to accelerate growth. The growth conditions were 27°C and 70% relative humidity (RH) in darkness. Adults and larvae were reared as described by Berry (1972) and the European Standard EN 47 (1988).

Two sawed Scots pine xylem blocks, from both H1 and H2, were placed against each other and held together with an elastic band. A groove (45×15×10 mm) was gouged into the point of contact. The double xylem block system was weighed, and one randomly selected second- (ca. 200 mg) or third-instar (ca. 355 mg) *H. bajulus* was enclosed into each groove under a glass plate, which was tightened by an elastic band to prevent the larvae from escaping. The double xylem block system (four replicates per tree per sampling height) with larvae were enclosed inside plastic boxes (volume 750 ml). The larvae were allowed to feed for 127 d in a dark culture room at 21°C and 60% RH. The mortality of the whole larval population feeding on xylem taken from H1 and H2 was 8.6% and 19.3% for I2L and 3.8% and 4.5% for I3L, with no statistical differences between sampling heights or instars (data not shown). After feeding, the relative growth rate (RGR) for the living larvae was calculated ($RGR = (\ln W_2 - \ln W_1) / (t_1 - t_2)$, where W_1 and W_2 were the fresh biomass at the beginning (t_1) and end (t_2) of the sampling period, and *ln* is the natural logarithm; Waldbauer 1964).

After the feeding tests, larvae were placed on the Scots pine wood to complete their development. Emerging adults were allowed to copulate. Females were allowed to lay eggs in Petri dishes with Scots pine wood disks, and the eggs hatched after about 9 d. The neonatal larvae (I1L), mean weight 0.24 mg) were transferred after hatching into a 5-mm deep hole. The hole was drilled into the top of the 50×20×20 mm size Scots pine xylem blocks taken from the trees of different fertilization treatments and sites described above, both at H1 and H2. Progenies of each female were randomized among the treatments. Larvae were allowed to feed for 194 d in a dark culture room at approximately 25°C and 85% RH. The relative growth rate for the I1L larvae was calculated as described.

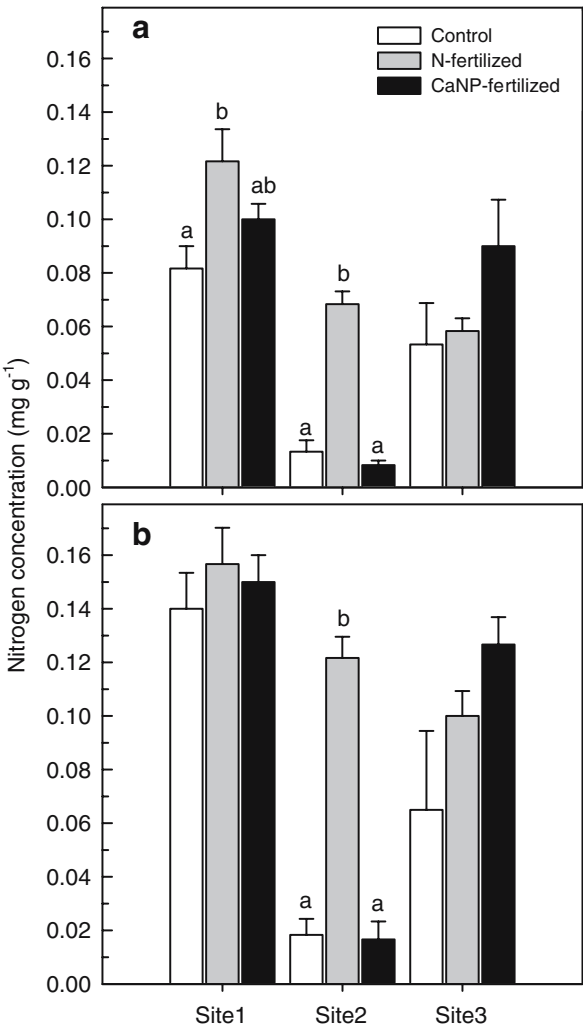

Fig. 1 The concentration of nitrogen (mean + SE) in Scots pine sapwood at breast- (H1; **a**) and canopy height (H2; **b**) in three experimental sites and fertilization treatments (control=no fertilization, N-fertilized=fertilization with nitrogen, CaNP-fertilized= fertilized with calcium, nitrogen and phosphorous). Means indicated with different letters within sites are significantly different according to ANOVA, $P<0.05$, $N=6$

Statistical Analyses The effect of fertilization treatments at each site was analyzed by analysis of variance (ANOVA) (GLM Univariate) and followed by the Tukey multiple range test (significance level $P<0.05$). *T* test (*t*) (or Chi-Square for larval mortality) was used when differences between sampling heights were tested. The relative growth rate (RGR) of *H. bajulus* larvae was calculated separately for the different larval stages. Correlation coefficients (*r*) between RGR and consumed xylem with different xylem properties were tested by Pearson correlations. Statistical analyses were carried out with SPSS 11.5.1 for Windows statistical software package.

Results and Discussion

It is known that nitrogen in plant tissue can positively affect host utilization by insects (Mattson 1980; Holopainen et al. 1995). In our study, *H. bajulus* larval performance was similar in wood taken from fertilized and non-fertilized trees (Table 1). Interestingly, we found that for 2nd- and 3rd-instar *H. bajulus*, RGR was lower for xylem from canopy height than that found in xylem collected from breast height SITE2 ($t_{10}=-2.343$, $P=0.041$ and $t_8=-2.893$, $P=0.020$) and SITE3 ($t_{10}=-2.884$, $P=0.018$ and $t_9=-4.482$, $P=0.002$). Conversely, the mean (± SD) final weight of neonatal larvae was marginally lower 0.58± 0.16 mg at H1 compared to 0.72±0.31 mg at H2 (Table 1). Similarly, Körting (1972) observed that neonatal larvae of *H. bajulus* had a three-fold higher growth rate on Scots pine canopy height xylem than at breast height xylem. The differences in neonatal larval growth rates at H1 and H2 may be a consequence of the within-tree variation in nitrogen concentration. As a consequence of its higher nitrogen concentrations, canopy height xylem may be a superior food source for neonatal *H. bajulus* larvae. The nitrogen concentration was significantly lower at H1 than at H2 in SITE1 in control ($t_{10}=-3.693$, $P=0.004$) and CaNP-fertilized trees ($t_{10}=-4.330$, $P=0.001$) and in SITE2 ($t_{10}=-5.766$, $P<0.001$) and SITE3 ($t_{10}=-3.983$, $P=0.003$) in N-fertilized trees (Fig. 1). Addition of nitrogen to Scots pine (*Pinus sylvestris* L.) plots increased the xylem nitrogen concentration (Fig. 1). This observed increase in nitrogen concentration is consistent with earlier studies (Helmisaari and Siltala 1989; Finér and Kaunisto 2000). We propose that for late instars, the nitrogen content of food is not as essential in the maintenance of daily metabolism as it is for neonatal larvae that are increasing their body mass.

Interestingly, we found that the performance of wood-boring *Hylotrupes bajulus* larvae was explained by the tree radial diameter; tree diameter correlated positively with the RGR of I2L in SITE2 ($r=0.609$, $P<0.001$, $N=34$) and in SITE3 ($r=0.430$, $P=0.010$, $N=35$). These results agree with Hanks et al. (2005) who reported that larvae of *P. semipunctata* (Cerambycidae) increased their body size more in large circumference eucalyptus host trees than in small trees. Furthermore, Ruel and Whitham (2002) found that pinyon pines (*Pinus edulis*) that had grown vigorously during the juvenile stage suffered greater herbivory when these trees matured and produced smaller growth rings. On the whole, these results provide evidence that some long-living wood-boring larvae may perform better in large host trees than in slow-growing trees. Future studies should examine larval performance in host trees undergoing different intrinsic growth patterns (e.g., provenance experiments).

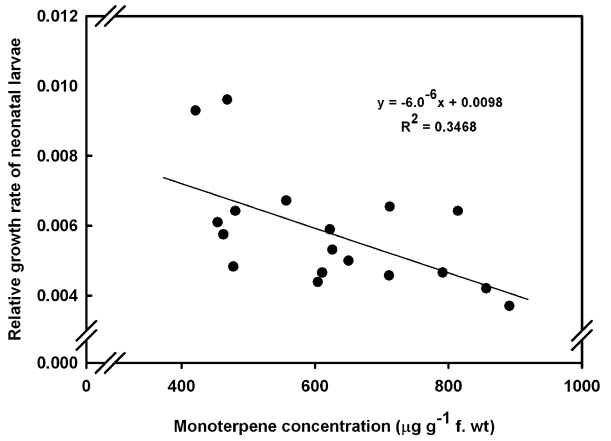

Fig. 2 Correlation between relative growth rate of neonatal *H. bajulus* larvae during 194-d feeding and total monoterpene concentration (μg g^{-1} fresh weight) at breast height of Scots pine sapwood in control trees. $P<0.05$, $N=18$

Although, the differences in the trunk diameter and volume of trees from fertilized and non-fertilized plots were significant (see Turtola et al. 2002; Heijari et al. 2005), fertilization treatments did not cause any changes in carbon allocation to secondary compounds in the xylem. Total resin acid concentration per volume of xylem (mean ± SE) in SITE3 trees (3.8±0.5 mg cm^{-3} dry weight) was significantly ($F_{2,\ 15}=7.568$, $P=0.005$) higher than in SITE2 trees (2.5±0.2 mg cm^{-3} dry weight) and also marginally ($P=0.061$) higher than in SITE1 trees (2.7±0.4 mg cm^{-3} dry weight). We observed that the total monoterpene concentration of sapwood had a negative effect on the growth rate of neonatal larvae (Fig. 2), whereas the concentration of resin acids did not affect larval growth. This result is consistent with earlier studies, which indicate that specific constitutive defense chemicals have effects on the performance of *H. bajulus* neonatal larvae and that early instars are more sensitive to host plant quality than late instars (Montgomery 1982), with high mortality occurring generally among early instars (Preszler and Price 1988). Furthermore, in SITE3, the generally lower growth and smaller diameter of the trees might be the reason for the high content of resin acids per volume of xylem. The inconsistent results among sites may also be caused by the fact that all of the sites were *Myrtillus* type forests, and thus might have been more nutrient-rich than the pines generally grown in Finland (Saarsalmi and Mälkönen 2001). Our study showed that the effect of fertilization on xylem constitutive defense was not significant. The genetic structure of the investigated stands might have differed substantially, and thus it is unlikely that the results would have differed in other tree-growing areas in Finland. Concomitantly, our study provides support for the conclusion of Herms (2002), who proposed that in general, fertilization treatments neither enhance nor reduce pest resistance in woody plants.

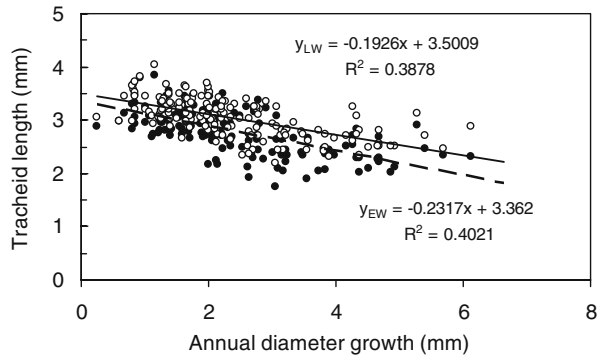

Fig. 3 Linear correlation between tracheid length (mm) and annual diameter growth (mm) at breast height in early (closed circles) and latewood (open circles) of Scots pine. Data (pooled from all sites) are from 12th, 30th, and 40th annual rings of xylem. Equations for relationships and R^2 are shown (y_{EW}=earlywood and y_{LW}=latewood), $P<0.05$

At the breast (H1) and canopy height (H2), the fertilization treatments had no significant effects on tracheid length (data not shown). In SITE3, tracheids in xylem collected from H1 and H2 were significantly ($F_{2,\ 15}>5.141$, $P<0.05$ and $F_{2,\ 15}>4.915$, $P<0.023$) longer than in trees growing at the other sites. In the pooled data, there was a negative correlation between the growth of annual rings and tracheid length (Fig. 3), but this was not associated with the fertilization treatments. Similarly, a fertilization experiment with Norway spruce (*Picea abies*) observed decreased tracheid length (Mäkinen et al. 2002). Lucas et al. (2000) detected differences between the mechanical properties of xylem, and this in turn had an impact on the performance of herbivores. We found that the RGR of I2L exhibited a significant positive correlation ($r=0.359$, $P=0.009$, $N=53$) with the latewood tracheid length, and xylem consumption of I3L showed a positive correlation ($r>0.542$, $P<0.030$, $N=16$) with both early- and latewood breast height tracheid lengths. These results indicate that cell properties could influence the growth of late instar larvae, but not the growth of the neonatal larvae. Furthermore, xylem cell properties may explain some of the differences occurring in growth performance of xylophagous *H. bajulus* larvae.

In summary, the nitrogen concentration of xylem had a positive effect on wood borer performance, but the impact was stronger on early instar than on late instar *H. bajulus* larvae. It is likely that fertilization treatments induce changes in the growth and nutrient concentrations of xylem, but that these do not lead to any major changes in tracheid properties or in constitutive defense level of xylem. The effect of long-term fertilization on the performance of wood-boring xylophagous *H. bajulus* larvae was notable,

and our results suggest that variation in the growth of Scots pine forests can have effects on the utilization of xylem by *H. bajulus* larvae.

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References

ALLISON, J. D., BORDEN, J. H., and SEYBOLD, S. J. 2004. A review of the chemical ecology of the Cerambycidae (Coleoptera). *Chemoecology* 14:123–150.

BERRY, R. W. 1972. A rearing procedure for the house longhorn beetle *Hylotrupes bajulus* L. *Int. Biodeterior. Biodegrad.* 8:141–144.

European Standard EN 47. 1988. Wood preservatives; determination of the toxic values against larvae of *Hylotrupes bajulus* (Linnaeus) (Laboratory method). European Committee for Standardization, Brussels.

FINÉR, L., and KAUNISTO, S. 2000. Variation in stemwood nutrient concentrations in Scots pine growing on peatland. *Scand. J. Forest Res.* 15:424–432.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, in G. A. Rosenthal and M. R. Berenbaum (eds.). Herbivores, their interactions with secondary plant metabolites. Academic Press, New York.

HANKS, L. M. 1999. Influence of the larval host plant on reproductive strategies of Cerambycid beetles. *Annu. Rev. Entomol.* 44:483–505.

HANKS, L. M., PAINE, T. D., and MILLAR, J. G. 2005. Influence of the larval environment on performance and adult body size of the wood-boring beetle *Phoracantha semipunctata*. *Entomol. Exp. Appl.* 114:25–34.

HEIJARI, J., NERG, A.-M., KAAKINEN, S., VAPAAVUORI, E., RAITIO, H., LEVULA, T., VIITANEN, H., HOLOPAINEN, J. K., and KAINULAINEN, P. 2005. Resistance of Scots pine wood to Brown-rot fungi after long-term forest fertilization. *Trees* 19:729–735.

HELMISAARI, H.-S., and SILTALA, T. 1989. Variation in nutrient concentration of *Pinus sylvestris* stems. *Scand. J. Forest Res.* 4:443–451.

HERMS, D. A. 2002. Effects of fertilization on insect resistance of woody ornamental plants: Reassessing an entrenched paradigm. *Environ. Entomol.* 31:923–933.

HOLM, C., and EKBOM, P. 1958. The significance of house longhorn beetle as a destroyer of buildings and its control. State Institute of Technical Research, Helsinki.

HOLOPAINEN, J. K., RIKALA, R., KAINULAINEN, P., and OKSANEN, J. 1995. Resource partitioning to growth, storage and defence in nitrogen fertilized Scots pine and susceptibility of the seedlings to the tarnished plant bug *Lygus rugulipennis*. *New Phytol.* 131:521–532.

KÖRTING, A. 1972. Über Wachstumsunterschiede von Eilarven des Hausbockes (*Hylotrupes bajulus* L.) bei Aufzucht in den rindennahen Holzzonen von Kiefernstämmen. *Z. Angew. Entomol.* 72:149–156.

KYTÖ, M., NIEMELÄ, P., and LARSSON, S. 1996. Insects on trees: population and individual response to fertilization. *Oikos* 75:148–159.

LINSLEY, E. G. 1959. Ecology of Cerambycidae. *Annu. Rev. Entomol.* 4:99–138.

LJUNGKVIST, H. 1983. The discovery of the house longhorn beetle in a natural biotope. *Entomol Tidskr* 104:35.

LUCAS, P. W., TURNER, I. M., DOMINY, N. J., and YAMASHITA, N. 2000. Mechanical Defence to Herbivory. *Annu. Bot. (Lond.)* 86:913–920.

MÄKINEN, H., SARANPÄÄ, P., and LINDER, S. 2002. Effect of growth rate on fibre characteristics in Norway spruce (*Picea abies* (L.) Karst.). *Holzforschung* 56:449–460.

MANNINEN, A.-M., TARHANEN, S., VUORINEN, M., and KAINULAINEN, P. 2002. Comparing the variation of needle and wood terpenoids in Scots pine provenances. *J. Chem. Ecol.* 28:211–228.

MATTSON, W. J. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MONTGOMERY, M. E. 1982. Life-cycle nitrogen budget for the gypsy moth, *Lymantria dispar*, reared on artificial diet. *J. Insect Physiol.* 28:437–442.

NERG, A.-M., HEIJARI, J., NOLDT, U., VIITANEN, H., VUORINEN, M., KAINULAINEN, P., and HOLOPAINEN, J. K. 2004. Significance of wood terpenoids in the resistance of Scots pine provenances against the old house borer, *Hylotrupes bajulus*, and brown-rot fungus, *Coniophora puteana*. *J. Chem. Ecol.* 30:125–142.

PRESZLER, R. W., and PRICE, P. W. 1988. Host quality and sawfly populations: a new approach to life table analysis. *Ecology* 69:2012–2020.

ROBINSON, W. H., and CANNON, K. F. 1979. The life history and habits of the old house borer, *Hylotrupes bajulus* (L.), and its distribution in Pennsylvania. *Melsheimer Entomol. Ser.* 27:30–34.

RUEL, J., and WHITHAM, T. G. 2002. Fast-growing juvenile pinyons suffer greater herbivory when mature. *Ecology* 83:2691–2699.

SAARSALMI, A., and MÄLKÖNEN, E. 2001. Forest fertilization research in Finland: A literature review. *Scand. J. Forest Res.* 16:514–535.

TURTOLA, S., MANNINEN, A.-M., HOLOPAINEN, J. K., LEVULA, T., RAITIO, H., and KAINULAINEN, P. 2002. Secondary metabolite concentrations and terpene emissions of Scots pine xylem after long-term forest fertilization. *J. Environ. Qual.* 31:1694–1701.

WALDBAUER, G. P. 1964. The consumption, digestion and utilization of solanaceous and non-solanaceous plants by larvae of the tobacco hornworm, *Protoparce sexta* (Johan). (Lepidoptera: Sphingidae). *Entomol. Exp. Appl.* 7:253–269.

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Relationship Between the Endophyte *Embellisia* spp. and the Toxic Alkaloid Swainsonine in Major Locoweed Species (*Astragalus* and *Oxytropis)*

M. H. Ralphs · R. Creamer · D. Baucom · D. R. Gardner · S. L. Welsh · J. D. Graham · C. Hart · D. Cook · B. L. Stegelmeier

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Abstract Locoweeds (*Astragalus* and *Oxytropis* spp. that contain the toxic alkaloid swainsonine) cause widespread poisoning of livestock on western rangelands. There are 354 species of *Astragalus* and 22 species of *Oxytropis* in the US and Canada. Recently, a fungal endophyte, *Embellisia* spp., was isolated from *Astragalus* and *Oxytropis* spp. and shown to produce swainsonine. We conducted a survey of the major locoweeds from areas where locoweed poisoning has occurred to verify the presence of the endophyte and to relate endophyte infection with swainsonine concentrations. Species found to contain the fungal endophyte and produce substantial amounts of swainsonine were *A. wootoni*, *A. pubentissimus*, *A. mollissimus*, *A. lentiginosus*, and *O. sericea. Astragalus* species generally had higher concentrations of swainsonine than *Oxytropis.* Swainsonine was not detected in *A. alpinus*, *A. cibarius*, *A. coltonii*, *A. filipes*, or *O. campestris*. The endophyte could not be cultured from *A. mollissimus* var. *thompsonii* or *A. amphioxys*, but was detected by polymerase chain reaction, and only 30% of these samples contained trace levels of swainsonine. Further research is necessary to determine if the endophyte is able to colonize these and other species of *Astragalus* and *Oxytropis* and determine environmental influences on its growth and synthesis of swainsonine.

Keywords Locoweed · *Astragalus wootoni* · *Astragalus pubentissimus* · *Astragalus mollissimus* · *Astragalus lentiginosus* · *Oxytropis sericea* · Swainsonine · Poisonous plant · Livestock poisoning

M. H. Ralphs (✉) · D. R. Gardner · D. Cook · B. L. Stegelmeier
USDA/ARS Poisonous Plant Research Laboratory,
1150 E. 1400 N.,
Logan, UT 84341, USA
e-mail: Michael.Ralphs@ars.usda.gov

R. Creamer · D. Baucom
Department Entomology, Plant Pathology and Weed Science,
New Mexico State University,
Las Cruces, NM 88003, USA

J. D. Graham
New Mexico State University,
Extension Service,
Clayton, NM 88415, USA

S. L. Welsh
Bean Natural History Museum, Brigham Young University,
Provo, UT 84602, USA

C. Hart
Texas A&M University Research and Extension Center,
Stephenville, TX 76401, USA

Introduction

Historically, locoweeds (*Astragalus* and *Oxytropis* spp. that contain the toxic alkaloid swainsonine) have caused widespread livestock losses on western rangelands (Kingsbury 1964), and they remain a major impediment to livestock production (Ralphs et al. 2002a). There are 354 species of *Astragalus* and 22 species of *Oxytropis* in the US and Canada (Welsh et al. 2007a), and *Astragalus* and *Oxytropis* plants occur in every major plant community on western rangelands. Only 24 species in North America have been verified to contain swainsonine or have a history of causing locoism (Ralphs et al. 2002a).

Recently, a fungal endophyte, *Embellisia* spp. Pleosporaceae, was isolated from *Astragalus* and *Oxytropis* spp. and

shown to produce swainsonine (Braun et al. 2003). Creamer et al. (2007) summarized recent research on *Embellisia* in locoweeds and its synthesis of swainsonine. The fungus was isolated from the stems, leaves, flowers, and seed of locoweed plants (Braun et al. 2003) and was shown to be solely responsible for the synthesis of swainsonine, and thus the toxicity of the plant (McLain-Romero et al. 2004a). Fungal isolates from *Oxytropis* species were similar, but those from *Astragalus* species were genetically and morphologically diverse (Belfon and Creamer 2003), suggesting they may be different species or subspecies. The fungus is passed to the next generation through the seed coat (Romero et al. 2002), and when the seed coat is removed and the embryo germinated alone, the plants are fungus- and swainsonine-free (McLain-Romero et al. 2004b). Environmental stresses, such as moderate water stress and a decrease in pH, appear to increase swainsonine concentration in *Embellisia* cultures (Oldrup 2005). An increase of the temperature or nutrient deficiency (N, P, K) did not influence swainsonine concentration. Gardner et al. (2004) reported a strong correlation (r=0.92) between swainsonine concentration and presence of the endophyte in plant parts.

There is a need to verify both the endophyte and its synthesis of swainsonine in major locoweed species. Previous studies have shown variability in the ability to culture the endophyte and detect swainsonine among varieties, populations, and individual plants. Of the three varieties of *O. lambertii*, the endophyte was cultured and swainsonine was measured only in var. *bigelovii*, and then only in its populations in the southwest U.S. (Ralphs et al. 2002b). Gardner et al. (2001) reported there were some individual plants within all surveyed populations of both *O. sericea* and *O. lambertii* where swainsonine concentration was below the level of detection, and that plant-to-plant variation in swainsonine concentration was the greatest source of variability in the statistical model.

The objective of this study was to collect the major locoweeds from areas where locoweed poisoning has occurred, verify the presence of the endophyte, and relate endophyte infection with swainsonine concentrations. Other *Astragalus* and *Oxytropis* species encountered in the survey were harvested for a random check of the endophyte in nonlocoweed species.

Methods and Materials

Collection and preparation of samples Locoweed species, varieties, and populations, along with locations are presented in Table 1. All plants at all locations were collected during the flower stage of growth during the spring and summer 2005. Five or 10 plants were collected at each location, and each plant was divided into three subsamples, each containing leaves, stems, and flowers. One subsample was pressed to quickly dry the intact plant for subsequent isolation and culturing of the endophyte. The other two subsamples were frozen immediately on dry ice. One frozen sample was used for polymerase chain reaction (PCR) detection of the endophyte, and the other was later freeze-dried and retained for swainsonine analysis. Two representative plants were pressed at each location; one for positive identification and a voucher specimen that is archived at the S.L. Welsh Herbarium in the Monte L. Bean Life Science Museum, Brigham Young University, Provo, UT, USA, and the other is archived at the Poisonous Plant Research Laboratory herbarium in Logan, UT, USA.

Swainsonine assay Samples were analyzed for the toxin swainsonine by the procedures described by Gardner et al. (2001). In brief, samples were freeze-dried, all parts were ground together, and 100 mg of plant material was extracted with chloroform and acetic acid. The acetic acid portion was passed through a cation exchange resin to retain the swainsonine, which was subsequently removed with a weak ammonium hydroxide solution. An aliquot of the final extract was quantitatively analyzed for swainsonine by liquid chromatography–mass spectrometry. The detection limit of swainsonine was 0.001% of dry weight. In samples with a low swainsonine concentration near the detection limit, the presence in the sample was confirmed by a second assay of the extract by using gas chromatography/mass spectrometry (GC/MS; Gardner et al. 2001). For GC/MS analysis, a 1.0-ml aliquot of the sample extract was evaporated to dryness under a flow of nitrogen at 60°C. To the dried sample was added 0.200 ml of *N*,*O*,-bis (trimethylsilyl) trifluoroacetamide with 1% trimethylchlorosilane (Pierce Chemical) and heated for 30 min at 60°C. The samples were then transferred to a 400-µl glass insert for the GC auto sample vial and analyzed by GC/MS (2 µl injection). Detection of swainsonine was confirmed by comparison of GC retention time and mass spectrum to that of a standard swainsonine sample $(TMS)_3$ derivative.

PCR detection of endophyte Frozen plant specimens were used for the detection of the endophyte with PCR. For each plant, 0.3 g of the frozen tissue was ground with liquid nitrogen. Following the protocol for the DNEasy Plant Mini kit (Qiagen, Valencia, CA, USA), DNA was extracted from the ground samples. The PCR reaction mixture contained a final concentration of 400 µM dNTP's (Promega, Madison, WI, USA), 3 mM $MgCl_2$, 0.25 µM each primer, 1.25 µ GoTaq Flexi DNA Polymerase (Promega, Madison, WI, USA), and 5X Green GoTaq Flexi buffer supplied with the enzyme. Sterile water and 5 µl of the DNA extract were added for a total volume of 50 µl. Primers used to amplify

Table 1 Locoweed species, varieties, locations, and plant community of collections

Species/Variety	Location	GPS	Plant Community	Voucher[a]
A. wootonii	Truth or Consequences, NM	N 33° 20′ 06.3″ W 107° 17′ 31.1″	Creosote bush	
	Ft. Davis, TX	N 30° 38′ 42.7″ W 103° 57′ 40.5″	Blue grama Sideoats grama	474326
A. mollissimus				
var. *earleii*	Alpine, TX	N 30° 32′ 10.4″ W 103° 57′ 13.2″	Blue grama	474331
	Ft. Davis, TX	N 30° 33′ 30.2″ W 103° 51′ 40.5″	Blue grama	474332
	Ft. Davis, TX	N 30° 38′ 18″ W 103° 55′ 20.1″	Blue grama	
var. *mollissimus*	Kenton, OK	N 36° 50′ 55″ W 103° 01′ 31″	Blue grama	474333
	Farley, NM	N 36° 21′ 52″ W 104° 00′ 27″	Blue grama	474334
	Capulin, NM	N 36° 40′ 59″ W 104° 01′ 04″	Blue grama Little bluestem	474335
var. *thompsonii*	Shiprock, NM	N 36° 48′ 18.7″ W 103° 52′ 18″	Salt desert shrub	474336
	Blanding, UT	N 37° 33′ 05″ W 109° 36′ 30.3″	Pinyon/juniper	474327
	Lisbon Valley, UT	N 38° 8′ 53.2″ W 109° 21′ 23.3″	Salt desert shrub	474329
	Price, UT	N 39° 24′ 37.4″ W 110° 25′ 55.9″	Pinyon/juniper	
	Sanders, AZ	N 35° 9′ 16.4″ W 109° 20′ 5.2″	Pinyon/juniper	474328
A. lentiginosus				
var. *diphysus*	St. Johns, AZ	N 34° 28′ 5.4″ W 109° 21′ 2.5″	Pinyon/juniper	474352
	St. Johns, AZ	N 34° 50′ 29.4″ W 109° 14′ 13.4″	Juniper	474346
var. *araneosus*	Wahwah Valley, UT	N 38° 24′ 10.804″ W 113° 16′ 39.62″	Wyo. Big sage	474349
	Wahwah Valley, UT	N 38° 23′ 45.6″ W 113° 17′ 28.66″	Pinyon juniper	
	Milford, UT	N 38° 26′ 6.17″ W 113° 0′ 32.76″	Big sage	
var. *wahweapensis*	Hanksville, UT	N 38° 11′ 36.9″ W 110° 44′ 52.5″	Pinyon juniper	
	Henry Mt, UT	N 38° 05′ 55.7″ W 110° 55′ 8.6″	Juniper/galletta grass	474351
var. *lentiginosus*	Juntura, OR	N 43° 46′ 57.5″ W 118° 14′ 1.7″	Big sage	473901
A. pubentissimus	Bonanza, UT	N 39° 59′ 6.7″ W 109° 10′ 38.8″	Black sage	474344
	Green River, WY	N 41° 16′ 40″ W 109° 37′ 27.1″	Wyoming big sage	474343
O. sericea	Chico, NM	N 36° 28′ 46″ W 104° 09′ 39″	Blue grama/buffalo grass	474340
	Des Moines, NM	N 36° 38′ 18″ W 103° 56′ 14″	Blue grama/little bluestem	474339
	Virginia Dale, CO	N 40° 54′ 13.6″ W 105° 18′ 14.8″	Midgrass Prairie	474341
	Green River, WY	N 41° 24′ 42.3″ W 109° 33′ 17.4″	Wyoming big sage	474342

Table 1 (continued)

Species/Variety	Location	GPS	Plant Community	Voucher[a]
	Raft River Mt., UT	N 41° 54′ 15.4″ W 113° 20′ 54.9′	Mountain grassland	
Nonlocoweed				
A. alpinus	Denali, AK	N 63° 43′ 56.69″ W 148° 55′ 8.96″	Boreal forest	
A. amphioxys	Truth or Consequences, NM	N 30° 20′ 06.3″ W 107° 17′ 31.1″	Creosote bush	
	Bernalillo, NM	N 35° 20′ 12.3″ W 106° 39′ 28.2″	Pinyon/juniper	
	Big Water, UT	N 37° 5′ 55.7″ W 111° 30′ 36.4″	Salt desert shrub	
A. cibarius	Nephi, UT	N 39° 44′ 37.4″ W 111° 54′ 28.6″	Wyoming big sagebrush	
A. coltonii	Monticello, UT	N 37° 46′ 16.4″ W 109° 21′ 23.3″	Oakbrush	
A. filipes	Juntura, OR	N 43° 46′ 57.5″ W 118° 14′ 1.7″	Big sagebrush	
O. campestris	Denali, AK	N 63° 43′ 56.69″ W 148° 55′ 8.96″	Boreal forest	

[a] Voucher specimens held in the S.L. Welch Herbarium, M.L. Bean Natural History Museum, Brigham Young University, Provo, UT.

the internal transcribed spacer (ITS) region were OR1 (5′ GTC AAA AGT TGA AAA TGT GGC TTG 3′) and ITS 5 (White et al. 1990). Conditions for PCR were 94°C for 3 min followed by 30 cycles of 94°C for 45 sec, 48°C for 1 min, and 72°C for 30 sec with a final extension at 72°C for 5 min.

For analyzing the PCR products, a 1.5% agarose gel that contained ethidium bromide was run at 100 V for 1.5 hr and visualized under UV illumination. Positive amplifications resulted in a band of approximately 580 base pairs (bp). Because the primer set can also amplify *Alternaria* species, all positive amplifications were digested with the restriction enzyme *Ava*II. For digestion, 5 μl of PCR product were added to 0.5 μl *Ava*II (Promega, Madison, WI, USA), 1 μl buffer C supplied with the enzyme, and sterile water for a total of 10 μl. After incubation at 37°C for at least 1 hr, restriction fragments were separated by gel electrophoresis according to the conditions above. A result of two fragments of lengths 200 and 380 bp confirmed the positive detection of the locoweed endophyte.

Isolation of endophyte Fungal isolation was conducted from four subsamples of leaves, stems, and flowers (if present) of dried plant specimens. The greenest tissues were selected and surface sterilized for 30 sec in 70% ethanol, followed by 3 min in 20% bleach, and then 30 sec in sterile water. Tissues were dried on sterile paper towels and plated onto water agar media. To expedite the growth of the fungus, the tissues were pressed into the agar. Plates were stored at room temperature and examined weekly for fungal growth. Any observed fungal growth was transferred to potato dextrose agar (PDA) plates and stored at room temperature for comparative morphological analysis.

Culture isolates were labeled positive when the morphology on PDA matched that of known locoweed endophytes. To confirm a positive result, a portion of the culture was dissected away from the media, ground with liquid nitrogen, and analyzed by PCR by using the same protocol as listed above for frozen plant specimens. If samples gave differing results by culture and PCR, PCR was carried out from the dried plant material or the cultured fungus.

Results

Swainsonine Astragalus locoweed species were higher in swainsonine concentration than *Oxytropis* species (Table 2). Within the *Astragalus* genus, *A. wootonii* had the highest concentration of swainsonine (0.37%) followed by *A. pubentissimus* (0.21%). Varieties of *A. mollissimus* (excluding *A. mollissiums* va. *thompsonii*) and *A. lentiginosus* were similar (0.19% vs. 0.15%, respectively). *O. sericea* contained the lowest amount of swainsonine within the locoweeds, 0.04%. Swainsonine was not detected in *A. alpinus*, *A. cibarius*, *A. coltonii*, *A. filipes*, or *O. campestris*.

Endophyte The endophyte was cultured from most samples that contained substantial quantities of swainsonine (>0.025%; Table 3). However, it could not be cultured from *A. m. thompsonii*, *A. amphioxys*, and a few of the individual plants of the other locoweeds, but its presence

Table 2 Swainsonine concentration (% of dry weight) in *Astragalus* and *Oxytropis* species and varieties

Species	Variety	*N*	Swainsonine %	SE	Min	Max
A. wootoni		11	0.37	0.06	0.25	0.43
A. pubentissimus		10	0.21	0.012	0.16	0.27
A. mollissimus	*earleii*	30	0.22	0.012	0.01	0.38
	mollissimus	15	0.14	0.007	0.11	0.21
	thompsonii	25	0.001	0.0004	0	0.008
A. lentiginosus	*diphysus*	10	0.23	0.018	0.18	0.39
	lentiginosus	5	0.15	0.016	0.11	0.19
	wahweapensis	10	0.15	0.019	0.002	0.20
	araneosus	15	0.11	0.011	0.001	0.14
O. sericea	*sericea*	26	0.04	0.005	0	0.09
Nonlocoweed species						
A. alpinus		1	0			
A. amphioxys		8	0.001		0	0.005
A. cibarius		5	0			
A. coltonii		5	0			
A. filipes		2	0			
O. campestris		1	0			

was detected by PCR in all these samples. Furthermore, swainsonine was not detected, or was near the detection limit (0.001%) in these samples. In *A. m. thompsonii*, swainsonine was not detected in collections from Price and Moab UT, and was low or at the detectable limit in collections from Blanding UT, Shiprock NM, and Sanders AZ. Likewise, only one sample of *A. amphioxys* contained swainsonine near the detection threshold (0.005%). A few samples of the other species and varieties did not contain swainsonine, or it was at the detection threshold: *O. sericea* (4/26 plants), *A. l. araneosus* (2/15 plants), and *A. l. wahweapensis* (1/10 plants; Table 3).

Discussion

The locoweed species selected in this study were taken from locations where locoweed poisoning is frequent. This study verified that the locoweed species *A. wootoni*, *A. pubentissimus*, *A. mollissimus* vars. *Earleii* and *mollissimus*, *A. lentiginosus* vars. *diphysus*, *lentiginosus*, *wahweapensis*, and *arenosus*, and *O. sericea* contain the fungal endophyte and produce substantial amounts of swainsonine. This was the first report of endophyte detection by culture or PCR from *A. wootoni*, *A. pubentissimus*, and the four varieties of *A. lentiginosus*.

Table 3 Number of samples in which detectable amounts of swainsonine was found, and the endophyte was detected from culture or PCR

Species	Variety	Swainsonine			Endophyte		
					Culture		PCR
		+	−	Low	+	−	
A. wootoni		11	0	0	11	0	11
A. pubentissimus		10	0	0	9	1	10
A. mollissimus	*earleii*	30	0	0	27	3	30
	mollissimus	15	0	0	12	3	15
A. lentiginosus	*diphysus*	10	0	0	8	2	10
	lentiginosus	5	0	0	5	0	5
	wahweapensis	9	0	1[a]	8	2	10
	araneosus	13	0	2[a]	14	1	15
O. sericea	*sericea*	22	1[a]	3[a]	22	4	26
Total		125	1[a]	6[a]	116	16	132
A. mollissimus	*thompsonii*	0	16[a]	9[a]	0	25	25
A. amphioxys		0	6[a]	1[a]	0	7	7

PCR detected the endophyte in all samples.
[a] Swainsonine near or below detection level (0.001).

Astragalus locoweeds were generally higher in swainsonine than the *Oxytropis* species. The endophytes associated with *Astragalus* spp. may be different species or strains of fungus from those that occur in *O. sericea* and *O. lambertii* (Belfon and Creamer 2003). Braun et al. (2003) reported that locoweed populations that had high concentrations of swainsonine produced endophyte cultures that were also high in swainsonine concentration, suggesting that fungal genetics influence swainsonine production. The amount of fungus within the plant, its growth rate, and environmental stress, may also affect swainsonine concentration of the plant.

We confirmed that samples of *A. alpinus*, *A. cibarius*, *A. coltonii*, *A. filipes*, and *O. campestris* did not contain swainsonine, and are, therefore, not likely poisonous.

The toxicity of two species and some individual plants were equivocal. *A. amphioxys* has not been considered to be a poisonous locoweed; however, we detected the endophyte by PCR in all its samples and found swainsonine near the detection threshold in one sample (0.005%). Swainsonine concentration of some samples of *A. m. thompsonii* were near the detection threshold and others were below it. Vallotton and Sterling (2002) also reported that *A. m. thompsonii* contained very low concentrations of swainsonine. In a phylogenetic analysis of locoweeds from New Mexico, *A. m. thompsonii* was grouped in a different cluster from *A. m. mollissimus* and *A. m. earleii* (Kulshreshta et al. 2004). Taxonomically, *A. m. thompsonii* was originally classified as a separate species based on its densely pubescent pods compared to glabrous pods in the other *A. mollissimus* varieties (Welsh 2007b).

There appears to be a discrepancy in the ability to culture the endophyte, its detection by PCR, and subsequent concentration of swainsonine. The endophyte was cultured from most samples where swainsonine concentration was greater than 0.025%. However, it could not be cultured from samples that contained swainsonine near or below the detection threshold (approximately 0.001%), although the endophyte was detected by PCR. The endophyte appears to be present in these plants, yet it apparently does not grow and produce swainsonine. The endophyte in individual plants of *O. sericea* (four), *A. l. areaneosus* (two), and *A. l. wahweapensis* (one), although present, simply may not have colonized these specific plants. Species and varieties, such as *A. amphioxys*, *A. m. thompsonii*, and *O. l. articulata* and *O. l. lambertii* from the previous study (Ralphs et al. 2002b), may contain an endophyte strain that does not colonize the plant effectively, or these plants may suppress its growth.

Fungal endophytes from the Clavicipitaceae family (*Claviceps*, *Neotyphodium*, *Epichloe*, *Balansia*, *Myriogenspora*) occur in temperate pasture grasses throughout the world and produce alkaloids that cause fescue toxicosis in livestock and insects (Porter 1997). Specific alkaloids and their metabolites vary across endophyte strains, grass varieties, and the environment. The endophyte is transferred from the seed of the maternal plant to the successive progeny, which defines the genetic potential of the endophyte (Adcock et al. 1997). However, the plant genotype exerts a large effect in suppressing the fungal growth and subsequent alkaloid synthesis (Agee and Hill 1994; Roylance et al. 1994). Symbiotic relationships of these endophytes increase the fitness of their host grasses (Omacini et al. 2005) and arid land plants (Barrow and Aaltonen 2001; Barrow 2003; Lulcero et al. 2006). Further research is necessary to determine if *Embellisia* spp. enhances the fitness of locoweeds.

The question remains as to the potential toxicity of plants having swainsonine concentrations near the detection threshold. Molyneux et al. (1994) suggested a conservative threshold of toxicity at 0.001%. Swainsonine doses at or above 0.2 mg/kg/day for at least 21 d produced irreversible neurologic disease (Stegelmeier et al. 1999). Less is known of the effect of lower doses of longer duration, but it has been suggested they produce transient weight loss and biochemical lesions (Stegelmeier et al. 1999). Swainsonine concentration in locoweeds of 0.004% would produce a dose of 0.2 mg/kg/day, based on the average locoweed consumption of 25% in diets (Ralphs 1993) and intake of 2% of body weight. Therefore, swainsonine concentrations near the detection threshold (approximately 0.001%) may be potentially toxic.

In summary, locoweed species that historically caused the majority of locoweed poisoning in the western U.S. were found to contain the fungal endophyte and produced toxic amounts of swainsonine. *Astragalus* species were generally higher in swainsonine concentration than *Oxytropis* species. The inability to culture the endophyte from *A. m. thompsonii* and *A. amphioxys*, and the inconsistency of detection of swainsonine in varieties of *O. lambertii* from the previous study, raises questions as to the genetic relationships among some species and varieties in the transmission of the endophyte, and its ability to colonize the plant and synthesize swainsonine. Further research is required to answer these questions.

References

ADCOCK, R. A., HILL, N. S., BOUTON, J. H., BOERMA, H. R., and WARE, G. O. 1997. Sympiont regulation and reducing ergot alkaloid concentration by breeding endophyte-infected tall fescue. *J. Chem. Ecol.* 23:691–704.

AGEE, C. S., HILL, and N. S. 1994. Ergovaline variability in *Acremonium*-infected tall fescue due to environmental and plant genotype. *Crop Sci.* 34:221–226.

BARROW, J. R. 2003. Atypical morphology of dark septate fungal root endophytes of *Bouteloua* in arid southwestern USA rangelands. *Mycorrhiza* 13:239–247.

BARROW, J. R., and AALTONEN, R. E. 2001. Evaluation of the internal colonization of *Atriplex canescens* (Pursh) Nutt. roots by dark septate fungi and the influence of host physiological activity. *Mycorrhiza* 11:199–205.

BELFON, R., and CREAMER, R. 2003. RAPD–PCR analysis of fungal endophytes of locoweed. *Phytopathology* 93:S7.

BRAUN, K., ROMERO, J., LIDDELL, C., and CREAMER, R. 2003. Production of swainsonine by fungal endophytes of locoweed. *Mycol. Res* 107:980–988.

CREAMER, R., MCLAIN-OMERO, J., and OLDRUP, E. 2007. Fungal endophytes of locoweed: an overview, pp. 338–344, in K. E. Panter, and J. A. Pfister (eds.). Poisonous Plants: Global Research and Solutions CABI, Wallingford, UK.

GARDNER, D. R., MOLYNEUX, R. J., and RALPHS, M. H. 2001. Analysis of swainsonine: extraction methods, detection, and measurement in populations of locoweeds (*Oxytropis* spp.). *J. Agric. Food Chem* 49:4573–4580.

GARDNER, D. R., ROMERO, J., RALPHS, M. H., and CREAMER, R. 2004. Correlation of an endophytic fungus (*Alternaria* spp.) with the presence of swainsonine in Lambert locoweed (*Oxytropis lambertii*), pp. 32–37, in T. Acamovic, C. S. Stewart, and T. W. Pennycott (eds.). Poisonous Plants and Related Toxins, CABI, Walligford, UK.

KINGSBURY, J. M. 1964. Poisonous Plants of the United States and Canada. Prentice-Hall, Englewood Cliffs, NJ.

KULSHRESHTHA, S., CREAMER, R., and STERLING, T. M. 2004. Phylogenetic relationships among New Mexico *Astragalus mollissimus* varieties and *Oxytropis* speices by restriction fragment analysis. *Weed Sci* 52:984–988.

LUCERO, M. E., BARROW, J. R., OSUNA, P., and REYES, I. 2006. Plant–fungal interactions in arid and semi-arid ecosystems: Large-scale impacts from microscale processes. *J. Arid Environ* 65:276–284.

MCLAIN-ROMERO, J., CREAMER, R., ZEPEDA, H., STRICKLAND, J., and BELL, G. 2004a. The toxicosis of *Embellisia* fungi from locoweed (*Oxytropis lambertii*) is similar to locoweed toxicosis in rats. *J. Anim. Sci* 82:2169–2174.

MCLAIN-ROMERO, J., PADILLA, A., and CREAMER, R. 2004b. Microscopic localization of *Embellisia* in seed and seedlings of southern speckle pod locoweed. *Phytopathology* 94:S69.

MOLYNEUX, R. J., JAMES, L. F., RALPHS, M. H., PFISTER, J. A., PANTER, K. E., and NASH, R. J. 1994. Polyhydroxylated glycosidase inhibitors from poisonous plants of global distribution: analysis and identification, pp. 107–112, in S.M. Colgate, and P.R. Dorling (eds.). Plant-associated Toxins, Agricultural, Phytochemical and Ecological Aspects, CABI, Wallingford, UK.

OLDRUP, W. O. 2005. Determining the influence of environmental parameters on swainsonine production in *Oxytropis sericea* and its fungal endophyte. MS thesis. New Mexico State University, Las Cruces, NM.

OMACINI, M., CHANETON, E. J., and GHERSA, C. M. 2005. A herarchial framework for understanding the ecosystem consequences of endophyte–grass symbioses, pp. 141–162, in C. A. Roberts, C. P. West, and D. E. Spiers (eds.). *Neotyphodium* in Cool-season GrassesBlackwell, Ames, IA.

PORTER, J. K. 1997. Endophyte alkaloids, pp. 51–62, in J. P. F. D'Mello (ed.). Handbook of Plant and Fungal Toxicants, CRC Press, Boca Raton.

RALPHS, M. H. 1993. Seasonal grazing of locoweeds by cattle in northeastern New Mexico. *J. Range Manag* 46:416–420.

RALPHS, M. H., GRAHAM, J. D., and JAMES, L. F. 2002a. A close look at locoweed poisoning on shortgrass prairies. *Rangelands* 24: 30–34.

RALPHS, M. H., WELSH, S. L., and GARDNER, D. R. 2002b. Distribution of locoweed toxin swainsonine in populations of *Oxytropis lambertii*. *J. Chem. Ecol* 28:701–707.

ROLYANCE, J. T., HILL, N. S., and AGEE, C. S. 1994. Ergovaline and peramine production in endophyte-infected tall fescue: Independent regulation and effects of plant and endophyte genotype. *J. Chem. Ecol* 20:2171–2183.

ROMERO, J., CREAMER, R., RALPHS, M. H., and GARDNER, D. R. 2002. Association of a fungal endophyte with seed tissue and locoweed toxicity. *Phytopathology* 92:S70.

STEGELMEIER, B. L., JAMES, L. F., PANTER, K. E., GARDNER, D. R., RALPHS, M. H., and MOLYNEUX, R. J. 1999. Dose response of sheep poisoned with locoweed (*Oxytropis sericea*). *J. Vet. Diagn. Invest* 11:446–454.

VALLOTTON, A. D., and STERLING, T. M. 2002. Variation in swainsonine content among extraction methods and between locoweed genera. *Proc. West. Soc. Weed Sci* 55:18.

WELSH, S. L., RALPHS, M. H., PANTER, K. E., PFISTER, J. A., and JAMES, L. F. 2007a. Locoweeds of North America; taxonomy and toxicity, pp. 20–29, in K. E. Panter, and J. A. Pfister (eds.). Poisonous Plants: Global Research and Solutions, CABI, Wallingford, UK.

WELSH, S. L. 2007b. North American Species of Astragalus Linnaeus (Leguminosae). A Taxonomic Revision. 932 p. M.L. Bean Life Science Museum. Brigham Young University, Provo, UT.

WHITE, T. J., BRUNS, T., LEE, S., and TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, pp. 315–322, in M. A. Innis, D. H. Gelfand, J. Sninsky, and T. J. White (eds.). PCR Protocols: A Guide to Methods and ApplicationsAcademic, San Diego.

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The Identification of 2,4-diacetylphloroglucinol as an Antifungal Metabolite Produced by Cutaneous Bacteria of the Salamander *Plethodon cinereus*

Robert M. Brucker · Cambria M. Baylor · Robert L. Walters · Antje Lauer · Reid N. Harris · Kevin P. C. Minbiole

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Abstract Beneficial bacteria that live on salamander skins have the ability to inhibit pathogenic fungi. Our study aimed to identify the specific chemical agent(s) of this process and asked if any of the antifungal compounds known to operate in analogous plant–bacteria–fungi systems were present. Crude extracts of bacteria isolated from salamander skin were exposed to HPLC, UV-Vis, GC-MS, and HR-MS analyses. These investigations show that 2,4-diacetylphloroglucinol is produced by the bacteria isolate *Lysobacter gummosus* (AB161361), which was found on the red-backed salamander, *Plethodon cinereus*. Furthermore, exposure of the amphibian fungal pathogen, *Batrachochytrium dendrobatidis* (isolate JEL 215), to different concentrations of 2,4-diacetylphloroglucinol resulted in an IC_{50} value of 8.73 μM, comparable to crude extract concentrations. This study is the first to show that an epibiotic bacterium on an amphibian species produces a chemical that inhibits pathogenic fungi.

Keywords Beneficial bacteria · Chytridiomycosis · 2,4-diacetylphloroglucinol · *Plethodon cinereus* · *Lysobacter gummosus* · Pathogenic fungi · Salamander

Introduction

Mutualisms between bacteria and metazoa are ubiquitous and ecologically important. Some well-studied mutualisms involve antifungal bacteria and their hosts, including bacteria that reside on plant roots and on the cuticle of leaf cutter ants (Currie et al. 2006; Raaijmakers et al. 2002). Amphibians, which inhabit moist surroundings hospitable to fungi, not only produce defensive secretions that inhibit fungi and other skin microbes (Simmaco et al. 1998; Rollins-Smith et al. 2005), but also harbor a distinct resident cutaneous bacterial community (Harris et al. 2006; Lauer et al. 2007) that resists removal by thorough rinsing. A specific chemical agent of fungal inhibition, however, has remained unidentified. Therefore, this study aimed to isolate an antifungal substance from the cutaneous bacterial community of an amphibian species and to test for its inhibitory activity against a pathogenic fungal species.

Our previous work demonstrated that bacteria isolated from skin of different amphibian species and grown in a low nutrient medium on a Petri dish inhibit fungi that are pathogenic to amphibian adults and embryos (Harris et al. 2006; Lauer et al. 2007). While these results suggest that antifungal bacteria produce an antibiotic, inhibition by resource competition can also occur. Crude extract removed from bacteria grown in broth culture, however, inhibited a fungal pathogen (Walters 2007), providing evidence that skin bacteria had produced one or more antifungal chemicals. Of the various antifungal species identified by sequencing of the 16S rRNA gene (~1,400 bp), an isolate with 99% genetic match to *Lysobacter gummosus* (AB161361, GenBank) was chosen for this study because of its consistently strong antifungal properties. This species was isolated from the red-backed salamander, *Plethodon cinereus*, and from the four-toed salamander, *Hemidactylium scutatum*.

To begin our study, we focused on antibiotics known to be produced by *Pseudomonas fluorescens*, a relative of

R. M. Brucker · C. M. Baylor · R. L. Walters · A. Lauer · R. N. Harris (✉) · K. P. C. Minbiole
Departments of Biology and Chemistry,
James Madison University, Harrisonburg, VA 22807, USA
e-mail: harrisRN@jmu.edu

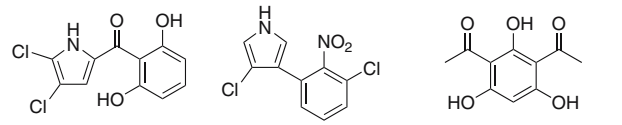

Fig. 1 The chemical structures of pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol

Lysobacter. The bacterium *P. fluorescens* is common in soil, on plant roots, and on amphibian skin, and is inoculated onto pea, wheat, and sugar beet roots to protect them from diseases (Timms-Wilson et al. 2005). This widespread bacterium *P. fluorescens* produces many different antibiotics. In this study, three of these were investigated and compared to the *L. gummosus* antibiotics: pyoluteorin, pyrrolnitrin, and 2,4-diacetylphloroglucinol (=2,4-DAPG; Whistler et al. 1998; Ligon et al. 2000; Sako et al. 2001; Brodhagen et al. 2004; Fig. 1).

Materials and Methods

Bacteria Isolates

Pure cultures of *L. gummosus* (AB161361) were obtained from the salamander *Plethodon cinereus* after a thorough rinsing of the animals to remove transient bacteria. Adult salamanders were obtained from James Madison University Arboretum, Harrisonburg, VA, USA. All *L. gummosus* isolates were stored in 20% glycerol solution and frozen at −20°C. When needed, *L. gummosus* was cultured onto R2A solid medium and allowed to grow at room temperature for 72 h. Isolates were cultured this way three times before extraction. Bacterial species identifications were made by sequencing a portion of the 16S rRNA gene and comparing the sequence with the GenBank database (www.ncbi.nlm.nih.gov/blast).

Preparation and Extraction of Crude Organic Samples

To extract bacterial products, broth cultures were grown. The *L. gummosus* isolate was inoculated into separate 250 ml flasks containing 1/10 strength Nutrient Broth medium (NB, Becton, Dickinson and Company, Sparks, MD, USA). Each liquid culture, as well as a 1/10 strength NB control, was kept at room temperature for 72 h in a Lab-Line incubator shaker. Bacterial cells were in stationary phase when they were removed from the nutrient broth to leave a "crude extract" as follows. The cultures were poured into 50 ml Falcon tubes and centrifuged at 5,000×*g* for 5–10 min. The supernatant was filtered through a 25 mm sterile syringe filter with 0.22 μm pores into new Falcon tubes to remove any remaining bacteria. The supernatant was acidified to pH<2 by using 1 M HCl. Each Falcon tube was frozen at −20°C until needed for chemical analysis.

To extract organic compounds, approximately 90 ml of crude extract supernatant from the *L. gummosus* broth culture were poured into a separatory funnel. The aqueous layer was extracted×4 with 35 ml of ethyl acetate (EtOAc). The combined organic layers were dried with Na_2SO_4 and filtered. The EtOAc solvent was evaporated in vacuo, and a crude recovery calculated. The crude samples (3–9 mg) were then kept frozen in a −20°C freezer until further analysis. A total of five samples were extracted.

HPLC Analysis of Extract

Each crude sample was dissolved in high-performance liquid chromatography (HPLC)-grade methanol (2 ml) before analysis. Standard solutions of 2,4-DAPG, pyrrolnitrin, and pyoluteorin were also prepared in HPLC-grade methanol (1 mg/ml). 2,4-DAPG and pyrrolnitrin were used as purchased (Sigma-Aldrich, Saint Louis, MO, USA and Maybridge Trevillett, Tintagel, UK); pyoluteorin was synthesized in three steps, slightly modifying a method described by Rao and Reddy (1990).

RP-HPLC analysis (Agilent Technologies, 1200 series, Wilmington, DE, USA) was used to determine the retention times of the antifungal standards as well as to analyze the components of bacterial samples. The HPLC diode array detector was programmed to observe and record absorbance at 270 and 310 nm. These two wavelengths best correlate to the λ_{max} of the standards: 2,4-DAPG at 270 nm, pyrrolnitrin at 252 nm (broad), and pyoluteorin at 310 nm (Sako et al. 2001; Brodhagen et al. 2004). The samples were injected (20 μl) into the HPLC equipped with a C18 reverse phase column (5 μm; 4.6×150 mm; Agilent Technologies) and eluted at 1 ml/min. The initial eluent, 10% acetonitrile/water (*v*/*v*, both acidified with 0.1% acetic acid), ran for 2 min. This was followed by a linear gradient to 100% acetonitrile (acidified with 0.1% acetic acid), over an 18-min-period. This final solvent was eluted for another 3 min. Samples showing absorbance at 270 or 310 nm were collected.

GC-MS Analysis of Extract

The standards and HPLC eluates were further analyzed by using coupled gas chromatography–mass spectrometry (Thermo/Finnigan Polaris Q, Somerset, NJ, USA; column: Restek, Rtx-5 crossbond 5% diphenyl-95%dimethyl polysiloxane, length: 30 m, i.d.: 0.25 mm; particle size 0.25 μm); 1.0 μl-samples in HPLC grade methanol were injected (injector temperature: 200°C; EI full scan, mass range 50–500 amu, ion source 200°C). Column temperature began

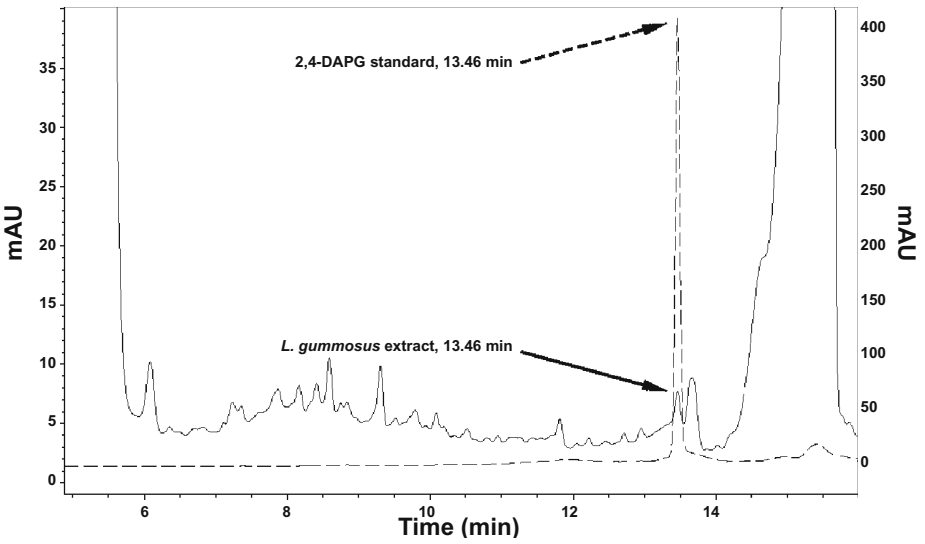

Fig. 2 HPLC chromatogram (270 nm) comparing *Lysobacter gummosus* crude extract (*solid line, left scale*) and 2,4-DAPG standard (*dashed line, right scale*). Both samples had similar retention times on the HPLC chromatograms (13.46 min). The *L. gummosus* extract had several additional peaks, including several very large peaks that were artifacts from the nutrient broth

at 180°C for 2 min and increased to 280°C within 10 min. Retention times and mass spectra of the substances were compared to chemical standards of pyoluteorin, pyrrolnitrin, and 2,4-DAPG.

HR-MS Analysis of Extract

The crude *L. gummosus* extract and 2,4-DAPG were analyzed by high resolution mass spectrometry (HR-MS). ESI mass spectra were obtained with an LCT-TOF mass spectrometer (Micromass, Harvard University Mass Spectrometry Facility).

Inhibitory Assay

To quantify the antifungal properties of 2,4-DAPG, a liquid growth assay was developed by using the fungal pathogen *Batrachochytrium dendrobatidis*. In a 96-well microtiter plate, 12 concentrations of 2,4-DAPG (ranging from 475 to 0.232 μM) as well as positive and negative controls were examined. Five replicates were performed per assay. Each well contained 125 μl of 1% tryptone medium plus 100 μl of fungal zoospores in 1% tryptone medium at a concentration of $\approx 1*10^6$ zoospores/ml (determined by using a hemocytometer). The plates were incubated for 1 day at 23°C before 25 μl of a solution of 2,4-DAPG in dimethyl sulfoxide (DMSO) were added. A volume of 25 μl neat DMSO was added to the positive control wells; heat-killed zoospores were used in the negative control. The optical density at 490 nm (OD_{490}) of each plate was measured with a BioRad microplate reader (model 680, Hercules, CA, USA), on days 0 and 8. Inhibition was determined by comparing the difference of the final and initial growth to the positive control by using a Dunnett's test. The logarithmic trend line was determined by linear regression and used to calculate the IC_{50}.

Results

RP-HPLC analysis of the organic extract of *L. gummosus* revealed a peak at 13.46 min, as observed at 270 nm (Fig. 2). This peak matched the HPLC retention time of the 2,4-DAPG standard (13.46 min). Furthermore, the UV spectra of extract and standard were similar, each showing a

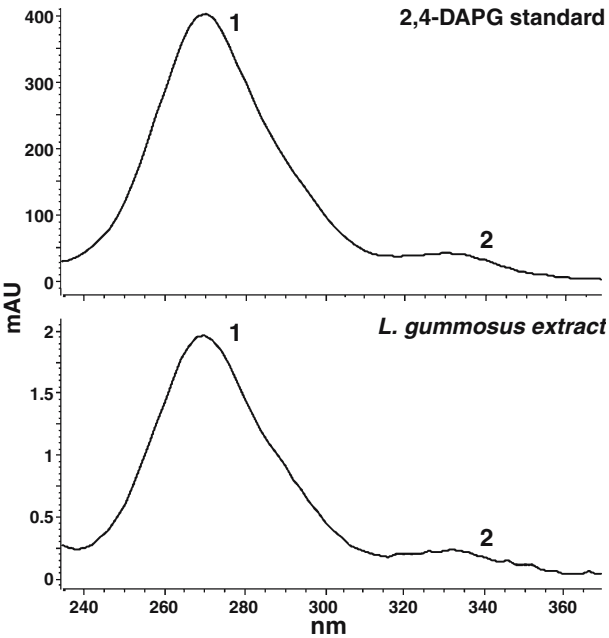

Fig. 3 Comparison of UV-Vis spectrum of 2,4-DAPG standard (*top*) and a *L. gummosus* sample (*bottom*) eluted at same HPLC retention time of 13.46 min. Both UV-Vis spectra share identical peak patterning; (1) a tall peak at 270 nm and (2) a smaller "shoulder" peak at 330 nm

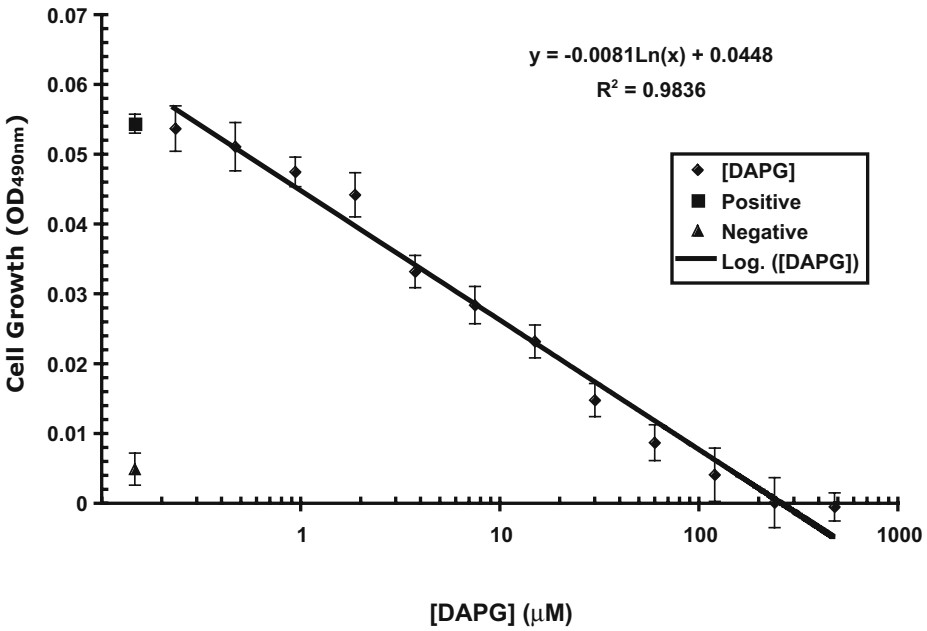

Fig. 4 Inhibitory Growth Assay. Cell growth of the fungal pathogen *B. dendrobatidis* measured in optical density at 490 nm wavelength (OD_{490}) versus concentrations of 2,4-DAPG in μM on a log scale. The trend line is displayed as $y=-0.0081\text{Ln}(x)+0.0448$; $IC_{50}=8.73$ μM; MIC= 136.13 μM; $P<0.001$

λ_{max} of 270 nm (Fig. 3). On GC–MS, the 2,4-DAPG standard displayed a retention time of 6.76 min; the corresponding mass spectrum indicated for M+: 210.1 *m/z* and 211.1 *m/z* (M+H). The extract of *L. gummosus* showed a peak with a retention time of 6.78 min, and a mass spectrum with 210.1 *m/z* (M+) and 211.2 *m/z* (M+H).

HR-MS analysis of the 2,4-DAPG standard showed a peak at 211.0603 *m/z*, corresponding to the calculated (M+H) peak of 211.0607 *m/z*. The *L. gummosus* extract showed a peak at 211.0612 *m/z*.

Analogous investigations (HPLC, UV-Vis, GC-MS) of *L. gummosus* extracts for pyoluteorin and pyrrolnitrin did not provide evidence for the presence of these compounds in the crude sample.

The growth assay (Fig. 4), which used *Batrachochytrium dendrobatidis*, indicated a 2,4-DAPG IC_{50} of 8.73 μM and a minimum inhibitory concentration (MIC) of 136.13 μM. These concentrations were calculated by using a regression of log-transformed OD_{490} values on 2,4-DAPG concentration ($F=1118.22$, $df=1,124$, $P<0.001$). All 2,4-DAPG concentration treatments differed significantly in OD_{490} values from the positive control with the exception of the 0.23 μM and the 0.46 μM concentrations (*a posteriori* Dunnett's *t* tests, $P<0.05$ for all comparisons).

Discussion

Four methods indicate that 2,4-DAPG was produced by the amphibian skin bacterium *L. gummosus*. RP-HPLC analyses repetitively resulted in nearly identical retention times of a peak of the bacterial extract and the 2,4-DAPG standard (Fig. 2); UV-Vis comparison demonstrated a nearly identical spectrum and λ_{max} (270 nm) of this peak and 2,4-DAPG (Fig. 3). Furthermore, GC-MS and HR-MS analyses support the presence of 2,4-DAPG in *L. gummosus* extracts. Although the exact concentration of 2,4-DAPG prevalent on an individual amphibian skin was not investigated in this study, the concentration calculated from the liquid cultures used in this study was about 2 μM. Estimation of 2,4-DAPG concentrations of amphibian skins will be the subject of further investigations.

Biologically, bacteria living on amphibian skin may act as part of the innate immune system of amphibia by preventing pathogens from colonizing. Amphibian species that attend their embryos, such as *H. scutatum*, may use skin bacteria and their antibiotics to keep their embryos free of fungus. Additional studies will be necessary to determine whether there are co-evolved mutualisms between amphibian hosts and skin bacteria. The investigation of other strong antifungal metabolites from a variety of epibiotic bacterial species obtained from frogs and salamanders are the focus of ongoing research. In addition, it would be of interest to determine the relative role of amphibian antimicrobial secretions and those of their bacterial symbionts in deterring pathogens. The study of skin bacteria and their antibiotics may also lead to an understanding of why some species succumb to an emerging infectious disease of the skin, chytridiomycosis, which is caused by *Batrachochytrium dendrobatidis*, and others do not.

References

BRODHAGEN, M., HENKELS, M. D., and LOPER, J. E. 2004. Positive autoregulation and signaling properties of pyoluteorin, an

antibiotic produced by the biological control organism *Pseudomonas fluorescens* Pf-5. *Appl. Environ. Microbiol.* 70:1758–1766.

CURRIE, C. R., POULSEN, M., MENDENHALL, J., BOOMSMA, J. J., and BILLEN, J. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311:81–83.

HARRIS, R. N., JAMES, T. Y., LAUER, A., SIMON, M. A., and PATEL, A. 2006. The amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3:53–56.

LAUER, A., SIMON, M. A., BANNING, J. L., ANDRÉ, E., DUNCAN, K., and HARRIS, R. N. 2007. Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia*. 2007:630–640.

LIGON, J. M., HILL, D. S., HAMMER, P. E., TORKEWITZ, N. R., HOFMANN, D., KEMPF, H. J., and VAN PEE, K. H. 2000. Natural products with antifungal activity from *Pseudomonas* biocontrol bacteria. *Pest Manage. Sci*. 56:688–695.

RAAIJMAKERS, J. M., VLAMI, M., and DE SOUZAL, J. T. 2002. Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* 81:537–547.

RAO, K. V., and REDDY, G. C. 1990. Synthesis and herbicidal activity of the halo analogues of pyoluteorin. *J. Agric. Food Chem.* 38: 1260–1263.

ROLLINS-SMITH, L. A., REINERT, L. K., O'LEARY, C. J., HOUSTON, L. E., and WOODHAMS, D. C. 2005. Antimicrobial peptide defense in amphibian skin. *Integrative and Comparative Biology* 45:137–142.

SAKO, M., KIHARA, T., OKADA, K., OHTANI, Y., and KAWAMOTO, H. 2001. Reductive cleavage of heteroaryl C-halogen bonds by iodotrimethylsilane: facile and regioselective dechlorination of antibiotic pyrrolnitrin. *J. Org. Chem.* 66:3610–3612.

SIMMACO, M., MIGNOGNA, G., and BARRA, D. 1998. Antimicrobial peptides from amphiban skin: what do they tell us? *Biopolymers* (*Peptide Science*) 47:435–450.

TIMMS-WILSON, T. M., KILSHAW, K., and BAILEY, M. J. 2005. Risk assessment for engineered bacteria used in biocontrol of fungal disease in agricultural crops. *Plant Soil* 266: 57–67.

WALTERS, R. L. 2007. Antibiotic production by cutaneous bacteria isolated from two species of salamanders. MSc thesis. James Madison University, Harrisonburg.

WHISTLER, C. A., CORBELL, N. A., SARNIGUET, A., REAM, W., and LOPER, J. E. 1998. The two-component regulators GacS and GacA influence accumulation of the stationary-phase sigma factor sigma σs and the stress response in *Pseudomonas fluorescens* Pf-5. *J. Bacteriol*. 180:6635–6641.

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Sex-Specific Tyrian Purple Genesis: Precursor and Pigment Distribution in the Reproductive System of the Marine Mollusc, *Dicathais orbita*

Chantel Westley · Kirsten Benkendorff

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Abstract Exploitation of Tyrian purple from muricid molluscs, since antiquity, has prompted much interest in its chemical composition. Nevertheless, there remains a paucity of information on the biosynthetic routes leading to observed sexual differences in pigmentation. A liquid chromatography-mass spectrometry (LQ-MS) method was developed to simultaneously quantify dye pigments and precursors in male and female *Dicathais orbita*. The prochromogen, tyrindoxyl sulfate, was detected for the first time, by using this method in hypobranchial gland extracts of both sexes. Intermediates tyrindoxyl, tyrindoleninone, and tyriverdin were detected in female hypobranchial glands, along with 6,6′-dibromoindigo, while males contained 6-bromoisatin and 6,6′-dibromoindirubin. Multivariate analysis revealed statistically significant differences in the dye composition of male and female hypobranchial glands (ANOSIM, P=0.002), thus providing evidence for sex-specific genesis of Tyrian purple in the Muricidae. Dye precursors were also present in male and female gonoduct extracts, establishing a mechanism for the incorporation of bioactive intermediates into muricid egg masses. These findings provide a model for investigating sex-specific chemical divergences in marine invertebrates and support the involvement of Tyrian purple genesis in muricid reproduction.

Keywords Brominated indoles · Hypobranchial gland · Muricidae · Reproduction · Sexual dimorphism

C. Westley · K. Benkendorff (✉)
School of Biological Sciences, Flinders University,
GPO Box 2100,
Adelaide, S.A. 5001, Australia
e-mail: kirsten.benkendorff@flinders.edu.au

Introduction

Tyrian purple, also known as Shellfish purple and Royal purple, is a historically important dye, traditionally obtained from the hypobranchial glands of the Muricidae (Neogastropoda: Mollusca). Exploitation of this dye from as early as the 17th Century BC, has attracted the ongoing interest of natural and cultural historians, archeologists, dyers and colorists, artists, chemists, and biologists (Baker 1974; Cooksey 2001a, 2006; Haubrichs 2004, 2006; Karapanagiotis and de Villemereuil 2006; Westley et al. 2006). In Historia Naturalis, Pliny the Elder provided the first detailed description of colors obtained by dyeing with different techniques and muricid species in ancient Rome (Bailey 1929). Much later, Cole (1685) described how the pigment develops in a series of color reactions under the influence of sunlight. This process can now be explained by a series of oxidation, dimerization, and photolytic cleavage reactions (Fig. 1). A major advance was made by Friedlander (1909), who resolved the structure of the principal pigment, 6,6′-dibromoindigo (**5**). Baker and Sutherland (1968) identified colorless tyrindoxyl sulfate (**1**), as the ultimate dye precursor in the Australian muricid, *Dicathais orbita*, and subsequent studies have revealed the intermediate precursors; tyrindoxyl (**2**), tyrindolinone, tyrindoleninone (**3**; Baker and Duke 1973, 1976), and tyriverdin (**4**; Christophersen et al. 1978; Fujise et al. 1980). Further investigations of dyed artifacts and hypobranchial gland secretions from various muricids have uncovered additional precursors, artifacts, and minor pigments (Michel et al. 1992; Wouters 1992; Koren 1995, 2006; Cooksey 2001a, b, 2006; Cooksey and Withnall 2001; Karapanagiotis and de Villemereuil 2006). These include the yellow oxidation by-product, 6-bromoisatin (**6**), the red structural isomer of (**5**), 6,6′-dibromoindirubin (**7**; Fig. 1), indigo (**8**), indirubin (**9**),

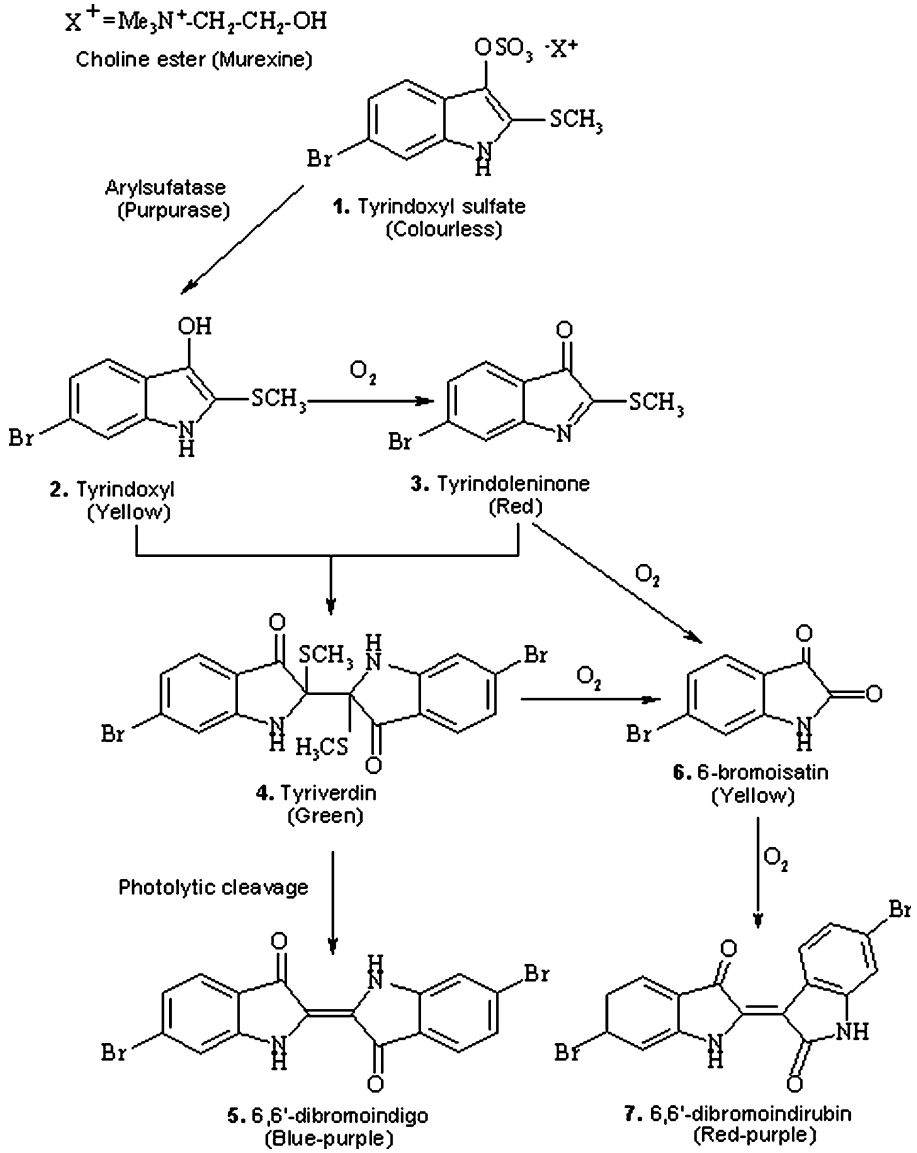

Fig. 1 Tyrian purple genesis from tyrindoxyl sulfate in the hypobranchial gland of *Dicathais orbita*

6-bromoindigo (**10**), 6-bromoindirubin (**11**), and 6′-bromoindirubin (**12**; Appendix).

The first indication of a link between dye production and reproduction in the Muricidae was provided by Aristotle's comments on "purpuras" in Historia Animalium ~350 BC (Peck 1970). He stated that, "When the purpuras have honeycombed (*sic* deposited egg capsules), their bloom (*sic* hypobranchial gland) is at its worst", a fact also reinforced by Pliny the Elder in first Century AD (Bailey 1929). However, this association was overlooked until more recent investigations on egg masses of the Muricidae revealed the presence of indigoid compounds (Palma et al. 1991; Benkendorff et al., 2000, 2001, 2004). In the egg masses of *D. orbita*, Benkendorff et al. (2000) not only reported relatively high concentrations of tyriverdin (**4**) and tyrindoleninone (**3**), but also demonstrated that these intermediates have potent bacteriostatic and mild cytotoxic activity, respectively. This discovery prompted the proposal of a novel biochemical role for indigoid compounds in the Muricidae, whereby they are incorporated into egg masses as a form of maternal investment in the chemical defense of developing embryos (Benkendorff et al. 2000; Westley et al. 2006). The potential for precursor transfer from the adult hypobranchial gland to the adjacent reproductive glands has been supported recently by accounts of deep red pigmentation in capsule and prostate glands of *D. orbita* (Benkendorff et al. 2004). Furthermore, preliminary experiments involving the excision of hypobranchial glands from reproductive organs suggests that the pallial gonoduct influences pigment synthesis (Benkendorff et al. 2004). However, this study was limited to visual accounts. Quantitative analysis is required to confirm the composition

of dye products and, hence, the biochemical relationship between these glandular structures.

Another question that remains to be resolved is whether dye composition and pigmentation differ between sexes of the Muricidae. Studies on hypobranchial gland secretions of *Murex trunculus* by Elsner and Spanier (1985), initially indicated that female glands produce predominantly purple dyes, while dyes of masculine origin gain blue pigmentation due to the presence of indigo (**8**). However, the method of specimen sexing was not reported, and subsequently, Verhecken (1989) proposed the opposite. Verhecken's proposal is consistent with recent visual accounts of red dye pigmentation in male *M. trunculus* and a more blue shade of purple in females (Fig. 2, Boesken Kanold, personal communication). Michel and colleagues (1992) failed to confirm any correlation between dye color and sex; however, a high incidence of pseudohermaphrodism may have influenced their results. Nevertheless, the final pigmentation of male samples after storage in the dark was described as predominantly purple, suggesting that male glands contain relatively less of the non-brominated indoxyl precursors and, hence, less indigo (Michel et al. 1992). An alternative hypothesis for the prevalence of red purple dyes in males could be related to the formation of red indirubins, structural isomers of indigoids that evolve in oxygen-rich environments (Fig. 1). Sensitive chemical analysis of male and female dye is required to assess objectively any sex-related differences in composition.

Over the last couple of decades, advances in chemical analysis by high performance liquid chromatography (HPLC) and mass spectrometry (MS) have facilitated the rapid and accurate detection of indigoid pigments (McGovern et al. 1990; Wouters and Verhecken 1991; Wouters 1992; Koren 1995, 2006; Szostek et al. 2003; Andreotti et al. 2004; Puchalaska et al. 2004; Karapanagiotis and de Villemereuil 2006; Polec-Pawlak et al. 2006). To date, however, no one has applied modern LC-MS techniques to analyze simultaneously the full suite of precursors and pigments that constitute Tyrian purple. In the current investigation, an effort was made to preserve glandular

Fig. 2 Extract preparations (**a**) and dried pigments (**b**) from *Hexaplex (Murex) trunculus* (photos provided by Inge Boesken Kanold, France). Male hypobranchial gland extracts, complete with prostate glands (*left*), display purple/red pigmentation in comparison to the purple/blue hue of female hypobranchial and capsule gland extracts (*right*)

biochemistry to examine dye genesis in the presence of precursors. Extracts from the hypobranchial and reproductive glands of *D. orbita* were employed, as this species possesses a comparatively simple biosynthetic pathway to Tyrian purple from a single brominated prochromogen (**1**; Fig. 1; Baker and Sutherland 1968) that is representative of most Muricidae (Cooksey 2006). Furthermore, previous studies on *D. orbita* provided the first evidence for dye precursors in egg capsules (Benkendorff et al. 2000) and observations of pigmentation in the reproductive organs (Benkendorff et al. 2004). This investigation aimed to quantify precursor and pigment composition in the female pallial gonoduct, specifically, the capsule, albumen, and ingesting glands, as a means for establishing maternal investment in embryonic chemical defense. Following from earlier observations (Benkendorff et al. 2004), analysis of dye composition in hypobranchial glands, attached and detached from male prostate and female capsule glands, was also undertaken to establish further the relationship between dye genesis, reproduction, and sex.

Methods and Materials

Six male and six female *D. orbita* specimens were sampled from subtidal rocky platforms along the metropolitan coastline of South Australia before the breeding season (July–August, 2005 and 2006). An additional three females were collected during breeding season (December, 2005) for dissection and extraction of ingesting glands. Females were identified by the presence of an albumen and capsule gland and the absence of a penis, posterior to the right eye tentacle, and sperm ducts, which occur in pseudohermaphrodites after exposure to tributylin tin (Gibson and Wilson 2003).

The shell of each specimen was removed by cracking with a vice at the junction of the primary body whorl and spire, and the soft body was removed by severing the columnar muscle. The soft body was transferred to a dissecting tray where visceral mass was separated from dorsal mantle by an incision along the lateral margins of the columnar muscle. The dorsal mantle was folded back to reveal the pallial gonoduct and pinned with the ventral surface facing up to expose the hypobranchial gland. In female specimens, care was taken to ensure that ingesting and albumen glands were dissected free of hypobranchial gland tissue. Prostate and capsule glands were dissected away from the medial and brachial regions of the hypobranchial gland in three male and three female specimens, respectively. Removal of the rectal gland and rectal hypobranchial gland from these reproductive structures was not possible. For the "detachment experiment", excised medial and branchial hypobranchial gland regions from both sexes were reserved for inclusion as "detached" replicates. In another three specimens of each sex, the pallial gonoduct and hypobranchial gland were left intact to give comparative "attached" replicates. To produce extracts, which represent the true biochemical composition of dye products from *D. orbita*, all dissected glands were left in their natural posture and exposed to ambient laboratory oxygen and lighting for 12 hr post dissection.

After dye development, each gland (24 in total) was transferred to an amber vial that contained enough dimethyl formamide (DMF) to submerge the tissue. Glands were then macerated and extracted for 48 hr, before being gravity filtered through glass wool. Before compositional analysis, all extracts were sonicated and centrifuged to precipitate tissue residues. Extracts were analyzed with HPLC (Waters Alliance), coupled to a mass spectrometer (MS, Micromass, Quatro micro™). HPLC separation was performed on a Phenomenex, Synergi, Hydro-RP C_{18} column (250× 4.6 mm×4 μm) with parallel UV/Vis diode-array detection (DAD) at 300 and 600 nm. The elution scheme was modified from Szostek et al. (2003) and Puchalaska et al. (2004) with a flow rate of 1 ml/min of 0.1% formic acid and a gradient of acetonitrile in water starting at 30% for 1 min followed by 60% for 3 min, then 100% for 15 min before returning to 30% for 15 min. Compounds were identified with electrospray ionization-mass spectrometry (ESI-MS) with a flow rate of 300 μl/min. Relative proportions of each compound were calculated from integrated absorption data in diode-array by using MassLynx 4.0 software. Proportions were expressed as a percentage of the total dye composition, including all detected precursors and end-products. To facilitate identification of the dye constituents, synthetic standards were analyzed by identical procedures.

Synthetic standards for all possible indole and indirubin end-products (Appendix; Karapanagiotis and de Villemereuil 2006) were prepared in DMF to a concentration of 40 μM. Standards included indigo (Sigma, 229296), 6-bromoindigo (MDPI, 19393), 6,6′-dibromoindigo (courtesy of Prof. P. Imming), indirubin (Apin Chemicals LTD, 20338I), and 6-bromoindirubin, 6′-bromoindirubin, and 6,6′-dibromoindirubin (courtesy of Prof. A. L. Skaltsounis). Apart from retention time (Table 1), discrimination between structural isomers was achieved by differences in visible absorption spectra at λ_{max} 600 nm for indigoids and λ_{max} 550 nm for indirubins. Indigo (**8**) and indirubin (**9**) were discriminated further by the registration of a doubly charged quasi-molecular ion $[M+2H]^{2+}$ at *m/z* 132, which allowed unequivocal identification of **8** (Puchalaska et al. 2004). Major ions in ESI-MS obtained at the apex of HPLC peaks for monobrominated compounds (**10**–**12**) produced duplet ion clusters at *m/z* 339, 341 $[M-H]^+$. Similarly, 6,6′-dibromoindigo (**5**) and 6,6′-dibromindirubin (**7**) were identified by triplet ion clusters at *m/z* 417, 419, 421 for Br^{79} Br^{79}, Br^{79} Br^{81}, Br^{81}, Br^{81}.

Synthetic standards were not available for precursors. However, mass spectra have previously been used to identify intermediate precursors (Michel et al. 1992; Benkendorff et al. 2000; Cooksey and Withnall 2001; Andreotti et al. 2004), based on expected mass and isotopic clusters for the mono- and dibrominated compounds. As the mass spectrum of tyrindoxyl sulfate has not yet been published, the presence of this compound in extracts was confirmed by thin layer chromatography (TLC). TLC was conducted on aluminum-backed silica gel plates (Merck), employing an *n*-butanol–EtOH–acetic acid–water (8:2:1:3) solvent system. Development in 1 M HCl results in the formation of a purple spot, characteristic of tyrindoxyl sulfate (Baker and Sutherland 1968). Presence of murexine and senecioylcholine was also investigated by TLC, as these choline esters are known to be associated with the prochromogen (Roseghini et al. 1996). Dipping plates in Dragendorff Reagent (Fluka-44578) allows visualization of alkaloids and quaternary ammonium bases and has been used to detect choline esters in several muricid hypobranchial gland extracts (Roseghini et al. 1996). Development of yellow and rose pigmentation in UV-active spots indicates the presence of senecioylcholine and murexine, respectively (Roseghini et al. 1996).

Statistical analyses of differences in male and female dye composition (N=6) were undertaken with Primer Version 5 Software. Multivariate analyses were undertaken on square root transformed data to increase the weighting of minor constituents. Nonmetric multidimensional scaling (nMDS) was performed on a Bray-Curtis similarity matrix (Clarke and Gorley 2001) and portrayed in a two-dimensional plot. Significant differences in dye composition between the sexes were then explored by using ANOSIM. SIMPER was undertaken to identify which indigoids contributed to compositional differences.

Results

Dyes and Precursors in Hypobranchial and Reproductive Glands LC-MS analysis revealed the presence of brominated indole derivatives in dimethyl formamide (DMF) extracts from all hypobranchial and reproductive glands (Table 1). The dominant compound in all samples registered an HPLC peak at 5.1 min (e.g., Fig. 3). Major ions in ESI-MS, obtained at the apex of this peak, were at *m/z* 338, 336, which correspond to the molecular ions of tyrindoxyl sulfate (**1**; Br^{79}, Br^{81}, Fig. 3). Fragment ions at *m/z* 240, 242 and *m/z* 224, 226 correlate with the loss of a sulfate ion [$MH–SO_4$], and a methyl group [$M^{-}H–SO_4–CH_3$], respectively, from the tyrindoxyl sulfate molecule. As this compound has not been characterized previously by using

Table 1 High performance liquid chromatography (HPLC) retention times (t_R) and electrospray ionization mass spectrum (*m/z*) values for the various indole derivatives[a] in dimethyl formamide extracts of male and female glandular extracts from *Dicathais orbita*

Dye Component	t_R (min)	Major Ions (m/z)	Males			Females		
			Hypobranchial	Prostate	Hypobranchial	Capsule	Albumen	Ingesting
Tyrindoxyl sulfate **1**	5.1–5.5 [b]	336, 338	89.66±2.42	82.06± 14.86	92.21±3.37	94.18± 0.55	100.00± 0.00	66.67± 57.74 [c]
Tyrindoxyl **2**/ Tyrindoleninone **3**	9.5–10.1	256, 258/255, 257 417, 419, 421	3.20±0.92	0.00	3.72±0.82	1.93± 0.91	0.00	0.00
Tyriverdin **4**	12.0–12.1	463, 465, 467 511, 513, 515	0.00	0.00	2.57±1.24	2.20± 0.49	0.00	0.00
6,6′-dibromo-indigo **5**	14.4–15.4 [d]	417, 419, 421	0.12±0.00	0.00	0.50±0.35	0.96± 0.02	0.00	0.00
6-bromoisatin **6**	6.4–6.6	224, 226	6.40±2.84	0.00	0.98±0.96	0.72± 0.08	0.00	0.00
6,6′-dibromo-indirubin **7**	16.7–16.9	417, 419, 421	0.70±0.35	17.94± 14.86	0.00	0.00	0.00	0.00

[a] The relative proportions of each compound were calculated from integration data taken at 300 and 600 nm using a diode array in the HPLC and expressed as the mean percent of total dye composition ±SD, where N=3. It should be noted that extinction coefficients are not available for all these compounds, thus prohibiting adjustment of integrated peak values for absolute quantification. Therefore, percentages of total dye composition should be viewed as relative for comparison between samples rather than actual compound proportions.

[b] Molecular ions corresponding to **1** in ingesting glands registered for peaks at 7.0 and 7.9 min. Despite shifts in t_R, TLC analysis confirmed the presence of **1** in these extracts at R_f 1.0.

[c] **1** was absent from one ingesting gland extract, but represented 100% dye composition in the two remaining replicates.

[d] The large t_R range for **5** is due to a shift downfield in some extracts after HPLC column replacement (Appendix 1).

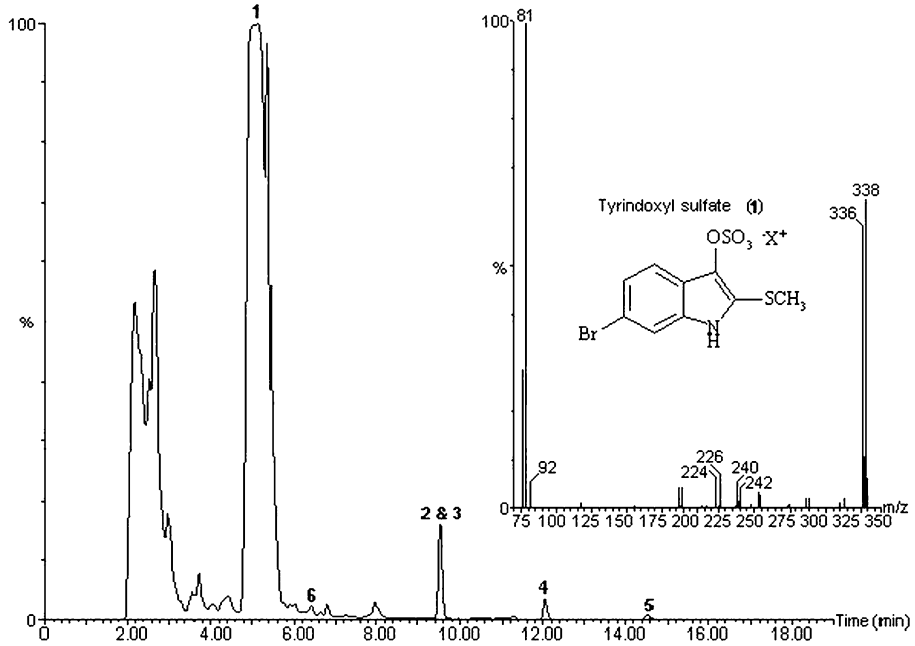

Fig. 3 Liquid chromatography-mass spectrometry analysis of a typical extract from the hypobranchial gland of a female *D. orbita*. The chromatogram obtained from the diode array at 300,600 nm shows the relative composition of the dye precursors and pigments. *Inset* is the electrospray ionization mass spectrum obtained from the apex of the major chromatographic peak obtained at 5.1 min showing dominant signals, which agree with the molecular mass (*m/z* 336, 338) and stable fragment ions of tyrindoxyl sulfate (**1**). Other peaks in the chromatogram correspond to tyrindoxyl/tyrindoleninone (**2**/**3**), tyriverdin (**4**), 6,6′-dibromoindigo (**5**), and 6-bromoisatin (**6**)

mass spectrometry, we used thin layer chromatography (TLC) to confirm the presence of **1**. A single spot (R_f 1.0) that turned purple after exposure to HCl was detected in all extracts except one ingesting gland, which also failed to produce a peak corresponding to tyrindoxyl sulfate in LC-MS (Table 1). TLC analysis also revealed a colorless UV-active spot (R_f 0.12) in all glandular extracts that contained tyrindoxyl sulfate. Application of the Dragendorff Reagent resulted in rose pigmentation, indicative of murexine (Roseghini et al. 1996).

Intermediate dye precursors were detected as minor components in LC-MS analyses (Fig. 3) of male and female hypobranchial and capsule gland DMF extracts (Table 1). Co-eluting peaks at 10.1 min with major ions in ESI-MS at *m/z* 255, 257 and 256, 258 correspond to the molecular mass of tyrindoleninone (**3**) and tyrindoxyl (**2**). Fragment ions at *m/z* 240, 242, formed by the elimination of a methyl group $[M–CH_3]^+$, are consistent with the mass spectrum data for **3**. The second duplet ion cluster at *m/z* 256, 258 and fragment ions at *m/z* 241, 243, correspond to those of **2** $[M–H]^+$ and the loss of a methyl group $[M–H–CH_3]^+$ during electron bombardment. The HPLC peak detected at 6.5 min with *m/z* 224, 226, in both male and female hypobranchial glands (Table 1) is attributed to the pseudomolecular ion $[M–H]^+$ of 6-bromoisatin (**6**), which has a molecular mass of 225, 227 (Br^{79}, Br^{81}).

An additional dye precursor, identified exclusively in female hypobranchial and capsule gland extracts, registered a chromatographic peak at 12.0 min (Table 1). Although major ions in ESI-MS at *m/z* 417, 419, and 421 correspond to the mass of dibrominated standards 5 and 7, the retention times disagree (Appendix). Minor peak isotopic clusters detected at 513, 515, and 517 correspond to the quasi-molecular ion of tyriverdin $[MH^+$; Br^{79} Br^{79}, Br^{79} Br^{81}, Br^{81}, $Br^{81}]$. Additional ion clusters at *m/z* 465 (Br^{79} Br^{81} $[M–SCH_3]^+$ from the elimination of a single methane thiol group) and *m/z* 419 (Br^{79} Br^{81} $[MH–2SCH_3]^+$ formed by the elimination of dimethyl disulphide) further confirm this peak as tyriverdin (**4**).

The dye pigments appear as relatively minor constituents in *D. orbita* extracts (Fig. 3, Table 1). The two dibrominated dye pigments **5** and **7** were detected in some extracts, with retention times and mass spectra that were consistent with synthetic standards (Appendix). No peaks corresponding to the mono- or non-brominated indigo or indirubin standards (Appendix) were detected in any *D. orbita* extracts.

Sex-Specific Pigment Genesis During the exposure period, hypobranchial glands sequentially developed yellow, red, and green pigmentation before gaining various shades of purple and blue, irrespective of sex. The major purple dye pigment extracted from female hypobranchial and capsules glands was identified as 6,6′-dibromoindigo (**5**; Table 1). One male hypobranchial gland extract was also found to contain trace amounts of 6,6′ dibromoindigo (**5**; Table 2).

Table 2 Liquid chromatography-mass spectrometry analyses of Tyrian purple precursors and pigments in dimethyl formamide extracts of attached and detached male and female hypobranchial glands

Dye component	Male		Female	
	Attached[a]	Detached[b]	Attached[a]	Detached[b]
Tyrindoxyl sulfate **1**	+++	+++	+++	+++
Tyrindoxyl **2**/ Tyrindoleninone **3**	+++	+++	+++	+++
Tyriverdin **4**	–	–	+++	+++
6,6′-dibromoindigo 5	+	–	+++	+++
6-bromoisatin 6	+++	+++	+++	+++
6,6′-dibromoindirubin 7	+++	+++	–	–

+++ Indicates presence in all three replicate extracts, + indicates presence in one of three replicate extracts, − indicates absence in all three replicate extracts

[a] Attached extracts represent glands in which connection with the pallial gonoduct was maintained (N=3).

[b] Detached extracts comprise hypobranchial glands excised from the prostate or capsule gland (N=3).

However, in contrast to females, the major dye pigment of male hypobranchial and prostate glands corresponded to 6,6′-dibromoindirubin (**7**; Tables 1 and 2). Within each sex, the dye and precursor composition was identical in all replicate hypobranchial glands whether "attached" or "detached" from the reproductive system. Male extracts were dominated by 6,6′-dibromoindirubin, and females by 6,6′-dibromoindigo (Table 2). Tyriverdin (**4**), was detected only in the female extracts (Table 2).

Multidimensional scaling (MDS) ordination on the dye compositions revealed a distinct separation between male and female hypobranchial gland extracts (Fig. 4). Multivariate analysis of similarities (ANOSIM) confirmed that these differences were statistically significant (Global R= 0.757, P=0.002). Similarity of percentage (SIMPER) analysis revealed an average dissimilarity of 21% in the dye composition between males and females. Intermediates and final pigments contributed substantially to the sexual differences, whereas the ultimate precursor, tyrindoxyl sulfate (**1**), occurred in similar abundance in both sexes. Tyriverdin (**4**) and 6,6′-dibromoindigo (**5**) were consistently more abundant in female samples, whereas 6-bromoisatin (6) and 6,6′-dibromoindirubin (**7**) characterized males. Blind examination of LC-MS chromatograms confirmed that we could reliably determine the sexual origin of *D. orbita* extracts based on these chemical compositions.

Discussion

Analysis of hypobranchial gland extracts by LC-MS, in conjunction with synthetic standards, confirmed the presence of dibrominated dyes generated from a single brominated

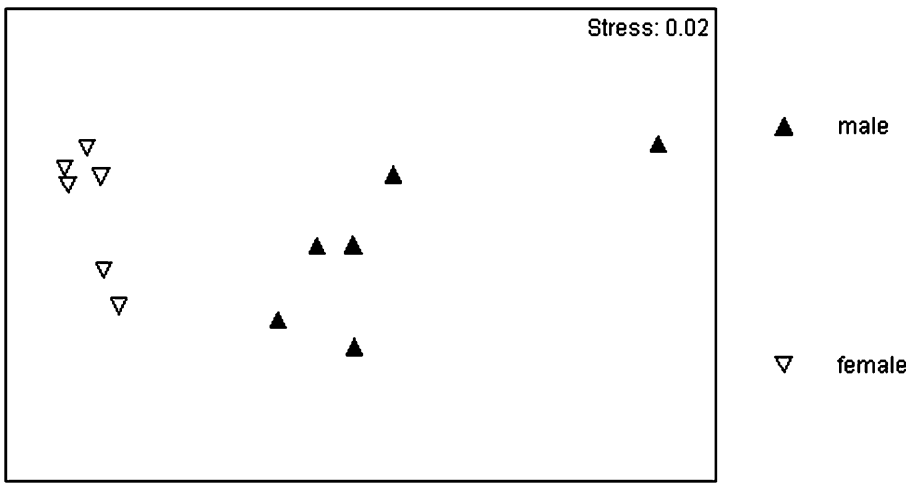

Fig. 4 Two-dimensional nMDS plot for the Tyrian purple composition found in extracts from the hypobranchial glands of male and female *D. orbita*. Data based on the percent composition of each precursor (**1**–**4**) and pigment (**5**–**7**) after square root transformation

prochromogen in *D. orbita*. Tyrindoxyl sulfate (**1**) was detected in hypobranchial gland extracts and also occurred throughout female reproductive glands and male prostate glands (Table 1). Comparison of male and female extracts provided evidence for clear sex-specific Tyrian purple genesis (Table 2, Fig. 4). Females dyes were composed of 6,6′-dibromoindigo (**5**), while male hypobranchial glands contained the red isomer (**7**). This is the first study in which the full suite of Tyrian purple precursors have been analyzed simultaneously alongside the final dye pigments. As suggested by Michel and colleagues (1992), precursors in the final dye product are most likely due to reduced light exposure resulting from the screening affect of mucoid glandular secretions or already formed dye. Consequently, this method of dye development may be viewed as somewhat incomplete compared to the majority of studies where muricid hypobranchial gland secretions were developed on filter paper. However, preservation of the complete glandular biochemistry can prove useful in deciphering the genesis of specific dye products under natural physiological conditions. This approach has enabled a significant advance in understanding the chemistry behind sex-specific color differences in Muricidae dyes (see Fig. 2).

Our findings of only one prochromogen corresponding to tyrindoxyl sulfate (Fig. 3) in *D. orbita* hypobranchial gland extracts are consistent with previous reports that **1** is the sole dye precursor in this species (Baker 1974). By using TLC developed in Dragendorff Reagent, we also detected only one choline ester corresponding to murexine, which is known to be associated with tyrindoxyl sulfate in *D. orbita* (Baker and Duke 1976). While murexine was not detected in our LC-MS analyses, the ability to detect the prochromogen **1** in muricid hypobranchial gland extracts should allow for identification of prochromogens in more complex extracts (e.g., *M. trunculus*), where substituted and un-substituted sulfate esters of indoxyl and 6-bromoindoxyl coexist (McGovern and Michel 1990). The absence of any peaks that correspond to the mono- or non-brominated indigo or indirubin standards (Appendix) in our *D. orbita* extracts (Fig. 3, Table 1), further confirms **1** as the sole ultimate dye precursor (Fig. 1) in this Australian species. This study provides new evidence for 6,6′-dibromoindirubin in glandular extracts of *D. orbita* (Table 1), as 6,6′-dibromoindigo was previously thought to be the sole dye component in secretions from this species (Baker 1974). It now appears that genesis of the structural isomer is also possible, similar to that reported for other Muricidae (Clark and Cooksey 1997; Cooksey 2001a, b, 2006; Cooksey and Withnall 2001; Naegel and Cooksey 2002; Withnall et al. 2003; Koren 2006).

The molecular weight (Table 1) and mass spectrum fragment ion data obtained for the intermediate precursors tyrindoxyl (**2**) and tyrindoleninone (**3**) are consistent with previous studies on Muricidae extracts. Both of these indole precursors have been detected by mass spectrometry of the hypobranchial gland extracts of *Nucella lapillus* (Cooksey and Withnall 2001), with **2** also detected in *Murex brandaris* (Michel et al. 1992), and *M. trunculus* (Andreotti et al. 2004), whereas **3** has been reported from egg mass extracts from *D. orbita* (Benkendorff et al. 2000). Also expected from previous studies was the HPLC peak corresponding to 6-bromoisatin (**6**), which is known to evolve from other dye precursors under oxidative conditions (Cooksey 2001a). This compound was detected in both male and female hypobranchial glands and in the female capsule glands (Table 1) and has been previously detected in the egg masses of *D. orbita* (Benkendorff et al. 2000). The immediate precursor tyriverdin, however, was detected for the first time by using ESI-MS. Field desorption/field ionization MS of tyriverdin has been successful previously in identifying this intermediate (Christophersen et al. 1978), while chemical ionization and electrospray (positive ion) MS failed to produce a molecular ion (Benkendorff et al. 2000). It appears that a change in ionization mode to negative in the ESI-MS facilitates detection of this compound.

Detection of Tyrindoxyl sulfate (**1**) in male prostate and all female capsule, albumen, and in the majority of ingesting gland extracts (Table 1) strongly supports a role for indole derivatives in muricid reproduction. Tyrian purple precursors are thought to be synthesized in the branchial and rectal regions of the hypobranchial gland, before being transported by muco-cilliary action to the medial region for storage (Roller et al. 1995). Similar to other muricids (Middlefart 1992a, b; Roller et al. 1995), the rectal region of the hypobranchial gland in *D. orbita* surrounds the ventral surface of the rectum, which is embedded in the prostate or capsule gland (Benkendorff et al. 2004). This apparent association could facilitate the transfer of dye precursors and pigments from their site of synthesis in the hypobranchial gland to the adjacent prostate and capsule glands. Detection of the prochromogen (**1**) in more posterior female reproductive glands could be explained by residual biosynthetic activity, as muricid genital ducts are thought to arise from an ancestral right hypobranchial gland (Kay et al. 1998). Failure to detect intermediates or pigments (**2**–**7**) in the albumen and ingesting glands of females (Table 1) implies the absence of arylsulfatase, which is required for the hydrolysis of **1** (Fig. 1; Dubois 1909; Baker and Sutherland 1968). By comparison, pigment production in the capsule and prostate glands (Table 1) suggests either the presence of arylsulfatase, in addition to **1**, or diffusion of hydrolyzed intermediates from the hypobranchial gland.

Detection of Tyrian purple precursors in the reproductive glands of *D. orbita* could provide a mechanism for incorporating bioactive intermediates into the egg masses of this species (see Benkendorff et al. 2001). Although the exact location of fertilization remains unclear, the capsule gland has been proposed in *N. lapillus* (Fretter 1941). This poses a practical site for precursor incorporation into egg capsules, as tyrindoxyl sulfate and arylsulfatase (Dubois 1909; Baker and Sutherland 1968) could be acquired from the adjacent hypobranchial gland. An alternative fertilization site in muricids is the albumen gland, where sperm are thought to pass from the duct of the ingesting gland into the albumen gland where eggs are received from the oviduct (Fretter 1941). If so, it may be possible that the prochromogen is incorporated into albuminous secretions before being passed into the capsule gland along with fertilized eggs. Detection of **1** in male prostate gland extracts (Table 1) could provide further means for transferring high concentrations of bioactive dye precursors to the egg masses of *D. orbita* (Benkendorff et al. 2001). As the prostate gland adds prostatic fluid to sperm within the pallial vas deferens during passage to the penis (Middlefart 1992a, b), the prochromogen could also be incorporated into seminal secretions. During copulation, semen is released into the bursa copulatorix or ventral channel of the female uterus (Fretter 1941), where it ultimately combines with albuminous secretions. Consequently, males feasibly could contribute tyrindoxyl sulfate to the intracapsular fluid constituent during capsule manufacture as an additional paternal investment. Previously, it was suspected that precursors in the egg masses of *D. orbita* were of adult origin (Benkendorff et al. 2004; Westley et al. 2006). However, the question remained as to how these compounds arrive in the egg capsules of this species. This investigation provides chemical evidence for Tyrian purple precursors and pigments in the reproductive system of a Muricidae.

Statistically significant sexual dimorphism was found in the chemical composition of Tyrian purple from the hypobranchial glands of *Dicathais orbita* (Fig. 4; $P=0.002$). Our results support previous observations of a more blue-purple pigmentation in female hypobranchial muricid secretions, compared to the red-purple dyes gained from males (Fig. 2, Boesken Kanold personal communication; Verhecken 1989; Michel et al. 1992). In the past, the blue hue of Tyrian purple secretions has been attributed to the presence of indigo (**8**) and/or 6-bromoindigo (**10**; Verhecken 1989; Michel et al. 1992; Benkendorff et al. 2004). However, neither of these blue compounds was detected during quantitative analysis of *D. orbita* extracts (Tables 1 and 2), including glands in which blue pigmentation was clearly observed. This demonstrates the unreliability of simple color observations for drawing conclusions about pigment composition in natural samples. The sex-specific dye pigmentation in *D. orbita* clearly is not due to variations in purple dibrominated vs blue non- or monobrominated indigoids, but is due rather to the presence of precursors and structural isomers of Tyrian purple. The blue-purple color of female secretions can be accounted for by the presence of blue-purple (**5**) and green (**4**), whereas the red hue of male dyes can be attributed to red-purple (**7**) and yellow (**6**; Tables 1 and 2). Previous observations have also suggested that dye pigmentation depends on whether mantle integrity is maintained between the pallial gonoduct and the hypobranchial gland (Benkendorff et al. 2004). Blue secretions in "detached" glands were speculated to result from the evolution of indigo (**8**) in the absence of bromoperoxidase or bromine in the rectal hypobranchial gland or pallial gonoduct. However, in this investigation, both "attached" and "detached" glands displayed consistent blue and purple pigment mixtures (Table 2), confirming that the sexual differences occur directly in the hypobranchial glands and are not dependant on the presence of reproductive glands.

The blue coloration observed in some of our *D. orbita* glands and extracts is most likely due to the molecular behavior of dibromoindigo. In solution or at low concentration, dibromoindigo can appear blue, while at high concentrations or as a textile dye, it develops a purple hue (Cooksey 2001b). The blue pigmentation arises from monomers of 6,6′-dibromoindigo, while the formation of dimers or higher polymers gives a purple color due to the van der Waals attraction between bromine atoms. This results in closer molecular stacking and shifts the absorption maximum towards the red (Cooksey 2001b). Furthermore, as dibromoindigo displays a strong anisotropy of light absorption in the solid state (Susse and Krampe 1979), the absorption maximum (λ_{max} 540 and 640 nm) depends greatly on molecular orientation (Cooksey and Withnall 2001). Consequently, the blue pigmentation in *D. orbita* secretions could result from the molecular arrangement and/or concentration of 6,6′-dibromoindigo, but is not due to isolation from bromoperoxidase or bromine.

Examination of dye genesis in male and female samples under natural conditions has interesting implications for differences in glandular physiology. For example, detection of the Tyrian purple isomer **7** and higher concentrations of 6-bromoisatin (**6**) in male hypobranchial glands suggests more aerobic conditions in comparison to female glands. It is hypothesized that **6** arises from the photochemical oxidation of the tyrindoxyl sulfate, tyrindoxyl, or tyrindoleninone, among other intermediates (Cooksey 2001a, 2006; Cooksey and Withnall 2001). 6-Bromoisatin (**6**) is considered a precursor to brominated indirubins (Clark and Cooksey 1997; Cooksey 2001a, 2006), which supports the

evolution of **7** in male hypobranchial glands, where the highest concentrations of **6** were detected (Table 1). Surprisingly, **6** was not detected in conjunction with **7** in male prostate glands, thus, suggesting a more complete reaction series in this gland. Conversely, in female extracts, the presence of sulfur compounds, such as dimethyl disulfide and the intermediate dye precursors tyrindoleninone (**3**) and tyriverdin (**4**), suggests the female glandular environment may be more reducing than oxidizing. The presence of **4**, in conjunction with comparatively low percentages of **6** explains the evolution of 6,6′-dibromoindigo (**5**) instead of **7**. In the presence of sunlight, photoliable tyriverdin undergoes cleavage to yield dimethyl disulfide (McGovern and Michel 1990; Cooksey 2001a; Cooksey and Withnall 2001) and **5**. The liberation of dimethyl disulfide would help maintain a reducing environment (McGovern and Michel 1990) that results in increased yields of **5** and a relative decrease in the oxidation by-products **6** and **7**. Although oxygen availability appears to explain differences in male and female dye composition, the reason for this divergence in glandular chemistry requires further investigation.

Our LC-MS analyses of hypobranchial gland secretions from *D. orbita* provide the first evidence for sex-specific genesis of Tyrian purple (Tables 1 and 2, Fig. 4) in the Muricidae. Sex-specific pigments that result in visual coloration occur in many species, including marine invertebrates (Bandaranayake 2006). These differences generally aid in mate selection. However, in the case of the Muricidae, the pigmentation from hypobranchial gland metabolites does not exist as an external visual cue and, thus, is more likely to occur as a by-product of their biologically active precursor compounds (Benkendorff et al. 2000; Westley et al. 2006). Sex is a known determinant in the synthesis of antibacterial ceratotoxins in the Mediterranean fruit fly, *Ceratitis capitata* (Marchini et al. 1993; Rosetto et al. 1996), the venom of spiders (Rash and Hodgson 2002), and the allelochemical, sarcophytoxin, in the soft coral, *Sarcophyton glaucum* (Fleury et al. 2006). However, this is the first account of sex-specific secondary metabolite synthesis in the Mollusca and provides a good model for exploring the driving forces for such biosynthetic divergences in marine invertebrates. Indole derivatives have been documented in a huge range of marine invertebrates (Christophersen 1983; Alvares and Salas 1991), including one report of dibromoindigo in the marine acorn worm, *Ptychodera flava laysanica* (Higa and Scheuer 1976). Unlike most other species, however, we now have a good understanding of the biosynthetic origin and anatomical distribution of these indole derivatives in *D. orbita*. This will facilitate future physiological and ecological investigations without confounding from inappropriately pooling between sexes or biosynthetic organs.

Understanding the sexual differences in muricid brominated indoles could also have useful implications for future development of natural medicines from these molluscs. The purple secretion from muricids is currently listed on the Homeopathic Materia Medica (Westley et al. 2006), although chemical and pharmacological research to substantiate the bioactivity of this remedy is lacking. Nevertheless, recent studies indicate that brominated indirubins in muricid Tyrian purple inhibit cell proliferation with selectivity towards GSK-3α/β receptors (Meijer et al. 2003; Magiatis and Skaltsounis 2006). Our results suggest that extracts from male muricids will yield the highest concentrations of these bioactive bromoindirubins. However, extracts from the females contain the intermediate precursors, tyrindoleninone and tyriverdin, with reported anticancer and bacteriostatic activity, respectively (Benkendorff et al. 2000; Westley et al. 2006; Vine et al. 2007). Consequently, there is much scope for future comparative studies to optimize extraction procedures for the development of novel natural remedies from these marine molluscs.

In summary, preservation of the glandular biochemistry, followed by quantification using HPLC-DAD coupled to ESI-MS, enables comparative investigations into the natural genesis of Tyrian purple in Muricidae molluscs. Tyrindoxyl sulfate (**1**) and the immediate precursor tyriverdin (**4**) were detected for the first time by LC-MS, thus providing a suitable procedure for the simultaneous analysis of all brominated precursors and pigments. The ultimate precursor **1** was detected throughout the female reproductive system, thus presenting a means for maternal investment in the chemical defense of *D. orbita* egg masses. A significant difference in the chemical composition of extracts from male and female hypobranchial glands provides evidence for sex-specific biosynthetic routes to Tyrian purple production. This sexual dimorphism is likely to be governed by glandular physiology, giving rise to an oxidizing and reducing environment in males and females, respectively. Together, these findings have useful implications for future investigations into the selective pressures that influence sex-specific metabolite biosynthesis in marine invertebrates and the development of bioactive muricid extracts.

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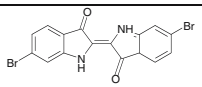
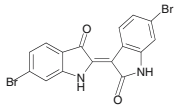
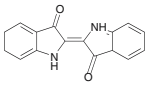
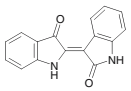
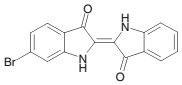
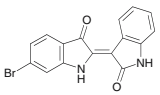
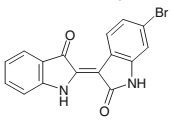
APPENDIX

Table 3 Characteristics of indigoid and indirubin standards and diagnostic parameters obtained by liquid chromatography-mass spectrometry

Compound Number	Standard	Structure	$[M-H]^+$	t_R
5	6,6'-dibromoindigo[a]		417, 419, 421	15.4
7	6,6'-dibromoindirubin		417, 419, 421	16.5
8	Indigo		261	10.7
9	Indirubin		261	10.5
10	6-bromoindigo		339, 341	12.7
11	6-bromoindirubin		339, 341	13.5
12	6'-bromoindirubin		339, 341	13.2

$[M–H]^+$ the pseuomolecular ion (Br^{79}, Br^{81}) registered as the dominant signal in ESI mass spectrums in the negative ionization mode
t_R = the retention time in minutes
[a] Female extracts displayed a shift in retention time compared to the synthetic standard (Table 1). This was attributed to HPLC column replacement (despite identical specifications). To confirm the identity of dye components, a female hypobranchial extract was spiked with the dibromoindirubin standard, which also contained trace amounts of the dibromoindigo isomer. In comparison to un-spiked extracts, an increase in relative peak intensity in the spiked extract at 14.5 min and an additional peak at 16.0 min confirmed the dominance of dibromoindigo in female extracts. Subsequent reanalysis of male extracts confirmed that the retention time shift was due to column replacement rather than any specific properties of the female extracts.

References

ALVARES, M., and SALAS, M. 1991. Marine, nitrogen-containing heterocyclic natural products- structures and syntheses of compounds containing indole units. *Heterocycles* 32:1391–1452.

ANDREOTTI, A., BONADUCE, I., COLOMBINI, M., and IBECHINI, E. 2004. Characterization of natural indigo and Tyrian purple by mass spectrometric techniques. *Rapid Commun. Mass. Spectrom.* 18:1213–1220.

BAILEY, K. 1929. The Elder Pliny's Chapters on Chemical Subjects, Part 1. Edward Arnold, London.

BAKER, J. 1974. Tyrian purple. Ancient dye, a modern problem. *Endeavour* 33:11–17.

BAKER, J., and DUKE, C. 1973. Isolation from the hypobranchial glands of marine molluscs of 6-bromo-2, 2-dimethylthioindolin-3-one and 6-bromo-2-methylthioindoleninone as alternative precursors to Tyrian purple. *Aust. J. Chem.* 26:2153–2157.

BAKER, J., and DUKE, C. 1976. Isolation of choline ester salts of tyrindoxyl sulphate from the marine molluscs *Dicathais orbita* and *Mancinella keineri*. *Tetrahedron Lett.* 15:1233–1234.

BAKER, J., and SUTHERLAND, M. 1968. Pigments of marine animals VIII. Precursors of 6,6′-dibromoindigotin (Tyrian purple) from the mollusc *Dicathais orbita* (Gmelin). *Tetrahedron Lett.* 1:43–46.

BANDARANAYAKE, W. 2006. The nature of pigments in marine invertebrates. *Nat. Prod. Rep.* 23:223–255.

BENKENDORFF, K., BREMNER, J., and DAVIS, A. 2000. Tyrian purple precursors in the egg masses of the Australian muricid, *Dicathais orbita*: A possible defensive role. *J. Chem. Ecol.* 26:1037–1050.

BENKENDORFF, K., BREMNER, J., and DAVIS, A. 2001. Indole derivatives from the egg masses of muricid molluscs. *Molecules* 6:70–78.

BENKENDORFF, K., WESTLEY, C., and GALLARDO, C. 2004. Observations of purple pigments in the egg capsules, hypobranchial and reproductive glands from seven species of the Muricidae (Gastropoda: Mollusca). Invertebr. *Reprod. Dev.* 46:93–102.

CHRISTOPHERSEN, C. 1983. Marine indoles, pp. 259–285, in P. J. Scheuer (ed.). Marine Natural Products, Chemical and Biological PerspectivesAcademic, New York, U.S.A.

CHRISTOPHERSEN, C., WATJEN, F., BUCHARDT, O., and ANTHONI, U. 1978. A revised structure of tyriverdin: The precursor to Tyrian purple. *Tetrahedron. Lett.* 34:2779–2781.

CLARK, R., and COOKSEY, C. 1997. Bromoindirubins: the synthesis and properties of minor components of Tyrian purple and the composition of the colourant from *Nucella lapillus*. *J. Soc. Dyers Colour* 113: 316–321.

CLARKE, K., and GORLEY, R. 2001. PRIMER v5: User Manual/ Tutorial. PRIMER-E Ltd, Plymouth, U.K.

COLE, W. 1685. Letter to the philosophical society of oxford containing observations on the purple fish. *Phil. Trans. R. Soc.* 15:1278.

COOKSEY, C. 2001a. Tyrian Purple: 6,6′-dibromoindigo and related compounds. *Molecules* 6:736–769.

COOKSEY, C. 2001b. The synthesis and properties of 6-bromoindigo: Indigo blue or Tyrian purple? The effect of physical state on the colours of indigo and bromoindigos. *Dyes in History and Archaeology* 16–17:97–104.

COOKSEY, C. 2006. Marine indirubins, Indirubin, the Red Shade of Indigo. pp. 23–30, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

COOKSEY, C., and WITHNALL, R. 2001. Chemical studies on *Nucella lapillus*. *Dyes in History and Archaeology* 16–17:91–96.

DUBOIS, R. 1909. Recherches sur la pourpre et sur quelques autres pigments animaux. *Arch. Zool. Expt.* 42:471–590.

ELSNER, D., and SPANIER, E. 1985. The dyeing with Murex extracts, an unusual dyeing method of wool to the biblical sky blue. Proceedings of the 7th International Wool and Textile Research Conference, Tokyo, Japan. 5:118–130.

FLEURY, B., COLL, J., and SAMMARCO, P. 2006. Complementary (secondary) metabolites in a soft coral: Sex-specific variability, inter-clonal variability and competition. *Mar. Ecol.* 27:204–218.

FRETTER, V. 1941. The genital ducts of some British stenoglossan prosobranchs. *J. Mar. Biol. Ass. U.K.* 25:173–211.

FRIEDLANDER, P. 1909. Ueber den Farbstoff des antiken Purpura aus *Murex brandaris*. *Ber.* 42:765–770.

FUJISE, Y., MIWA, K., and ITO, S. 1980. Structure of tyriverdin, the intermediate precursor to Tyrian purple. *Chem. Let.* 6:631–632.

GIBSON, C., and WILSON, S. 2003. Imposex still evident in Australia 10 years after tributyltin restrictions. *Mar. Environ. Res.* 55:101–112.

HAUBRICHS, R. 2004. L'etude de la pourpre: Histoire d'une couleur, chimie et experimentations. *Preist. Alp* 20:133–160.

HAUBRICHS, R. 2006. Natural history and iconography of purple shells, Indirubin, the Red Shade of Indigo. pp. 55–70, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

HIGA, T., and SCHEUER, P. 1976. Bisindoxyl-derived blue marine pigments. *Heterocycles* 4:227–230.

KARAPANAGIOTIS, I., DE, and VILLEMEREUIL, V. 2006. Identification of the colouring constituents of four natural indigoid dyes. *Liq. Chromatogr. Relat. Technol.* 29:1491–1502.

KAY, E., WELLS, F., and PONDER, W. 1998. Class Gastropoda, Mollusca the Southern Synthesis Part B. p. 161, in P. Beesley, G. Ross, and A. Wells (eds.). CSIRO, Melbourne, Australia.

KOREN, Z. 1995. High-performance liquid chromatographic analysis of an ancient Tyrian purple dyeing vat from Israel. *Isr. J. Chem.* 35:117–124.

KOREN, Z. 2006. HPLC-PDA analysis of brominated indirubinoid, indigoid and isatinoid dyes, Indirubin, the Red Shade of Indigo. pp. 45–53, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

MAGIATIS, P., and SKALTSOUNIS, A. L. 2006. From *Hexaplex trunculus* to new kinase inhibitory indirubins, Indirubin, the Red Shade of Indigo. pp. 147–156, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

MARCHINI, D., GIORDANO, P., AMONS, R., BERNINI, L., and DALLAI, R. 1993. Purification and primary structure of ceratotoxins A and B, two antibacterial peptides from the female reproductive accessory glands of the medfly *Ceratitis capitata* (Insecta: Diptera). *Insect Biochem. Mol. Biol.* 5:591–598.

MCGOVERN, P., and MICHEL, R. 1990. Royal purple dye: The chemical reconstruction of the ancient Mediterranean industry. *Acc. Chem. Res.* 23:152–158.

MCGOVERN, P., LAZAR, J., and MICHEL, R. 1990. The analysis of indigoid dyes by mass-spectrometry. *J. Soc. Dyers Colour* 106:22–25.

MEIJER, L., SKALTSOUNIS, A. L., MAGIATIS, P., POLYCHRONOPOULOS, P., KNOCKAERT, M., LEOST, M., YAN, X., VONICA, C., BRIVANLOU, A., DAJANI, R. et al. 2003. GSK-3-Selective inhibitors derived from Tyrian purple indirubins. *Chem. Biol.* 10:1255–1266.

MICHEL, R., LAZAR, J., and MCGOVERN, P. 1992. The chemical composition of indigoid dyes from the hypobranchial glandular secretions of Murex molluscs. *J. Soc. Dyers Colour* 108:150–154.

MIDDLEFART, P. 1992a. Morphology and anatomy of *Chicoreus brunneus* (Link, 1807): Description of shell and soft part. *T. M. M. P.* 11:54–60.

MIDDLEFART, P. 1992b. Morphology and anatomy of *Chicoreus ramosus* (Linnaeus, 1758) soft parts. *T. M. M. P.* 11:54–60.

NAEGEL, L., and COOKSEY, C. 2002. Tyrian purple from marine muricids, especially from *Plicopurpura pansa* (Gould, 1853). *J. Shellfish Res.* 21:193–200.

PALMA, H., PAREDES, J., and CRISTI, E. 1991. 6,6′-dibromoindigotin en capsulas de embriones di *Concholepas concholepas* (Bruguiere, 1789). *Medio. Ambiente.* 11:93–95.

PECK, A. 1970. Aristotles' Historia Animalium. Harvard University Press, Great Britain.

POLEC-PAWLAK, K., PUCHALASKA, M., WITIWSKA-JAROSZ, J., and JAROSZ, M. 2006. Mass spectrometry: An efficient tool for the identification of indirubin and indigo related dyestuffs, Indirubin, the Red Shade of Indigo. pp. 115–125, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

PUCHALASKA, M., POLEC-PAWLAK, K., ZANDRONZA, I., HRYSZKO, H., and JAROSZ, M. 2004. Identification of natural indigoid dyes in natural organic pigments used in historical art objects by high-performance liquid chromatography coupled to electrospray ionization mass spectrometry. *J. Mass Spectrom.* 39:1441–1449.

RASH, L., and HODGSON, W. 2002. Pharmacology and biochemistry of spider venoms. *Toxicon* 40:225–254.

ROLLER, R., RICKETT, J., and STICKLE, W. 1995. The hypobranchial gland of the estuarine snail *Stramonita haemastoma canaliculata* (Gray) (Prosobranchia: Muricidae): a light electron microscopical study. *Am. Malacol. Bull.* 11:177–190.

ROSEGHINI, M., SEVERINI, C., FALCONIERI, E., and ERSPAMER, V. 1996. Choline esters and biogenic amines in the hypobranchial gland of 55 molluscan species of the neogastropod Muricidae Superfamily. *Toxicon* 34:33–55.

ROSETTO, M., MANETTI, A., GIORDANO, P., MARRI, L., AMOS, R., BALDARI, C., MARCHINI, D., and DALLAI, R. 1996. Molecular characterization of ceratotoxin C, a novel antibacterial female-specific peptide of the ceratotoxin family from the medfly *Ceratitis capitata*. *Eur. J. Biochem.* 241:330–337.

SUSSE, P., and KRAMPE, C. 1979. 6,6′-dibromo-indigo, a main component of Tyrian purple. Its crystal structure and light absorption. *Naturwissenschaften* 66:110.

SZOSTEK, B., ORSKA-GAWRYS, J., SUROWIEC, I., and TROJANOWICZ, M. 2003. Investigation of natural dyes occurring in historical Coptic textiles by high-performance liquid chromatography with UV-Vis and mass spectrometric detection. *J. Chromatogr.* 1012:179–192.

VERHECKEN, A. 1989. The indole pigments of Mollusca. *Annales de la Societe Royale Zoologique de Belgique* 119:181–197.

VINE, K., LOCKE, J., ANSON, M., BENKENDORFF, K., PYNE, S., and BREMNER, J. 2007. *In vitro* cytotoxicity evaluation of some substituted isatin derivatives. *Bioorg. Med. Chem.* 15:931–938.

WESTLEY, C., VINE, K., and BENKENDORFF, K. 2006. A proposed functional role for indole derivatives in reproduction and defense of the Muricidae (Neogastropoda: Mollusca), Indirubin, the Red Shade of Indigo. pp. 31–44, in L. Meijer, N. Guyard, A. L. Skaltsounis, and G. Eisenbrand (eds.). Life in Progress Editions, Roscoff, France.

WITHNALL, R., PATEL, D., COOKSEY, C., and NAEGEL, L. 2003. Chemical studies of the purple dye of *Purpura pansa*. *Dyes in History and Archaeology* 1617:91–96.

WOUTERS, J. 1992. A new method for the analysis of blue and purple dyes in textiles. *Dyes in History and Archaeology* 10:17–21.

WOUTERS, J., and VERHECKEN, A. 1991. High-performance liquid chromatography of blue and purple indigoid natural dyes. *J. Soc. Dyers Colour.* 107:266–269.

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Variation of Phlorotannins Among Three Populations of *Fucus vesiculosus* as Revealed by HPLC and Colorimetric Quantification

R. Koivikko · J. K. Eränen · J. Loponen · V. Jormalainen

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Abstract In ecological studies, phlorotannins have conventionally been quantified as a group with similar functionality. Since this group consists of oligo- and polymers, the quantification of their pooled contents alone may not sufficiently describe the variation of these metabolites. Genetic variation, plastic responses to environment, and the ecological functions of separate phlorotannin oligo- and polymers may differ. Two analyses, i.e., the colorimetric Folin–Ciocalteu assay and a normal-phase high-performance liquid chromatographic (HPLC) method were used to study genetic and environmental variation in phlorotannins of the brown alga *Fucus vesiculosus* (L.). The colorimetric method provides the total phlorotannin content, the latter a profile of 14 separate traces from the phenolic extract that represent an individual or groups of phlorotannins. We reared the algae that originated from three separate populations in a common garden for 3 months under ambient and enriched-nutrient availability and found that they differed in both their total phlorotannin content and in phlorotannin profiles. Some individual traces of the profiles separated the populations more clearly than the colorimetric assay. Although nutrient enrichment decreased total phlorotannin content, it did not show a significant influence on the phlorotannin profile. This implies that plastic responses of compounds other than phlorotannins may interfere with the determination of total phlorotannins. However, the phlorotannin profile and the total content showed genetic variation among local populations of *F. vesiculosus*; therefore, phlorotannins may respond to natural selection and evolve both quantitatively and qualitatively.

Keywords Brown algae · Common garden · Folin–Ciocalteu · High-performance liquid chromatography · Phenolic compounds

R. Koivikko (✉) · J. K. Eränen · V. Jormalainen
Section of Ecology, Department of Biology, University of Turku,
FIN-20014 Turku, Finland
e-mail: riitta.koivikko@utu.fi

R. Koivikko · J. Loponen
The Laboratory of Organic Chemistry and Chemical Biology,
Department of Chemistry,
University of Turku,
FIN-20014 Turku, Finland

Introduction

Phlorotannins are polyphenols formed by the polymerization of phloroglucinol (1,3,5-trihydroxybenzene) units and are known only from brown algae (Ragan and Glombitza 1986; Amsler and Fairhead 2006). The variation in total phlorotannin content has been intensely studied, particularly due to the putative adaptive nature of these compounds as deterrents against herbivores (reviews by Targett and Arnold 1998, 2001; Amsler and Fairhead 2006). Total phlorotannin content varies as a response to environmental factors such as salinity, nutrient and light availability, ultraviolet radiation, and the intensity of herbivory (reviews by Targett and Arnold 1998; Amsler and Fairhead 2006; Jormalainen and Honkanen 2008). Due to this high plasticity with respect to the number of distinct environmental cues, the several putative primary and secondary functions, and various methodological concerns, the ecological roles of phlorotannins have been considered somewhat controversial (Amsler and Fairhead 2006).

Total phlorotannin contents also commonly vary among local populations of algae (Pavia et al. 1999, 2003; Honkanen et al. 2002; Hemmi and Jormalainen 2004b). This variation may arise either from genetic divergence

among populations, or from plastic responses of algae to environmental variation, or both. Although the role of genetic differentiation in the among-population variation of phlorotannins has not been directly tested, such differentiation is possible. Phlorotannin concentration in *Fucus vesiculosus* (L.) is genetically variable (Jormalainen and Honkanen 2004). Based on neutral molecular markers, genetic differentiation has been found among adjacent *F. vesiculosus* populations in the Baltic Sea (Bergström et al. 2005; Tatarenkov et al. 2007) and among populations of *F. serratus* (L.), in which species there is substantial genetic substructuring at a scale of less than 2 km along the Atlantic and southern Baltic Sea coastlines (Coyer et al. 2003). While the neutral markers indicate differentiation due to genetic drift, spatial variation in natural selection may, in addition, select for among-population differences in the contents of total phlorotannins (Jormalainen and Honkanen 2004).

Nutrient availability is an environmental factor that is commonly found to influence the resource allocation of plants (e.g., Koricheva et al. 1998), and thus, the contents of total phlorotannins also (review by Amsler and Fairhead 2006). Nutrient enrichment causes a decrease of total phlorotannin content in the growing tips of *F. vesiculosus* (Hemmi et al. 2004; Koivikko et al. 2005). Such an effect can arise due to growth increase at the expense of soluble phlorotannins production, or in eutrophic conditions, due to an increase of epibiota and consequent shading of the thallus. The resource-based theories on this allocation between growth and defense, e.g., the carbon-nutrient balance (CNB) and the growth-differentiation balance (GDB) models, have been used successfully as the contextual framework to understand such plasticity in the contents of total phlorotannins (reviews by Cronin 2001; Pavia and Toth 2008).

In ecological studies, phlorotannin content has practically always been quantified by using colorimetric methods, e.g., Folin–Ciocalteu, Folin–Denis, or DMBA, mainly because individual phlorotannins are difficult to measure with common analytical techniques because of their large size, polarity, and water solubility, and the relative similarity of structures of separate oligo- and polymers (Stern et al. 1996; Targett and Arnold 1998; Koivikko et al. 2007). Such a total quantification, however, may conceal the variation of individual compounds present. In the analyses of total phenolics, the variation in the reactivity of structurally different phlorotannins is combined with that variation caused by different amounts of compounds. For example, phlorotannins of different molecular size differ in their bioactivity (Boettcher and Targett 1993). Therefore, more specific methods to separate individual phlorotannins are needed to measure all the variation of phlorotannins and to evaluate their role in ecological interactions. It may also be possible that genetic variation exists in the phlorotannin profile or that separate phlorotannins respond to different environmental cues in distinct ways.

To study both genetic and environmental variation in the phlorotannin profile of the brown alga *Fucus vesiculosus*, we applied the high-performance liquid chromatographic (HPLC) method for separation of phlorotannins, previously described by Koivikko et al. (2007). Specifically, we tested the occurrence of genetic among-population phlorotannin differentiation by rearing algae that originated from three separate populations in a common environment and by analyzing their phlorotannins. We also examined within-population genetic variance by measuring the variation among cloned genotypes. To examine the environmental plasticity of phlorotannins, algae were reared in both ambient and nutrient-enriched conditions. We compared the phlorotannin variation revealed by using two assay methods which generated total phlorotannin content and phlorotannin profiles, i.e., the Folin–Ciocalteu and HPLC assays, respectively.

Methods and Materials

Study Organism F. vesiculosus is a perennial brown alga that dominates the shallow littoral rocky bottoms in the nontidal study area in the Archipelago Sea, SW Finland. In the naturally fragmented environment of the archipelago, local populations of *F. vesiculosus* are somewhat isolated owing to the low dispersal ability of fucoid propagules (Chapman 1995). The chemical contents of several metabolites (Hemmi and Jormalainen 2004a) and the food quality for herbivores (Honkanen and Jormalainen 2002) vary among populations, even at a scale of a few kilometers. The populations of *F. vesiculosus* in our study area face a gradient of ambient nutrient availability, from high, close to the mainland, to low, in the outer archipelago (Hänninen et al. 2000).

Sampling and Experimental Growing Conditions of Algae We collected 15 *F. vesiculosus* genotypes from three different populations (Aaviikki: 60°21.48′ N, 22°32.45′ E; Jurmo: 59°49.36′ N, 21°36.57′ E; Säppi: 61°28.80′ N, 21° 20.80′ E), located 80–185 km apart, from depths of 0.5–2 meters in May 2002. Genotypes were defined as entities growing from a single branch of thallus on a holdfast. Each of the 45 genotypes was split into four 3- to 5-cm long apical pieces (hereafter "algal individual"). To eliminate among population differences caused by environmental plasticity, these algal individuals were transferred to grow in the common littoral environment of Katava island (60° 14.03′ N, 21°57.51′ E). Algal individuals were attached with a clothes-peg to four plastic grid mats, one piece of each genotype to each mat. Two mats were assigned to the

nutrient enrichment treatment (nutr) and two to ambient nutrient conditions (ctrl). The mats were settled randomly to a depth of 1.5 m, at a minimum distance of 4 m apart. Nutrient enrichment consisted of a nitrogen, phosphorous, and potassium mixture (16:5:9) in a controlled-release fertilizer (Osmocote exact standard 3–4 M). Fabric packages containing nutrients (ca 500 g) were attached to each nutrient enrichment mat and replaced monthly during the experiment to ensure the fertilizer's sufficiency. Nutrient enrichment of this magnitude under comparable conditions in the same area has been shown to increase the concentration of DIN and DIP, 5 and 1.2 times higher, respectively, compared to ambient nutrient concentration (Korpinen et al. 2007). After 3 mo (May 27–August 27), we took samples of the algal individuals, from the apical parts grown during the experiment, for analysis of phlorotannins. We took a sample from each individual, but due to the loss of some individuals, did not get samples from every genotype (total N=138; Aaviikki: 27 ctrl, 21 nutr; Jurmo: 23 ctrl, 19 nutr; Säppi: 24 ctrl, 24 nutr). Samples were frozen immediately, freeze–dried, homogenized, and stored at −20°C until further analyses.

Phlorotannin Analysis The total phlorotannin content for all 138 samples (74 ctrl and 64 nutr) was analyzed according to the procedure reported by Koivikko et al. (2005). We used an extraction procedure for soluble phlorotannins and a quantification protocol with a modification of the Folin–Ciocalteu method with phloroglucinol as the standard agent. Due to the lack of sufficient thallus biomass (200 mg) from all samples, only 84 were analyzed by HPLC (Aaviikki: 18 ctrl, 16 nutr; Jurmo: 9 ctrl, 13 nutr; Säppi: 8 ctrl, 10 nutr). Samples for HPLC analysis were prepared and analyzed by the protocol and conditions described in Koivikko et al. (2007). In the liquid chromatographic analysis of the phenolic fraction, the contents of total phlorotannins were split into fragments. This separation procedure with a selected wavelength of 280 nm, characteristically revealed those phlorotannins which are produced via the polyketide pathway, the only known biosynthetic route for phenolic compounds in brown algae. Each separate trace in the chromatogram represents either individual phlorotannins or groups of phlorotannin molecules of similar size (Koivikko et al. 2007). The HPLC profile gave 14 clearly distinguishable traces (14 of 16 traces described in Koivikko et al. (2007) were used). The size of each individual trace is expressed as the integrated area of the peak and numbered 1 to 14 in the figures and tables, according to Fig. 1.

Statistical Methods To explore the repeatability of the individual traces in the HPLC profile, we analyzed 18 samples (including a random sample of individuals from the experiment and 6 additional samples from the study populations) twice. The whole process from sample preparation to the calculation of the results from HPLC analysis was replicated. Repeatability measures the consistency of repeated measures within a subject (here, a sample), and obtains values from 0 to1, the latter occurring where the repeated measures within a subject are identical (Krebs 1999). A one-way analysis of variance (ANOVA) was carried out for each trace, i.e., the integrated areas of peaks, to obtain the variance components. The repeatability was calculated as a coefficient of intra-class correlation according to Krebs (1999). As a criterion of repeatability, we used the 95% confidence interval of the intra-class correlation coefficient; the measurement was considered non-repeatable when the confidence interval included zero.

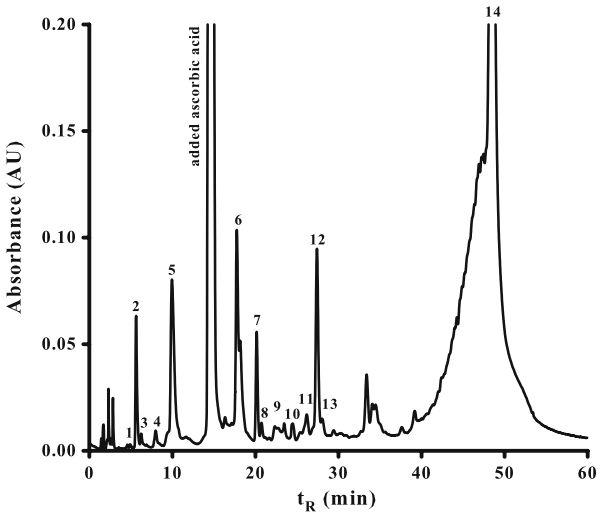

Fig. 1 Traces of soluble phenolics from polar extract of *F. vesiculosus*. *Numbers* indicate the 14 separate traces analyzed in this study

To study the contribution of the HPLC profile to the total phlorotannin content, we calculated the Pearson correlation coefficients between individual traces of the chromatogram and the contents of total phlorotannins. We further estimated how well the variation in the phlorotannin profile can explain the variation of the content of total phlorotannins by conducting a multiple regression analysis, where the total phlorotannin content was explained with all possible combinations of those seven traces that we found to be repeatable (nos. 3, 7, 8, 11–14; Fig. 1, Table 1). We did this both for the pooled data and separately for each population. The best-fitting model was found on the basis of the Akaike Information Criterion.

By using mixed-model ANOVAs, we tested both population and nutrient enrichment effects on the total phlorotannin contents and the area of each individual trace (nos. 1–14). The population and nutrient enrichment treatments were included as fixed effects, and the genotype was treated as a random effect, nested under population. To

Table 1 Repeatability of the peak area for each trace of the phlorotannin profile and the Pearson correlation coefficients of the trace area with the total phlorotannin contents (*N*= *84*)

Trace Number	Repeatability		Correlation with Total Phlorotannins	
	(+95% Confidence Interval)		*r*	*P*
1	0.19	(−0.3 to 0.6)	0.07	ns
2	0.43	(−0.03 to 0.7)	0.005	ns
3	0.72	(0.4 to 0.9)	−0.05	ns
4	−0.09	(−0.5 to 0.4)	−0.05	ns
5	0.14	(−0.3 to 0.6)	−0.03	ns
6	−0.26	(−0.6 to 0.2)	0.02	ns
7	0.69	(0.4 to 0.9)	0.23	<0.05
8	0.79	(0.5 to 0.9)	0.22	<0.05
9	0.06	(−0.4 to 0.5)	0.14	ns
10	−0.02	(−0.5 to 0.4)	−0.05	ns
11	0.90	(0.8 to 1.0)	0.28	<0.01
12	0.81	(0.6 to 0.9)	0.32	<0.01
13	0.93	(0.8 to 1.0)	0.31	<0.01
14	0.56	(0.2 to 0.8)	0.26	<0.05

estimate the variation of the individual HPLC traces attributable to algal origin, we calculated the among-population variance components. In addition, to quantify the effect of nutrient enrichment on both the individual HPLC traces and total phlorotannin content, we calculated the effect sizes (Gurevitch and Hedges 2001) of nutrient enrichment for each trace and total phlorotannins. We further tested the effects of population, nutrient enrichment, and genotype on the phlorotannin profile (including only the repeatable traces) by using a multivariate analysis of variance (MANOVA). To visualize the among-population differences in the phlorotannin profile, we conducted a principal component analysis (PCA) and plotted the values of the first and second principal component axes. In the PCA, we used genotypic means calculated over both nutrient treatments. All statistics were calculated with the SAS statistical package (SAS Institute 1999).

Results

From the HPLC profile, seven individual traces were repeatable (Table 1). According to the Pearson correlation coefficients, 6 of the 14 traces from the HPLC chromatogram correlated positively and significantly with the total phlorotannin content (Table 1). Multiple regression analysis revealed that the best-fitting model, which explained 22.9% of the variation in total phlorotannin content, included two traces of the phlorotannin profile, i.e., traces nos. 13 and 14. However, the within population analyses explained the variation in total phlorotannins better for some populations, while the profile trace patterns included in the bets-fitting model varied among populations. The best-fitting models for Säppi, Aaviikki, and Jurmo included 1 (no. 7), 2 (nos. 13 and 14), and 5 (nos. 8 and 11–14) traces, respectively, which in turn successively explained 15.3, 25.9, and 44.7% of the variation in the contents of total phlorotannins.

After rearing in a common environment for 3 mo, the three *F. vesiculosus* populations diverged with respect to both their total phlorotannin contents (Fig. 2, Table 2) and phlorotannin profiles (Fig. 2, Table 3). The MANOVA analysis of the HPLC profiles showed a significant among-population difference, particularly in the population from Aaviikki (Table 3). Although some genotypes mixed with those from other populations, the PCA analysis separated the Aaviikki population quite well (Fig. 3), the first two principal components explaining 79% of the total variance in the phlorotannin profile. By contrast, the populations that originated from Jurmo and Säppi did not differ in either the MANOVA (Table 3) or the PCA analyses (Fig. 3).

The among-population tests of the individual traces from the phlorotannin profile revealed a high variability in divergence among the separate traces; some showed none, while others showed a higher degree of divergence than what was found in the total phlorotannin content (Fig. 2). The average areas of individual traces (Fig. 4) showed similar among-population trends to those of the total phlorotannin contents: the population from Aaviikki had the highest (7.47±0.22, percent of DW and SE), with intermediate and lowest total phlorotannin contents from Jurmo (6.92±0.23) and Säppi (6.01±0.18), respectively. However, the amounts of total phlorotannins from the Säppi

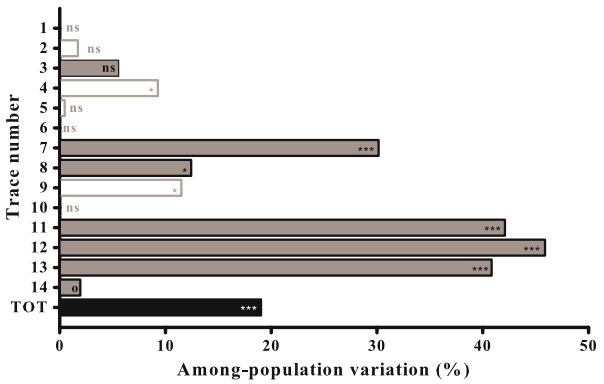

Fig. 2 The proportion of among-population variation of the total variation in the area of the 14 traces of the phlorotannin profile analyzed with HPLC (1–14, *white and light gray bars*) and in the total phlorotannin content measured with the Folin–Ciocalteu assay (TOT, *black bar*). Significances are based on the univariate ANOVA *F* statistics from the tests of among population variation. Traces are numbered as in Fig. 1. *White bars* are traces with poor repeatability. *ns* Nonsignificant, $P>0.1$, o=$0.05<P<0.1$; *=$0.01<P<0.05$, ***$P<0.001$

Table 2 Mixed model ANOVA test statistics on the effects of nutrient manipulation and original population on the total contents of phlorotannins and the variation among genotypes (N=138)

Source of Variation	Variance Estimates						
	Total Content of Phlorotannins						
	S^2	SE	χ^2	P	ndf, ddf	F	P
Random effects							
Genotype (population)	0.31	0.19	3.8	0.05			
Residual	1.64	0.24					
Fixed effects							
Population					2, 40.8	9.02	<0.001
Aaviikki vs Säppi					1, 38.5	17.93	<0.001
Aaviikki vs Jurmo					1, 42.6	3.13	0.084
Jurmo vs Säppi					1, 41.5	5.62	<0.05
Nutrient enrichment					1, 106	6.53	<0.05
Population × Nutrient enrichment					2, 106	0.34	ns

population deviated most from the other populations (Table 2), while the phlorotannin profile of the Aaviikki showed the greatest variation (Table 3). The area of the peak did not correlate with the magnitude of the among-population response: a rather small trace had an equally high among-population divergence, similar to one that was ten times higher in intensity (traces nos. 13 vs 7, Figs. 2 and 4).

Although the contents of total phlorotannins were significantly lower in the nutrient enrichment (6.47%±0.19, SE) than in the control treatment (7.07%±0.18, SE; Fig. 5, Table 2), the HPLC profile showed no differences between them (Fig. 5, Table 3). In the nutrient enrichment treatment, only the poorly repeatable trace no. 6 was higher than in the control (Fig. 5).

The genotypic variation within the population, in both the content of total phlorotannins (Table 2) and the phlorotannin profile (Table 3) was marginally significant.

Table 3 MANOVA test statistics on the effect of population, nutrient enrichment and genotype on the HPLC profile of phlorotannins (areas of the traces 3, 7, 8, 11–14) in *F. vesiculosus* (N=84)

Source of variation	Pillai's trace	ndf, ddf	F	P
Population	0.83	14, 60	3.05	<0.01
Aaviikki vs Säppi	0.70	7, 29	9.72	<0.001
Aaviikki vs Jurmo	0.55	7, 29	4.98	<0.001
Jurmo vs Säppi	0.32	7, 29	1.96	0.095
Nutrient enrichment	0.14	7, 29	0.65	ns
Population × Nutrient enrichment	0.41	14, 60	1.12	ns
Genotype (population)	3.48	245, 301	1.21	0.055

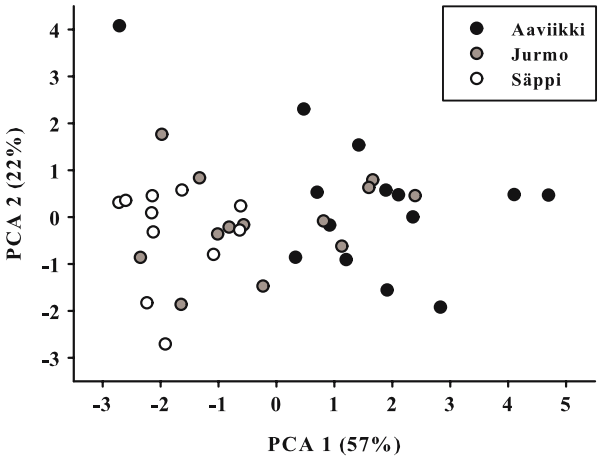

Fig. 3 The scattering of algal genotypes from three populations according to the values of the first and second principal components. The PCA axes represent linear combinations of the seven variables (nos. 3, 7, 8, 11–14) from the phlorotannin profile analyzed with HPLC. Each dot represents a mean of a genotype (N=38)

Discussion

Our main finding was that the three populations of *F. vesiculosus* differed in both their phlorotannin profiles and

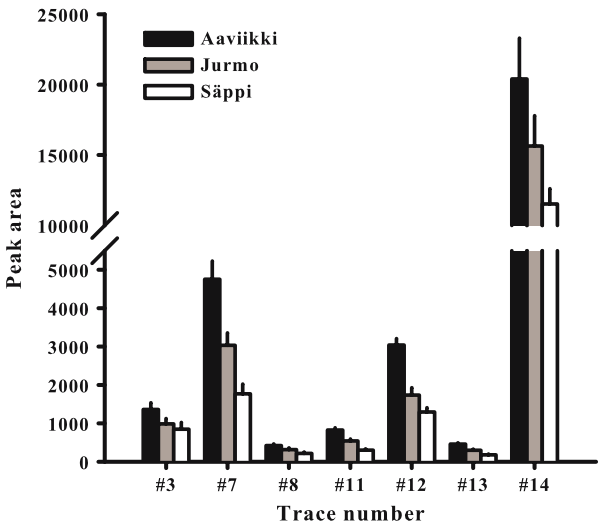

Fig. 4 The mean area (and SE) of the repeatable traces of the phlorotannin profile in different population after a 3-mo growth period in a common environment

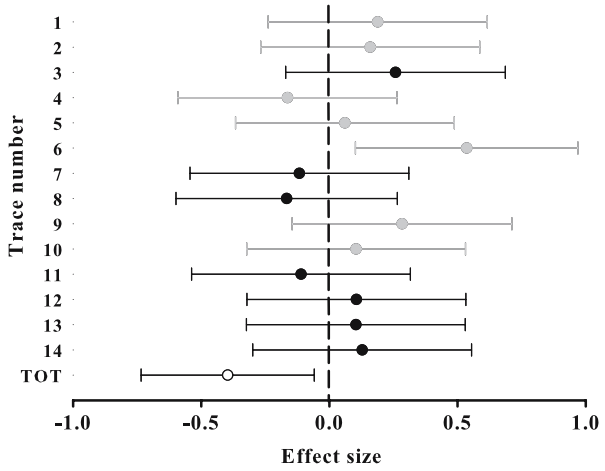

Fig. 5 The effect size (standardized mean difference between nutrient enrichment and control treatment levels±95% confidence intervals) of nutrient enrichment separately for each of the 14 traces of the phlorotannins profile analyzed with HPLC and the total contents of phlorotannins measured using the Folin–Ciocalteu assay (TOT, *white circle*). *Gray bars* indicate traces with poor repeatability

in the total phlorotannin content after the algae had grown for 3 mo in a common environment. A period of 3 mo is plenty of time for the phlorotannin pool to turn over and respond to the new common environment: in brown algae, metabolic turnover of phlorotannins takes place in days under field conditions (Arnold and Targett 2000), and in *F. vesiculosus*, plastic responses to environmental change occur in less than 1.5 mo (Hemmi et al. 2004; Honkanen and Jormalainen 2005; Koivikko et al. 2005). Thus, preservation of differences shows that the among-population variation in phlorotannins is genetic in origin. Further support for the genetic variation came from the marginally significant variation among the cloned genotypes within populations. Although genetic variation in total phlorotannins has also been found previously (Jormalainen et al. 2003; Jormalainen and Honkanen 2004), this is the first report on genetic variation of the phlorotannin profile.

The formation of phlorotannin oligo- and polymers in plants from their precursors necessitates enzyme activity (Waterman and Mole 1994). Those enzymes involved are transcription products of the plant genome (Waterman and Mole 1994); thus, the genetic variability in the profile and amount of phenolic compounds in a plant must reflect evolutionary differentiation among populations. Such a microevolutionary change may be caused either by natural selection or random changes in gene frequencies. We cannot exclude the role of genetic drift in the differentiation of phlorotannins, but they have been shown not to be selectively neutral; the form and intensity of natural selection for total phlorotannins varies among different environments (Jormalainen and Honkanen 2004). Therefore, the geographic variation in environmental conditions among local populations, i.e., in abiotic and biotic selective agents, may explain the observed differentiation.

The populations from Aaviikki and Jurmo were more alike in their contents of total phlorotannins but differed in their phlorotannin profiles, whereas the populations from Jurmo and Säppi differed in their total phlorotannin contents but resembled each other in their HPLC profiles. Thus, how the different compounds appeared varied among populations. Some individual traces differed among the populations. It might be suspected that such traces could be under differential selection, i.e., having ecological functions whose roles vary among populations. As with total phlorotannins, the phlorotannin profile also seems to reflect the quantitative differences among populations to some extent. However, the among-population differences in phlorotannin profile are also qualitative, as the magnitude of divergence varied among the separate traces.

Nutrient enrichment of the growing environment caused a decrease in total phlorotannins but did not change the composition of the profile. Similar effects of nutrient enrichment on total phlorotannins have been found previously in manipulative and correlative studies in *F. vesiculosus* (Peckol et al. 1996; Pavia and Toth 2000; Jormalainen et al. 2003; Hemmi et al. 2004) and in *Ascophyllum nodosum* (Svensson et al. 2007), but not in all studies of brown algae (Cronin and Hay 1996; Pavia and Toth 2000). The negative influence of nutrient enrichment on the total phlorotannin contents is predicted by both the CNB and GDB hypotheses.

The lack of differences in phlorotannin profiles among nutrient levels may indicate that such plasticity includes neither qualitative nor quantitative changes, but that nutrient enrichment induced a plastic response in compounds other than phlorotannins. As the colorimetric method gives the total contents of all compounds that have a similar functional group, it also measures some compounds not classified as phenolic but that include an oxidizable group. For example, amino acids and proteins, ascorbic acid, urea, and diethyl ether are known to cause such inaccuracy (Ragan and Glombitza 1986; Steinberg 1989; Waterman and Mole 1994; Van Alstyne 1995; Stern et al. 1996). It has been suggested that the concentrations of compounds other than phlorotannins in the Folin–Ciocalteu assay range between 3 and 5% (Van Alstyne 1995). Thus, although an increase of about 10% in the value of the FC assay as a response to nutrient enrichment is unlikely to result from non-phlorotannin compounds alone, the lack of any response in the phlorotannin profile may indicate that plasticity in non-phlorotannin compounds may interfere with the determination of total phlorotannins more than has been conventionally assumed. However, there remains the possibility that the profile does not include those phlorotannins that increase with the nutrient level. Some compounds might remain undetected or be underrepresented in liquid chroma-

tography because of low UV response in relation to the concentration, large molecular structure, overlapping of isomers, or an unsuitable polarity for chromatographic determination (Adamson et al. 1999; Lazarus et al. 1999). As the area of a trace is affected by the sensitivity of the compound to detection, the stability of the molecule and its amount can at best, only be an indication of the actual quantity of the compound. However, it is likely that oligomers or polymers up to hexamers are in fact detectable by NP-HPLC (Koivikko et al. 2007).

Six of the fourteen traces of the phlorotannin profile correlated positively with the contents of total phlorotannins. However, when using pooled data, the best multiple regression model of total phlorotannins on the phlorotannin profile explained less than a quarter of the observed variation. In the within-population analyses, the amount of explained variation differed among populations; in one, it increased to 45%. Such variability and increase in the amount of explained variance at the within population level was expected because the phlorotannin profiles of the populations differed. At least two factors contribute to the relatively poor consistency of the total phlorotannins and phlorotannins profile, i.e., the HPLC method may leave some phenolic compounds undetected, while the Folin–Ciocalteu assay might measure some compounds not classified as phlorotannins.

To summarize, the phlorotannin profile shows genetic variation among local populations of *F. vesiculosus*. Thus, not only the total contents of phlorotannins but also the amounts of separate phlorotannins may respond to natural selection and evolve. This implies that local populations of brown algae may become qualitatively different with respect to their major group of secondary metabolites. While understanding the functions and variability of separate phlorotannins remains a challenge, the HPLC assay of the phenolic fraction provides a method to explore their responses to both the abiotic and biotic factors. To better understand the suggested multiple roles of phlorotannins, the phlorotannin profile can give more detailed data than the total phlorotannin content.

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References

ADAMSON, G. E., LAZARUS, S. A., MITCHELL, A. E., PRIOR, R. L., CAO, G. H., JACOBS, P. H., KREMERS, B. G., HAMMERSTONE, J. F., UCKER, R. B., ITTER, K. A., and SCHMITZ, H. H. 1999. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. *J. Agric. Food Chem* 47:4184–4188.

AMSLER, C. D., and FAIRHEAD, V. A. 2006. Defensive and sensory chemical ecology of brown algae. *Adv. Bot. Res* 43:1–91.

ARNOLD, T. M., and TARGETT, N. M. 2000. Evidence for metabolic turnover of polyphenolics in tropical brown algae. *J. Chem. Ecol* 26:1393–1410.

BERGSTRÖM, L., TATARENKOV, A., JOHANNESSON, K., JONSSON, R. B., and KAUTSKY, L. 2005. Genetic and morphological identification of *Fucus radicans* sp Nov (Fucales, Phaeophyceae) in the brackish Baltic Sea. *J. Phycol* 41:1025–1038.

BOETTCHER, A. A., and TARGETT, N. M. 1993. Role of polyphenolic molecular-size in reduction of assimilation efficiency in *Xiphister mucosus*. *Ecology* 74:891–903.

CHAPMAN, A. R. O. 1995. Functional ecology of fucoid algae–23 years of progress. *Phycologia* 34:1–32.

COYER, J. A., PETERS, A. F., STAM, W. T., and OLSEN, J. L. 2003. Post-ice age recolonization and differentiation of *Fucus serratus* L. (Phaeophyceae; Fucaceae) populations in Northern Europe. *Mol. Ecol* 12:1817–1829.

CRONIN, G. 2001. Resource allocation in seaweeds and marine invertebrates. Marine Chemical Ecology. pp. 325–353, in J. B. McClintock, and B. J. Baker (eds.). CRC Press, Boca Raton, FL.

CRONIN, G., and HAY, M. E. 1996. Effects of light and nutrient availability on the growth, secondary chemistry, and resistance to herbivory of two brown seaweeds. *Oikos* 77:93–106.

GUREVITCH, J., and HEDGES, L. V. 2001. Meta-analysis - combining the results of independent experimentsDesign and Analysis of Ecological Experiments. pp. 347–369, in S. M. Scheiner, and J. Gurevitch (eds.). Oxford University Press, New York.

HÄNNINEN, J., VUORINEN, I., HELMINEN, H., KIRKKALA, T., and LEHTILÄ, K. 2000. Trends and gradients in nutrient concentrations and loading in the Archipelago Sea, Northern Baltic, in 1970–1997. *Estuar. Coast. Shelf S* 50:153–171.

HEMMI, A., HONKANEN, T., and JORMALAINEN, V. 2004. Inducible resistance to herbivory in *Fucus vesiculosus* - duration, spreading and variation with nutrient availability. *Mar. Ecol. Prog. Ser* 273:109–120.

HEMMI, A., and JORMALAINEN, V. 2004a. Genetic and environmental variation in performance of a marine isopod: Effects of eutrophication. *Oecologia* 140:302–311.

HEMMI, A., and JORMALAINEN, V. 2004b. Geographic covariation of chemical quality of the host alga *Fucus vesiculosus* with fitness of the herbivorous isopod *Idotea baltica*. *Mar. Biol* 145:759–768.

HONKANEN, T., and JORMALAINEN, V. 2002. Within-alga integration and compensation: Effects of simulated herbivory on growth and reproduction of the brown alga, *Fucus vesiculosus*. *Int. J. Plant Sci* 163:815–823.

HONKANEN, T., and JORMALAINEN, V. 2005. Genotypic variation in tolerance and resistance to fouling in the brown alga *Fucus vesiculosus*. *Oecologia* 144:196–205.

HONKANEN, T., JORMALAINEN, V., HEMMI, A., MÄKINEN, A., and HEIKKILÄ, N. 2002. Feeding and growth of the isopod *Idotea baltica* on the brown alga *Fucus vesiculosus*: Roles of inter-population and within-plant variation in plant quality. *Ecoscience* 9:332–338.

JORMALAINEN, V., and HONKANEN, T. 2004. Variation in natural selection for growth and phlorotannins in the brown alga *Fucus vesiculosus*. *J. Evol. Biol* 17:807–820.

JORMALAINEN, V., and HONKANEN, T. 2008. Macroalgal chemical defenses and their roles in structuring temperate marine communities. Algal Chemical Ecology. pp. 57–89, in C. D. Amsler (ed.). Springer, Berlin.

JORMALAINEN, V., HONKANEN, T., KOIVIKKO, R., and ERÄNEN, J. 2003. Induction of phlorotannin production in a brown alga: defense or resource dynamics? *Oikos* 103:640–650.

KOIVIKKO, R., LOPONEN, J., HONKANEN, T., and JORMALAINEN, V. 2005. Contents of soluble, cell-wall-bound and exuded phlorotannins in the brown alga *Fucus vesiculosus*, with implications on their ecological functions. *J. Chem. Ecol* 31:195–212.

KOIVIKKO, R., LOPONEN, J., PIHLAJA, K., and JORMALAINEN, V. 2007. High-performance liquid chromatographic analysis of phlorotannins from the brown alga *Fucus vesiculosus*. *Phytochem. Anal* 18:326–332.

KORICHEVA, J., LARSSON, S., HAUKIOJA, E., and KEINÄNEN, M. 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. *Oikos* 83:212–226.

KORPINEN, S., JORMALAINEN, V., and HONKANEN, T. 2007. Effects of nutrients, herbivory, and depth on the macroalgal community in the rocky sublittoral. *Ecology* 88:839–852.

KREBS, C. J. 1999. Ecological Methodology. pp. 554–559. Addison-Wesley Longman, California, USA.

LAZARUS, S. A., ADAMSON, G. E., HAMMERSTONE, J. F., and SCHMITZ, H. H. 1999. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. *J. Agric. Food Chem* 47:3693–3701.

PAVIA, H., and TOTH, G. 2008. Macroalgal models in testing and extending defense theories. Algal Chemical Ecology. pp. 147–172, in C. D. Amsler (ed.). Springer, Berlin.

PAVIA, H., and TOTH, G. B. 2000. Influence of light and nitrogen on the phlorotannin content of the brown seaweeds *Ascophyllum nodosum* and *Fucus vesiculosus*. *Hydrobiologia* 440:299–305.

PAVIA, H., TOTH, G. B., LINDGREN, A., and ÅBERG, P. 2003. Intraspecific variation in the phlorotannin content of the brown alga *Ascophyllum nodosum*. *Phycologia* 42:378–383.

PAVIA, H., TOTH, G., ÅBERG, P. 1999. Trade-offs between phlorotannin production and annual growth in natural populations of the brown seaweed *Ascophyllum nodosum*. *J. Ecol.* 87:761–771.

PECKOL, P., KRANE, J. M., and YATES, J. L. 1996. Interactive effects of inducible defense and resource availability on phlorotannins in the North Atlantic brown alga *Fucus vesiculosus*. *Mar. Ecol. Prog. Ser* 138:209–217.

RAGAN, M. A., and GLOMBITZA, K. W. 1986. Phlorotannins, brown algal polyphenols. Progress in Phycological Research. pp. 129–241, in F. E. Round, and D. J. Chapman (eds.). Biopress Ltd, Bristol.

SAS Institute 1999. SAS/STAT User's Guide, Version 8. SAS Institute Inc., Cary, NC, USA.

STEINBERG, P. D. 1989. Biogeographical variation in brown algal polyphenolics and other secondary metabolites—Comparison between temperate Australasia and North-America. *Oecologia* 78:373–382.

STERN, J. L., HAGERMAN, A. E., STEINBERG, P. D., WINTER, F. C., and ESTES, J. A. 1996. A new assay for quantifying brown algal phlorotannins and comparisons to previous methods. *J. Chem. Ecol* 22:1273–1293.

SVENSSON, C. J., PAVIA, H., and TOTH, G. B. 2007. Do plant density, nutrient availability, and herbivore grazing interact to affect phlorotannin plasticity in the brown seaweed *Ascophyllum nodosum*. *Mar. Biol* 151:2177–2181.

TARGETT, N. M., and ARNOLD, T. M. 1998. Predicting the effects of brown algal phlorotannins on marine herbivores in tropical and temperate oceans. *J. Phycol* 34:195–205.

TARGETT, N. M., and ARNOLD, T. M. 2001. Effects of secondary metabolites on digestion in marine herbivores. Marine Chemical Ecology. pp. 391–412, in J. B. McClintock, and B. J. Baker (eds.). CRC Press, Boca Raton, FL.

TATARENKOV, A., JONSSON, R. B., KAUTSKY, L., and JOHANNESSON, K. 2007. Genetic structure in populations of *Fucus vesiculosus* (Phaeophyceae) over spatial scales from 10 m to 800 km. *J. Phycol* 43:675–685.

VAN ALSTYNE, K. L. 1995. Comparison of 3 methods for quantifying brown algal polyphenolic compounds. *J. Chem. Ecol* 21:45–58.

WATERMAN, P. G., and MOLE, S. 1994. Analysis of Phenolic Plant Metabolites. Blackwell Scientific Publications, Oxford, Great Britain.

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Helikauranoside A, a New Bioactive Diterpene

Francisco A. Macías · Adriana López · Rosa M. Varela · Ascensión Torres · José M. G. Molinillo

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Abstract A new *ent*-kaurane glucoside, named helikauranoside A (**4**), was isolated from the aerial parts of *Helianthus annuus* L. together with three known *ent*-kaurane-type diterpenoids: (−)-kaur-16-en-19-oic acid (**1**), grandifloric acid (**2**), and paniculoside IV (**3**). The structure of 4 was determined by using a combination of 1D (^{1}H-NMR and ^{13}C-NMR) and 2D (COSY, HSQC, and HMBC) NMR techniques. Bioactivity spectra of isolated compounds were tested by using the etiolated wheat coleoptile bioassay in aqueous solutions at concentrations ranging from 10^{-3} to 10^{-6}M. Helikauranoside A (**4**) was the most active (−84%, 10^{-3}M; −56%, 10^{-4}M). These results suggest that this new compound may be involved in defense mechanisms of *H. annuus*.

Keywords Allelopathic agents · Kaurane · Glycoside · Sunflower · Diterpene

Introduction

Chemical studies of *Helianthus annuus* L. (Spring et al. 1981; Melek et al. 1985; Alfatafta and Mullin 1992; Spring et al. 1992; Macías et al. 1993a, b, 1994, 1996) have shown that this species is a rich source of terpenoids, particularly sesquiterpenoids. A wide spectrum of biological activities has been reported for sunflower extracts and components (Beale et al. 1983; Rieseberg et al. 1987; Spring et al. 1991; Ghisalberti 1997). These activities include potential allelopathic effects of extracts from different sunflower cultivars (Macías et al. 1998, 1999a, b).

The isolations of diterpenoids from *H. annuus* L. that have different skeleton types include the following: trachylobanes (Ferguson et al. 1982; Pyrek 1984; Melek et al. 1985); atisane (Morris et al. 2005); gibberellins (Hutchinson et al. 1988); and the kaurenoid carboxilic acids (−)-kaur-16-en-19-oic acid (**1**) (Fig. 1) (Pyrek 1970), grandifloric acid (**2**) (Fig. 1) (Panizo and Rodriguez 1979; Ferguson et al. 1982), angeloylgrandifloric acid, 15β-acetoxy-kaur-16-en-19-oic acid, 15β-*i*-valeroxy-kaur-16-en-19-oic acid, 15β-angeloxy-kaur-16-en-19-oic acid (Ferguson et al. 1982), *ent*-kauran-16α-ol (Morris et al. 2005), 2β,16β-*ent*-kauranediol, and 15α,16α-epoxy-17β-al-*ent*-kaurane-19-oic acid (Suo et al. 2006).

A bioassay-guided fractionation of *H. annuus* L. extracts was carried out to isolate, identify, and characterize the allelopathic constituents. Four kaurene diterpenes were isolated from SH-222 and Stella cultivars of sunflowers. Helikauranoside A (**4**) (Fig. 1) is characterized by a glycosylated kaurane-type skeleton and is described for the first time. The etiolated wheat coleoptile bioassay in aqueous solutions at concentrations between 10^{-3} and 10^{-6}M were used to complete the bioactivity spectrum of the isolated compounds.

F. A. Macías (✉) · A. López · R. M. Varela · A. Torres · J. M. G. Molinillo
Grupo de Alelopatía, Departamento de Química Organica, Facultad de Ciencias, Universidad de Cádiz, C/ República Saharaui s/n, Apdo. 40, 11510 Puerto Real, Cádiz, Spain
e-mail: famacias@uca.es

Methods and Materials

General IR spectra (KBr) were recorded on a Perkin Elmer FT-IR Spectrum 1000, Matton 5020 spectrophotometer. NMR spectra were run on Varian INOVA-400 and Varian INOVA 600 spectrometers. Chemical shifts are given in

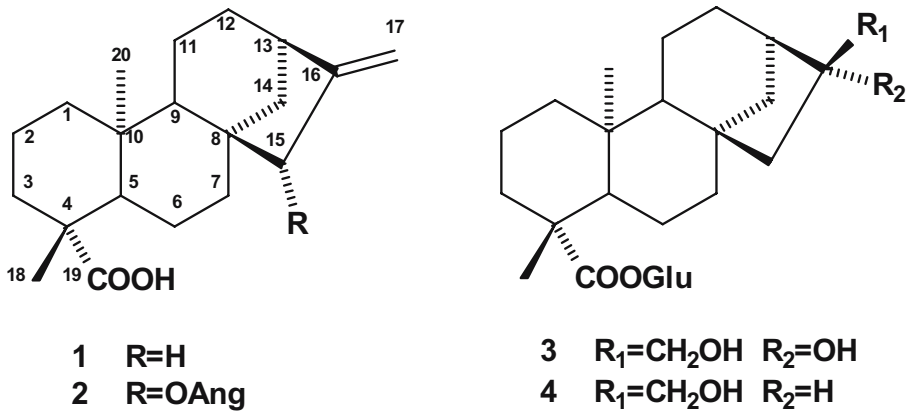

Fig. 1 Isolated diterpenes from *H. annuus* cv. Stella and SH-222

parts per million with respect to residual ^{1}H signals of MeOD-d_4 and py-d_5 (δ 3.30 and 7.55, respectively), as well as ^{13}C with those of the solvent (δ 49.0 and 135.5, respectively). Optical rotations were determined with a Perkin Elmer polarimeter model 241 (on the sodium D line). High-resolution mass spectroscopy (HRMS) was carried out on VG AUTOESPEC mass spectrometer (70eV).

Plant Material Helianthus annuus cv. SH-222 (commercialized by Semillas Pacífico) and Stella were collected during the third plant development stage (Macías et al. 1999c) (i.e., plants were 1.2m tall with flowers, 1month before harvest) and were provided by Rancho de la Merced, Agricultural Research Station (CIFA), Junta de Andalucía, Jerez, Spain.

Extraction and Isolation Fresh leaves of *H. annuus* cv. SH-222 (6kg) were extracted in water (18l) for 24h in the dark at room temperature. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc at room temperature. The organic layer was removed by reduced pressure evaporation and yielded two extracts of 16g (CH_2Cl_2-W) and 6g (EtOAc-W), respectively. EtOAc-W was chromatographed by using hexane–ethyl acetate mixtures of increasing polarity. The most polar fraction was chromatographed on a reverse-phase column (C-18) with a water–methanol (1:4 to 0:1) solvent system to elute compounds **3** (2mg) and **4** (3mg) (Fig. 1).

Similarly, 4kg of *H. annuus* cv. Stella was extracted and yielded 77g (CH_2Cl_2-W) and 7g (EtOAc-W). CH_2Cl_2-A was chromatographed with hexane–ethyl acetate mixtures of increasing polarity. The fraction eluting with hexane–ethyl acetate (1:1) was chromatographed and yielded two compounds—**1** (28mg) and **2** (1mg).

Known compounds were identified by comparison of their physical and spectroscopical data (m.p., [α], IR, MS, ^{1}H-NMR and ^{13}C-NMR) with those previously reported in the literature. *Helikauranoside A* (**4**) crystalline solid mp 260°C; IR ν_{max} (KBr) cm^{-1}: 3,500 (OH, carboxylic acid) 1,700 (C=O, acid). ^{1}H-NMR data (600MHz) MeOD-d_4: δ 0.95 (s, H-20), 1.20 (s, H-18), 1.91 (m, H-16), 2.17 (m, H-3α), 3.32–3.37 (m, H-4′, H-3′, H-2′), 3.35 (m, 2H, H-17), 3.40 (ddd, 8.0, 1.7, 4.6, H-5′), 3.68 (dd, 11.9, 4.6, H-6′a), 3.81 (dd, 11.9, 1.7, H-6′b), 5.39 (d, 8.1, H-1′); py-d_5: δ 6.26 (d, 7.9, H-1′), δ 4.45 (brd, 11.2, H-6′β), δ 4.38 (brd, 11.2, H-6′α), δ 4.34 (dd, 10, 8.8, H-3′), δ 4.25 (dd, 8.8, 8.8, H-4′), δ 4.22 (dd, 8.1, 7.9, H-2′), δ 3.94 (ddd, 8.8, 4.4, 2.4, H-5′), δ 3.61 (m, 2H, H-17), δ 2.38 (brd, 13.2, H-3a), δ 2.29 (brs, H-13), δ 2.16 (m, H-16), δ 1.79 (brd, 12.3, H-1a), δ 1.61 (dd, 9, 12.1, H-15a), δ 1.44 (m, H-7a), δ 1.27 (s, 3H, H-18), δ 1.22 (s, 3H, H-20), δ 1.08 (dd, 5.1, 12.1, H-15b), δ 0.96 (brd, 8.0, H-3a), δ 0.76 (m, H-7b); ^{13}C NMR data (150MHz): see Table 1; HREIMS *m/s* 482.2884 [M^{+}] (cal. for 482.2880).

Coleoptile Bioassay Wheat seeds (*Triticum aestivum* L. cv. Duro) were sown in Petri dishes (15cm diameter), misted with water, and grown in the dark at 22 ± 1°C for 3days (Hancock et al. 1964). The roots and caryopsis were removed from the shoots. The apical 2mm of the caryopsis was cut off and discarded with a Van der Weij guillotine. The next 4mm of the coleoptiles was removed and used for bioassay. All manipulations were performed under a green safelight (Nitsch and Nitsch 1956). Compounds were predisolved in DMSO and diluted to the final bioassay concentration with a maximum of 0.1% DMSO. Parallel controls treated with water and DMSO were also run at the same concentrations.

Crude extracts, fractions, or pure compounds to be assayed for biological activity were added to test tubes. Assays were run in duplicate. Phosphate–citrate buffer (2ml) containing 2% sucrose (Nitsch and Nitsch 1956) at pH5.6 was added to each test tube. Following the placement of five coleoptiles in each test tube (three tubes per dilution), the tubes were rotated at 0.25rpm in a roller

Table 1 ^{13}C NMR data for compound 4 in deuterated methanol and pyridine

C	$\delta^{13}C$ MeOD-d_4	$\delta^{13}C$ py-d_4
1	42.1 *t*	41.0
2	20.0 *t*	19.3
3	39.1 *t*	38.5
4	45.1 *s*	44.1
5	58.8 *d*	57.5
6	23.5 *t*	22.9
7	43.0 *t*	42.2
8	45.9 *s*	45.0
9	56.9 *d*	55.6
10	40.9 *s*	40.0
11	20.2 *t*	19.6
12	32.5 *t*	
13	39.5 *d*	38.7
14	37.9 *t*	37.2
15	46.3 *d*	45.7
16	44.5 *s*	44.3
17	67.7 *t*	67.1
18	29.1 *q*	28.7
19	178.3 *s*	175.6
20	16.5 *q*	16.0
1′	95.6 *d*	95.8
2′	74.1 *d*	74.1
3′	78.7 *d*	71.1
4′	71.1 *d*	79.2
5′	78.7 *d*	79.4
6′	62.4 *d*	62.1

tube apparatus for 24h in the dark at 22°C. Coleoptiles were measured following digitalization of their images. Data were statistically analyzed by using Welch's test (Martín Andrés and Luna del Castillo 1990). Data are presented as percentage differences from control. Thus, zero represents the control, positive values represent stimulation of the studied parameter, and negative values represent inhibition.

Results and Discussion

Fresh *H. annuus* cv SH-222 leaves were extracted in water in the dark at room temperature. The aqueous solution was extracted with CH_2Cl_2 and then with EtOAc. Following chromatography, the EtOAc extract was separated into eight fractions. Fraction H contained the compounds paniculoside IV (**3**) (2mg) and helikauranoside A (**4**) (3mg) described for the first time in this report (Fig. 1). *Helianthus annuus* cv. Stella was extracted similarly. The fraction eluting with hexane-EtOAc (1:1) yielded compounds **1** (28mg) and **2** (1mg). The spectroscopic data for **1**, (Pyrek 1970; Ohno et al. 1979) **2** (Panizo and Rodriguez 1979; Herz et al. 1983), and **3** (Fig. 1)(Ohno and Mabry 1980) were identical to those previously reported.

Table 2 Selected ^{13}C NMR data for α- and β-isomers of aglycone in $CDCl_3$ and those for compound 4 in methanol

C	α-CH_2OH	β-CH_2OH	4
12	20.0	31.4	32.5
13	36.9	38.1	39.5
15	44.2	45.0	46.3
17	64.2	67.4	67.7

Helikauranoside A (**4**) was isolated as white crystals (mp 260°C) with $[\alpha]_D^{25}$ −6.4 (*c* = 0.14) from fraction H. The molecular formula was deduced to be $C_{26}H_{42}O_8$ on the basis of HRMS that showed an ion peak $[M]^+$ at *m/z* 482.2884. Intense absorption bands at 3,500 and 1,700cm^{-1} were observed in the IR spectrum of **4**, in accordance with the presence of hydroxyl and carbonyl functions, respectively. The ^1H NMR spectrum of **4** was run in methanol (CD_3OD) and pyridine (C_5D_5N). Both spectra exhibited signals for a β-glucopyranosyl moiety in the structure [CD_3OD: δ 5.39 (H-1′, d, *J* = 8.1), δ 3.81 (H-6′β, dd, *J* = 11.9, 1.7), δ 3.68 (H-6′α, dd, *J* = 11.9, 4.6), δ 3.40 (H-5′, ddd, *J* = 8.0, 4.6, 1.7), δ 3.32–3.37 (H-2′, H-3′, H-4′, m); C_5D_5N: δ 6.26 (H-1′, d, *J* = 7.9), δ 4.45 (H-6′β, brd, *J* = 11.2), δ 4.38 (H-6′α, brd, *J* = 11.2), δ 4.34 (H-3′, dd, *J* = 10, 8.8), δ 4.25 (H-4′, dd, *J* = 8.8, 8.8), δ 4.22 (H-2′, dd, *J* = 8.1, 7.9), δ 3.94 (H-5′, ddd, *J* = 8.8, 4.4, 2.4)] (Agrawal 1992; Rakotondraibe et al. 2002; Harinantenaina et al. 2002a, b). This was confirmed by signals in the ^{13}C NMR spectrum [CD_3OD: δ 95.6 (C-1′), δ 78.7 (C-3′), δ 78.7 (C-5′), δ 74.1 (C-2′), δ 71.1 (C-4′), δ 62.4 (C-6′)].

Additionally, the ^1H NMR in methanol exhibited a signal for the protons of two methyl groups at δ 1.20 (3H, s, H-18) and δ 0.95 (3H, s, H-20). The H-17 protons resonate in MeOD at δ 3.35 (2H, m) and suggest that **4** is an ent-kaurane-type diterpene glycoside. The ^{13}C NMR and HSQC spectra of **4** showed the presence of two methyls, 11 methylenes, nine methines, and four quaternary carbons

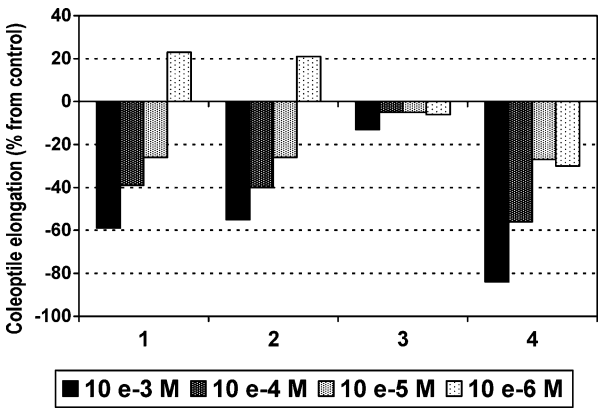

Fig. 2 Bioactivities of compounds **1**–**4** in wheat coleoptile bioassay

containing an ester carbonyl group. We found analogies in the chemical shifts of signals from the ^{1}H NMR and ^{13}C NMR spectra of **4** with those previously described for the epimeric diterpenes at C-16 that correspond to the aglycone (−)-17-hydroxy-kauran-19-oic acid (Table 1). On the basis of the stereochemical studies performed by Yang et al. (2002), the chemical shift of H-17 in methanol (δ 3.35) suggested a β-orientation for the hydroxymethylene moiety (δ 3.40 and 3.72 of the aglycone in $CDCl_3$ for the β and α isomers, respectively). This was confirmed by comparison of the chemical shifts observed in ^{13}C NMR spectrum with those found for the both isomers described by Wu et al. (1996; see Table 2). According to the chemical shift observed for C-17 in the pyridine-d_5 spectrum (δ 67.7), the β-glucopyranosyl moiety must be attached to the carboxylic ester at C-19; otherwise, a glycosylic moiety attached at C-17 should resonate between δ 74 and 75ppm (Shao et al. 1996). These data allow us to propose the structure *O*-β-D-glucopyranosyl-17-hydroxy-16β-kauran-19-oate. This is the first report of this structure, which was named helikauranoside A (**4**).

Bioactivity—Coleoptiles Bioassay There are many antecedents of bioactivity of kaurane diterpenes. Some of them are for compounds described in this report or for structures related to them. Thus, (−)-kaur-16-en-19-oic acid (**1**) has an inhibitory effect on the growth of *Bacillus subtilis* and *Staphylococus aureus* (Phan et al. 2005) and has been tested on the aggregation of Washed Rabbit Platelets, showing complete inhibition induced by collagen at 200μM (Yang et al. 2002). This compound was active in the brine shrimp test (BST LC_{50} 16μg/ml) and was selective for MCF-7 (breast cancer, ED_{50} 1.0μg/ml) cells among six human solid tumor cell lines (Majekodunmi and Oladimeji 1996). Compound **1** and the aglycone of **4** have strong inhibitory effects against cyclooxygenase (COX) (Phan et al. 2005).

The aglycone of **3** gave 46% and was inhibited by HIV reverse transcriptase at a concentration of 33μg/ml (Chang et al. 1998). Epimers at C-16 inhibited HIV replication in H9 lymphocyte cells with EC_{50} of 0.8μg/ml (Wu et al. 1996).

To complete the bioactivity spectrum of the isolated compounds, isolated compounds were tested with the etiolated wheat coleoptile bioassay (Hancock et al. 1964) in a range of 10^{-3}–10^{-6}M. This bioassay has been used to estimate plant growth regulation, herbicide, or phytotoxic activities (Cutler 1984).

The growth of etiolated wheat coleoptiles (Fig. 2) was inhibited ($P<0.01$) by solutions of **1** (−59%, 10^{-3} M; −39%, 10^{-4} M), **2** (−55%, 10^{-3} M; −40%, 10^{-4} M), and **4** (−84%, 10^{-3} M; −56%, 10^{-4} M). The most active compound was **4**, whereas compounds **1** and **2** presented similar activities, but **3** did not show any significant effect.

Chemical differences among diterpenes **1**, **2**, **3**, and **4**, are centered at C-16. Therefore, the differences in the activity must be explained by the different substitutions in this position. Compound **4** has a hydroxymethylene group at C-16 and is highly active, whereas the presence of an additional hydroxyl group (compound **3**) decreases activity dramatically. On the other hand, if a double bond (between C-16 and C-17) is present instead of the hydroxymethylene group, activity also decreases (compounds **1** and **2**). Other positions in the kaurane skeleton seem to be less important in relation to activity, such as those compounds that present an angelate at C-15 or a sugar at C-19. The presence of angelate at C-15 only changes the activity from slightly stimulatory to inhibitory at the lowest concentration (compound **2** vs. compound **1**).

Although diterpenoids are not usually reported as phytotoxic agents, in this case, the most active compound is the glycosylated diterpenoid **4**. The ecological role of these compounds has been associated more with antifeedant, insecticidal, and deterrent activity. Moreover, diterpenoids as giberrellins act as important plant hormones involved in growth regulation (Macías et al. 2007). In contrast, the glycosidic form of active natural products is less active than the aglycones (Macías et al. 2005). Unfortunately, the lack of sufficient quantities of compound did not allow us to hydrolyze it and to bioassay the aglycone to compare their activities.

These results suggest that compound **4** should be involved in defense mechanisms of *H. annuus*. The shown activity indicates that its role may be related with the allelopathic behavior shown by this species.

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References

AGRAWAL, P. K. 1992. NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides. *Phytochemistry* 31:3307–3330.

ALFATAFTA, A. A., and MULLIN, C. A. 1992. Epicuticular terpenoids and an aurone from flowers of *Helianthus annuus*. *Phytochemistry* 31:4109–4113.

BEALE, M. H., BEARDER, J. R., MACMILLAN, J., MATSUO, A., and PHINNEY, B. O. 1983. Diterpene acids from *Helianthus* species and their microbiological conversion by *Gibberella fujikurol* mutant B1-41 a. *Phytochemistry* 22:875–881.

CHANG, F., YANG, P., LIN, J., LEE, K., and WU, Y. 1998. Bioactive kaurane diterpenoids from *Annona glabra*. *J. Nat. Prod.* 61:437–439.

CUTLER, H. G. 1984. Fresh look at the wheat coleoptile bioassay, pp. 1–9, in Proceedings of the 11th Annual Meeting of the Plant Growth Regulator Society of America. PGRSA, Boston, MS.

FERGUSON, G., MCCRINDLE, R., MURPHY, S. T., and PARVEZ, M. 1982. Further diterpenoid constituents of *Helianthus annuus* L. Crystal and molecular structure of methylent-15b-hydroxytrachyloban-19-oate. *J. Chem. Res. Synop.* 8:200–201.

GHISALBERTI, E. L. 1997. The biological activity of naturally occurring kaurene diterpenes. *Fitoterapia* 68:303–325.

HANCOCK, C. R., BARLOW, H. W., and LACEY, H. J. 1964. The east malling coleoptiles straight growth test method. *J. Exp. Bot.* 15:166–176.

HARINANTENAINA, R., KASAI, R., and YAMASAKI, K. 2002a. A new e*nt*-kaurane diterpenoid glycosides from the leaves of *Cussonia bojeri*, a Malagasy endemic plant. *Chem. Pharm. Bull.* 50:1122–1123.

HARINANTENAINA, R., KASAI, R., and YAMASAKI, K. 2002b. *Ent*-kaurane diterpenoid glycosides from a Malagasy endemic plant, *Cussonia vantsilana*. *Phytochemistry* 61:367–372.

HERZ, W., KULANTHAIVEL, P., and WATANABE, K. 1983. *Ent*-kauranes and other constituents of three *Helianthus* species. *Phytochemistry* 22:2021–2025.

HUTCHINSON, M., GASKIN, P., MACMILLAN, J., and PHINNEY, B. O. 1988. Gibberellins in seeds of *Helianthus annuus*. *Phytochemistry* 27:2695–2701.

MACÍAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1993a. Novel sesquiterpenes from bioactive fractions of cultivar sunflowers. *Tetrahedron Lett.* 34:1999–2002.

MACÍAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1993b. Potential allelopathic guaianolides from cultivar sunflowers leaves var. SH-222. *Phytochemistry* 34:669–674.

MACÍAS, F. A., MOLINILLO, J. M. G., VARELA, R. M., and TORRES, A. 1994. Structural elucidation and chemistry of a novel family of bioactive sesquiterpenes: heliannuols. *J. Org. Chem.* 59:8261–8266.

MACÍAS, F. A., TORRES, A., MOLINILLO, J. M. G., VARELA, R. M., and CASTELLANO, D. 1996. Potential allelopathic sesquiterpene lactones from sunflower leaves. *Phytochemistry* 43:1205–1215.

MACÍAS, F. A., VARELA, R. M., TORRES, A., OLIVA, R. M., and MOLINILLO, J. M. G. 1998. Bioactive norsesquiterpenes from *Helianthus annuus* with potential allelopathic activity. *Phytochemistry* 48:631–636.

MACÍAS, F. A., MOLINILLO, J. M. G., GALINDO, J. C. G., VARELA, R. M., TORRES, A., and SIMONET, A. M. 1999a. Terpenoids with potential use a natural herbicide templates, pp. 15–31, in H. G. Cutler, and S. J. Cutler (eds.). Biologically Active Natural ProductsAgrochemicals CRC Press LLC, Boca Raton, FL.

MACÍAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1999b. Bioactive compounds from the genus *Helianthus*, pp. 121–134, in F. A. Macías, J. C. G. Galindo, J. M. G. Molinillo, and H. G. Cutler (eds.). Recent Advances in Allelopathy, vol I. A Science for the FutureServicio de Publicaciones, Universidad de Cádiz, Cádiz.

MACÍAS, F. A., VARELA, R. M., TORRES, A., and MOLINILLO, J. M. G. 1999c. Potentiality of cultivar sunflowers (*Helianthus annuus* L.) as source of natural herbicide templates, pp. 531–550, in Inderjit, K. M. M. Dakshini, and C. L. Foy (eds.). Principles and Practices in Plant Ecology. Allelochemical InteractionsCRC Press LLC, Boca Raton, FL.

MACÍAS, F. A., MARIN, D., OLIVEROS-BASTIDAS, A., CASTELLANO, D., SIMONET, A. M., and MOLINILLO, J. M. G. 2005. Structure–Activity Relationships (SAR) studies of Benzoxazinones, their degradation products and analogues. Phytotoxicity on Standard Target Species (STS). *J. Agric. Food Chem.* 53:538–548.

MACÍAS, F. A., MOLINILLO, J. M. G., VARELA, R. M., and GALINDO, J. C. G. 2007. Allelopathy—a natural alternative for weed control. *Pest Manag. Sci.* 63:327–348.

MAJEKODUNMI, O. F., and OLADIMEJI, T. A. 1996. Bioactive *ent*-kaurane diterpenoids from *Annona senegalensis*. *J. Nat. Prod.* 59:301–303.

MARTÍN ANDRÉS, A., and LUNA DEL CASTILLO, J. DE D. 1990. Bioestadística para las Ciencias de la Salud, . rd ed. 3rd edn. Norma, Madrid.

MELEK, F. R., GAGE, D. A., GERSHENZON, J., and MABRY, T. J. 1985. Sesquiterpene lactones and diterpene constituents of *Helianthus annuus*. *Phytochemistry* 24:1537–1539.

MORRIS, B. D., FOSTER, S. P., GRUGEL, S., and CHARLET, L. D. 2005. Isolation of the diterpenoids, *ent*-atisan-16α-ol, from sunflowers, as oviposition stimulants for the banded sunflower moth, *Cochylis hospes*. *J. Chem. Ecol.* 31:89–102.

NITSCH, J. P., and NITSCH, C. 1956. Studies on the growth of coleoptiles and first internodes sections. A new sensitive, straight-growth test for auxins. *Plant Physiol.* 31:94–111.

OHNO, N., and MABRY, J. T. 1980. Sesquiterpene Lactones and diterpene carboxylic acids in *Helianthus* niveussubspecies *Canescens*. *Phytochemistry* 19:609–614.

OHNO, N., MABRY, J. T., ZABEL, V., and WATSON, W. H. 1979. Tetrachirin a new rearraged kaurenoid lactone, and diterpene acids from *Tetrachyron orizabaensis* and *Helianthus debilis*. *Phytochemistry* 18:1687–1689.

PANIZO, F. M., and ODRIGUEZ, B. 1979. Algunos componentes diterpenicos del girasol. *An. Quím.* 75:428–430.

PHAN, V. K., MINH, V., NGUYEN, T. D., LEE, J. J., and KIM, Y. H. 2005. Bioactivities of compounds isolated from *Acanthopanax trifoliatus*. *Tap Chi Hoa Hoc* 43:51–55.

PYREK, J. S. 1970. New pentacyclic diterpene acid. Trachylobane-19-oic acid from sunflowers. *Tetrahedron* 26:5029–5032.

PYREK, J. S. 1984. Neutral diterpenoids of *Helianthus annuus*. *J. Nat Prod.* 47:822–827.

RAKOTONDRAIBE, L., HARINANTENAINA, R., KASAI, R., and YAMASAKI, K. 2002. *Ent*-kaurane diterpenoid glycosides from the leaves of *Cussonia racemosa*, a Malagasy endemic plant. *Chem. Pharm. Bull.* 50:268–271.

RIESEBERG, L. H., SOLTIS, D. E., and ARNOLD, D. 1987. Variation and localization of flavonoid aglycones in *Helianthus annuus* (Compositae). *Am. J. Bot.* 74:224–233.

SHAO, Y., BAI, N., and ZHOU, B. 1996. Kaurane glycosides from *Inula britannica*. *Phytochesmitry* 42:783–786.

SPRING, O., ALBERT, K., and GRADMANN, W. A. 1981. A new biologically active germacranolide from *Helianthus annuus*. *Phytochemistry* 20:1883–1885.

SPRING, O., BENZ, A., and FAUST, V. 1991. Impact of downy mildew (*Plasmopara halstedii)* infection on the development and metabolism of sunflower. *J. Plant Dis. Protect.* 98:597–604.

SPRING, O., ODON, U., and MACÍAS, F. A. 1992. Sesquiterpenes from noncapitate glandular trichomes of *Helianthus annuus*. *Phytochemistry* 31:1541–1545.

SUO, M. R., YANG, J. S., LU, Y., WU, L., and ZHENG, Q. T. 2006. Two new diterpenes from *Helianthus annuus* L. *Chin. Chem. Lett.* 17:45–48.

WU, Y., HUNG, Y., CHANG, F., COSENTINO, M., WANG, H., and LEE, K. 1996. Identification of *ent*-16β,17.dihydroxykauran-19-oico acid an anti-HIV principle and islation of the new diterpenoids annosquamosins A and B from *Annona squamosa*. *J. Nat. Prod.* 59:635–637.

YANG, Y., CHANG, F., WU, C., WANG, W., and WU, Y. 2002. New *ent*-kaurane diterpenoids with anti-platelet aggregation activity from *Annona squamosa*. *J. Nat. Prod.* 65:1462–1467.

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Solid-Phase Microextraction Method For In Vivo Measurement of Allelochemical Uptake

Rebecca X. Loi · Marissa C. Solar · Jeffrey D. Weidenhamer

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Abstract Solid-phase microextraction (SPME) was used to measure allelochemical uptake by tomato plants in vivo. Exogenously applied 1,8-cineole was rapidly taken up by tomato, with the first traces of cineole being detected in the tomato stem just 1–2 h after a single application of 0.5 mM cineole to soil. The pulse of cineole persisted in the tomato stem for 72 h. When cineole concentrations were measured 24 h after a single application, trace amounts of cineole could be detected in tomato stem fluid at application concentrations as low as 50 μM. Tomato was also found to take up camphor, menthol, and coumarin, but not carveol. In preliminary tests with common ragweed (*Artemisia annuifolia* L.) and purslane (*Portulaca oleraceae* L.) plants growing in garden beds, both ragweed and purslane took up 1,8-cineole, and purslane also took up camphor. The quantitation of allelochemical uptake by plants is considered to be a crucial test of hypotheses of allelopathic effects, but demonstration of allelochemical uptake has had to be inferred based on observed toxicity due to the lack of methods to measure uptake in vivo. This new technique now provides a means of tracking compounds within target plants. Furthermore, the demonstrated rapid uptake of 1,8-cineole by plants suggests a potential mechanism whereby brief pulses of allelochemicals over an extended period of time might be able to exert an allelopathic effect on plants.

Keywords Allelopathy · SPME · Translocation · Camphor · Carveol · 1,8-cineole · Coumarin · Menthol · Xylem chemistry

R. X. Loi · M. C. Solar · J. D. Weidenhamer (✉)
Department of Chemistry, Ashland University,
Ashland, OH 44805, USA
e-mail: jweiden@ashland.edu

Introduction

One of the methodological barriers to the study of allelopathy is the lack of techniques to measure the uptake of allelochemicals by target plants. Fuerst and Putnam (1983) asserted that quantification of the amount of toxin released to the environment and taken up by the target plant was crucial to proving a hypothesis of allelopathy. The focus of most investigations has been measurement of allelochemical concentrations in soil by various approaches (Tang and Young 1982; Ponder and Tadros 1985; Dalton et al. 1987; Gallet and Pellissier 1997; Weidenhamer 2005; Blair et al. 2006). Uptake of allelochemicals is typically inferred based on observed toxic effects (e.g., Bais et al. 2003). Because radiolabeled analogs of plant natural products are generally unavailable, studies of allelochemical uptake with the radiotracer techniques commonly employed to monitor herbicide translocation (e.g., Kalnay and Glenn 2000; Chun et al. 2001; Shoup and Al-Khatib 2005) are not feasible.

Arthur and Pawliszyn (1990) introduced solid-phase microextraction (SPME) as a nondestructive, solvent-free sampling technique that is now widely used to monitor volatiles in headspace (Deng et al. 2004a, b; Custódio et al. 2006) and trace organics in aqueous solutions (Boussahel et al. 2002), and it has numerous other applications. Recently, Lord et al. (2004) demonstrated the use of SPME to monitor herbicide levels in living plant tissues. Triazine herbicides were applied to soil and then detected in tomato plants by placement of an SPME fiber directly inside the plant stem. Provided that allelochemicals are translocated into target plants rather than exerting their toxic effects at the root surface or by other means, in vivo SPME could be a tool to monitor the uptake of suspected allelopathic agents by target plants. The objective of this study was to test the

applicability of this method by using tomato plants and several candidate allelochemicals.

Methods and Materials

Chemicals and Supplies (1*R*)-(+)-Camphor (99%), carveol (97%), and 1,8-cineole (99%) were obtained from Aldrich Chemical (Milwaukee, WI, USA). Coumarin (reagent grade) was purchased from Eastman Chemical (Rochester, NY, USA). Menthol (USP grade) was purchased from Mallinckrodt Chemicals (St. Louis, MO, USA). SPME fiber assemblies and holders were obtained from Supelco (Bellefonte, PA, USA). The fibers chosen were coated with a 100-μm layer of polydimethylsiloxane (PDMS).

Plants and Treatment Tomato (*Lycopersicon esculentum* L. cv. "Big Boy") plants were obtained from a local garden store and transplanted into large styrofoam cups that contained a 50:50 mix of sand and potting soil. Common ragweed (*Artemisia annuifolia* L.) and purslane (*Portulaca oleraceae* L.) plants were growing in flower beds as weeds. Allelochemical solutions were applied in aqueous solution. Concentrations of cineole ranged from 0.05 to 0.5 mM. Other compounds were applied at a concentration of 0.5 mM. The volume of solution application was 40 ml with tomato plants and 300 ml for common ragweed and purslane. All analyses of tomato plants were based on duplicate sampling of two identically treated plants, and plants were not reused. Preliminary measurements with common ragweed and purslane were made on single plants.

Sampling Method The SPME sampling method was modeled after that of Lord et al. (2004). Plants were sampled at a stem height of 6.0 cm above the soil, except for the study of concentration with sampling height, in which tomato plants were sampled at heights ranging up to 30 cm. One to 72 h after treatment application, a SPME fiber was inserted into the stem of the test plant. The SPME fiber was preconditioned in phosphate-buffered saline (PBS) for 30 min. The retracted fiber (without spring and unattached to the SPME fiber holder) was then placed into a small 1.5-cm hole, which was made in the stem with a 22-gauge needle and filled with PBS. The outer protective needle was carefully retracted while holding the fiber support wire in place, so that the fiber was exposed to the stem fluid. The SPME fiber assembly was supported during the sampling process (either by leaning against a metal spatula inserted into the soil for the 6-cm sampling point, or by using a loop of masking tape at higher points) so that no stress was put on the fiber. The fiber was equilibrated in the stem for 1 h. It was removed by reversing these steps. The fiber was rinsed with distilled water and then installed into an SPME holder for immediate GC-MS analysis. With care, it was possible to reuse fibers 10 or more times.

Quantitation Cineole was quantified by comparison to direct injections of cineole standard solutions (linear over a range of 1 ng–1 μg; r^2=0.999) and by comparison to static 1-h extractions of cineole solutions in PBS buffer (linear over a range of 0.25–60 μM, r^2=0.978). Stem fluid concentrations were estimated by comparison to the static PBS buffer calibrations.

Chromatographic Methods Samples were analyzed on an Agilent 6890 gas chromatograph equipped with a model 5975 quadrupole mass spectrometer. An HP-5MS column (30 m, 0.25 mm ID×0.25 μm film thickness) was used for separation. SPME fibers were desorbed manually at 250°C for 2 min in splitless injection mode. All injections of liquid standards were made in splitless mode. Two basic temperature gradients were used. With tomato, optimal separation was achieved by holding column temperature at 50°C for 2 min, then increasing at 10°C min^{-1} to 110°C. For preliminary investigations with common ragweed and purslane, column temperature was held at 50°C for 2 min, then increased at 25°C min^{-1} to 200°C. Compound identities were confirmed with the National Institute of Standards and Technology (NIST) Mass Spectral Search Program (Washington, DC, USA).

Results

Cineole was taken up rapidly by tomato plants (Fig. 1). Just 1–2 h after a single application of 0.5 mM cineole to soil, the first traces of cineole were detected in the tomato stem. Cineole concentrations in the stem increased up to 12 h

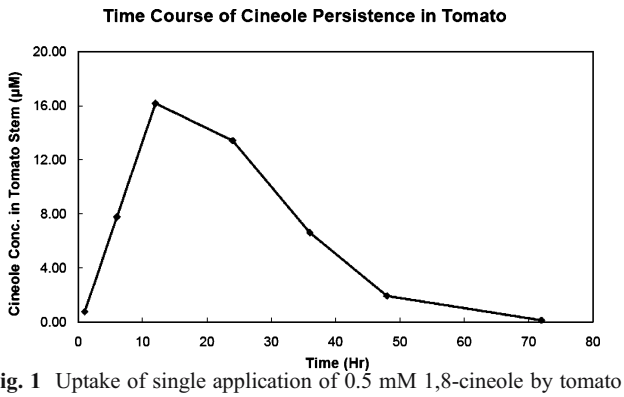

Fig. 1 Uptake of single application of 0.5 mM 1,8-cineole by tomato plants, measured by SPME of stem fluid. Concentration of cineole in stem fluid was estimated by comparison to SPME calibration in PBS solution. Values are the average of separate measurements on two plants

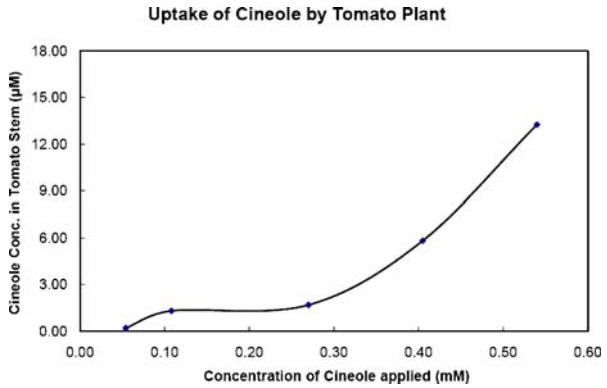

Fig. 2 Uptake of a single application of 1,8-cineole by tomato plants as a function of cineole concentration. Values are the average of separate measurements on two plants

after the initial application, and slowly declined until only a trace amount was detected at 72 h.

When cineole concentrations were measured 24 h after a single application, trace amounts could be detected in tomato stem fluid at application concentrations as low as 50 µM (=0.05 mM, Fig. 2). Concentrations in stem fluid increased with application concentration. Cineole concentrations in tomato stem decreased linearly with sampling height (Fig. 3). Cineole could still be detected in very low concentrations to a height of 30 cm above the soil surface.

Applications of other compounds (0.5 mM solutions except for carveol, which was applied at 0.6 mM) gave variable results. Camphor and menthol, but not carveol, were detected in tomato. Coumarin was also detected after application to tomato, but only after 72 h. 1,8-Cineole was detected following application to both ragweed and purslane. Camphor was detected following application to purslane, but not ragweed. Coumarin, menthol, and carveol were not detected following application to ragweed and purslane.

Chromatograms of cineole-treated tomato and common ragweed are presented in Fig. 4a and b, respectively. Cineole and other exogenously supplied monoterpenes were not detected in control plants. However, several additional monoterpenes and other tentative compounds were detected in stem fluid of control and treated plants. Compounds identified on the basis of their mass spectral NIST library matches in tomato included *p*-mentha-1,4(8)-diene, α-phellandrene, β-phellandrene, and methyl salicylate. In common ragweed, compounds identified included β-myrcene, limonene, and ocimene. Several sesquiterpenes were also detected (data not shown).

Discussion

Analytical Methodology Methyl salicylate and other plant volatiles play important roles in signaling by herbivore-damaged plants that attract herbivore parasitoids (Turlings et al. 1990; Demoraes et al. 1998) and by pathogen-infected plants that induce pathogen resistance in other plants (Shulaev et al. 1997). Therefore, a number of methods have been developed for the analysis of monoterpenes and other plant volatiles. These have included in-plant as well as headspace analyses. Deng et al. (2004a) analyzed methyl salicylate and other volatiles in tomato leaves by HS-SPME/GC-MS after tomato leaves were ground in liquid nitrogen. The analysis required 2.0 g fresh weight of tissue. Deng et al. (2005) removed 1.2-cm-diameter pieces of leaves, placed them in a GC injection liner, and thermally desorbed volatiles directly into GC-MS. The analytical method reported here is a novel approach to measuring compounds *in planta*. Our results demonstrate for the first time the possibility of quantifying the amount of at least some allelochemicals taken up by target plants, a crucial test for hypotheses of allelopathy. The size of current SPME fibers will prevent in vivo sampling of most leaves. Thus, techniques such as those used by Deng et al. (2004a, 2005) may be useful complements to in vivo sampling of stems or for analysis of plants where plant structure does not permit direct insertion of an SPME fiber. We also note that one potential limitation of this method is that it requires that allelochemicals be translocated into target plants. It is conceivable that some compounds may exert their toxic effects at the root surface or within the root (e.g., Bais et al. 2003) and not be translocated to the upper part of the plant. In such cases, other analytical techniques will be needed.

The presence and relative amounts of native mono- and sesquiterpenes and other compounds detected in stem fluid (Fig. 4) varied among tested plants. In particular, methyl salicylate was sporadically found in tomato stem fluid, and levels of methyl salicylate did not change in response to

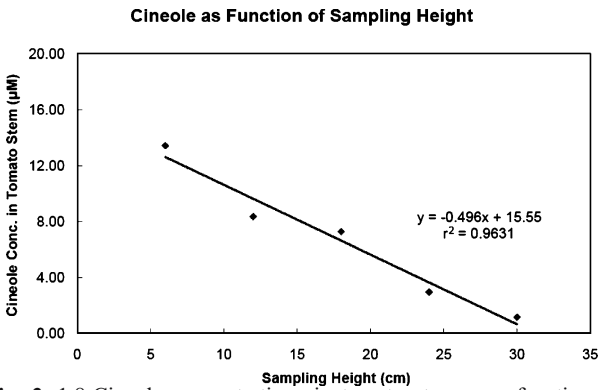

Fig. 3 1,8-Cineole concentrations in tomato stem as a function of sampling height. Samples were taken 24 h after a single application of 0.5 mM cineole to soil. Values are the average of separate measurements on two plants

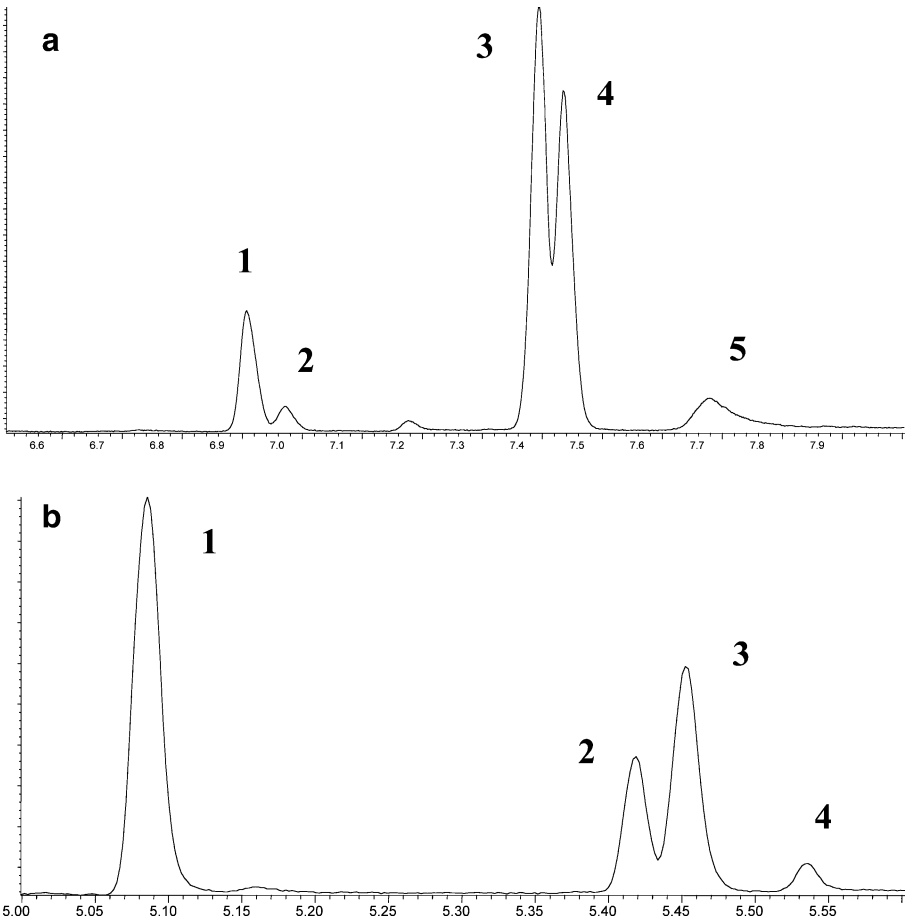

Fig. 4 **A** In vivo SPME total ion chromatogram of tomato stem from cineole-treated plant. Compounds identified by their mass spectra are: *1* *p*-mentha-1,4 (8)-diene, *2* α-phellandrene, *3* β-phellandrene, *4* 1,8-cineole, and *5* methyl salicylate. **B** In vivo SPME total ion chromatogram of common ragweed stem from cineole-treated plant. Compounds identified by their mass spectra are: *1* β-myrcene, *2* limonene, *3* 1,8-cineole, and *4* ocimene. Chromatographic conditions are described in Methods and Materials

exogenous monoterpene application. However, our results suggest that in vivo SPME may be a useful tool for the study of these compounds within plants.

The 1-h equilibration time used in this study was based on the results of Lord et al. (2004), who also used a 1-h equilibration time. They found that two of three triazine herbicides tested did not equilibrate within 3 h, but that the slope of the extraction profile was not steep. Given the dynamic nature of cineole concentrations, a 1-h sampling time was chosen for this study. Quantitation of stem extracellular fluid concentrations by external calibration with static PBS buffer extraction is probably not valid given the difficulty of preparing a buffer that matches the composition of extracellular fluid (Lord et al. 2004). The results obtained should, thus, be regarded as semiquantitative, but they do provide useful information on the relative amounts of monoterpenes taken up by plants over time. A possible approach to quantitation that could overcome these issues would be to use the newly developed kinetic calibration method in which a standard is preloaded on the SPME fiber and the concentrations of analyte are estimated based on the isotropy of absorption of analyte and desorption of standard (Musteata et al. 2007).

Monoterpenes and Allelopathy Monoterpenes have been implicated in allelopathic interactions in a number of systems. Muller et al. (1964) and Muller (1965) identified several monoterpenes, including 1,8-cineole and camphor, from the California chaparral shrub *Salvia leucophylla* and provided evidence for their role in maintaining bare zones around these shrubs. In the Florida scrub, several fire-sensitive shrubs have allelopathic activity against grasses and herbs that would provide fuel for fires if they were not excluded from the scrub. In two species, oxygenated monoterpenes have been identified as the presumed allelopathic agents. A mixture of compounds including 1,8-cineole, camphor, borneol, mytenal, and several other oxygenated monoterpenes was identified in leaf washes of *Conradina canescens* (Williamson et al. 1989), while derivatives of menthofuran were identified as potential allelopathic compounds from *Calamintha ashei* (Tanrisever et al. 1988; Weidenhamer et al. 1994). In conifer forests, there is experimental evidence that monoterpenes, probably from roots, may play a role in reducing nitrogen mineralization and nitrification (White 1991, 1994; Paavolainen et al. 1998). Recently, Barney et al. (2005) presented evidence that the invasive characteristics of mugwort

(*Artemisia vulgaris*), which can form dense, monospecific stands, can be attributed in part to allelopathic effects derived from a mixture of monoterpenes that include 1,8-cineole and camphor.

Numerous studies have shown that monoterpenes are potent toxins and exert a number of toxic effects on plant cells and seedlings (Vaughn and Spencer 1993; Weidenhamer et al. 1993; Peñuelas et al. 1996; Koitabashi et al. 1997; Abrahim et al. 2000; Romagni et al. 2000). Nishida et al. (2005) found that the volatile monoterpenes of *S. leucophylla*, including camphor and 1,8-cineole, inhibited germination and growth of *Brassica campestris* and decreased cell proliferation in the root apical meristem. Zunino and Zygadlo (2005) showed that monoterpenes, including camphor and 1,8-cineole, affected the phospholipid fatty acid and sterol composition of maize root. These changes in membrane lipids could interfere with seedling growth.

One of the questions about the plausibility of allelopathic mechanisms that involve monoterpenes, as with other proposed allelopathic compounds, is their generally short persistence in the environment. The results of this study that show rapid uptake of 1,8-cineole and camphor by plants suggests a potential mechanism whereby brief pulses of allelochemicals over an extended period of time might be able to exert an allelopathic effect on plants. The mechanism by which certain compounds are taken up by plants, while others with similar functional groups (e.g., carveol) are not, is in need of further study, as is the extent to which monoterpenes are taken up by other species. It is interesting to note that with two of the monoterpenes most widely implicated in allelopathic interactions, 1,8-cineole and camphor, 1,8-cineole was taken up by all three species tested and camphor by two of the three species tested.

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References

ABRAHIM, D., BRAGUINI, W. L., KELMER-BRACHT, A. M., and ISHII-IWAMOTO, E. L. 2000. Effects of four monoterpenes on germination, primary root growth, and mitochondrial respiration of maize. *J. Chem. Ecol* 26:611–624.

ARTHUR, C. L., and PAWLISZYN, J. B. 1990. Solid-phase microextraction with thermal-desorption using fused-silica optical fibers. *Anal. Chem* 62:2145–2148.

BAIS, H. P., VEPACHEDU, R., GILROY, S., CALLAWAY, R. M., and VIVANCO, J. M. 2003. Allelopathy and exotic plant invasion: From molecules and genes to invasive success. *Science* 301: 1377–1380.

BARNEY, J. N., HAY, A. G., and WESTON, L. A. 2005. Isolation and characterization of allelopathic volatiles from mugwort (*Artemisia vulgaris*). *J. Chem. Ecol* 31:247–265.

BLAIR, A. C., NISSEN, S., BRUNK, G. R., and HUFBAUER, R. A. 2006. A lack of evidence for an ecological role of the putative allelochemical (±)-catechin in spotted knapweed invasion success. *J. Chem. Ecol* 32:2327–2331.

BOUSSAHEL, R., BOULAND, S., MOUSSAOUI, K. M., BAUDU, M., and MONTIEL, A. 2002. Determination of chlorinated pesticides in water by SPME/GC. *Water Res* 36:1909–1911.

CHUN, J. C., LEE, H. J., LIM, S. J., KIM, S. E., and GUH, J. O. 2001. Comparative absorption, translocation, and metabolism of foliar-applied oxyfluorfen in wheat and barley. *Pestic. Biochem. Phys* 70:118–125.

CUSTÓDIO, L., SERRA, H., NOGUEIRA, J. M. F., GONÇALVES, S., and OMANO, A. 2006. Analysis of volatiles emitted by whole flowers and isolated flower organs of the carob tree using HS-SPME-GC/MS. *J. Chem. Ecol* 32:929–942.

DALTON, B. R., WEED, S. B., and BLUM, U. 1987. Plant phenolic acids in soils: a comparison of extraction procedures. *Soil Sci. Soc. Am. J* 51:1515–1521.

DEMORAES, C. M., LEWIS, W. J., PARÉ, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DENG, C. H., QIAN, J., ZHU, W. M., YANG, X., and ZHANG, X. M. 2005. Rapid determination of methyl salicylate, a plant-signaling compound, in tomato leaves by direct sample introduction and thermal desorption followed by GC-MS. *J. Sep. Sci* 28:1137–1142.

DENG, C. H., ZHANG, X. M., ZHU, W. M., and QIAN, J. 2004a. Gas chromatography-mass spectrometry with solid-phase microextraction method for determination of methyl salicylate and other volatile compounds in leaves of *Lycopersicon esculentum*. *Anal. Bioanal. Chem* 378:518–522.

DENG, C. H., ZHANG, X. M., ZHU, W. M., and QIAN, J. 2004b. Investigation of tomato plant defence response to tobacco mosaic virus by determination of methyl salicylate with SPME-capillary GC-MS. *Chromatographia* 59:263–268.

FUERST, E. P., and PUTNAM, A. R. 1983. Separating the competitive and allelopathic components of interference: theoretical principles. *J. Chem. Ecol* 18:1683–1691.

GALLET, C., and PELLISSIER, F. 1997. Phenolic compounds in natural solutions of a coniferous forest. *J. Chem. Ecol* 23:2401–2412.

KALNAY, P. A., and GLENN, S. 2000. Translocation of nicosulfuron and dicamba in hemp dogbane (*Apocynum cannabinum*). *Weed Technol* 14:476–479.

KOITABASHI, R., SUZUKI, T., KAWAZU, T., SAKAI, A., KUROIWA, H., and KUROIWA, T. 1997. 1,8-Cineole inhibits root growth and DNA synthesis in the root apical meristem of *Brassica campestris* L. *J. Plant Res* 110:1–6.

LORD, H. L., MÖDER, M., POPP, P., and PAWLISZYN, J. B. 2004. *In vivo* study of triazine herbicides in plants by SPME. *Analyst* 129: 107–108.

MULLER, C. H. 1965. Inhibitory terpenes volatilized from *Salvia* shrubs. *Bull. Torrey Bot. Club* 92:38–45.

MULLER, C. H., MULLER, W. H., and HAINES, B. L. 1964. Volatile growth inhibitors produced by aromatic shrubs. *Science* 143:471–473.

MUSTEATA, F. M., MUSTEATA, M. L., and PAWLISZYN, J. 2007. Fast *in vivo* microextraction: A new tool for clinical analysis. *Clin. Chem* 52:708–715.

NISHIDA, N., TAMOTSU, S., NAGATA, N., SAITO, C., and SAKAI, A. 2005. Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: Inhibition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. *J. Chem. Ecol* 31:1187–1203.

PAAVOLAINEN, L., KITUNEN, V., and SMOLANDER, A. 1998. Inhibition of nitrification in forest soil by monoterpenes. *Plant Soil* 205:147–154.

PEÑUELAS, J., IBAS-CARBO, M., and GILES, L. 1996. Effects of allelochemicals on plant respiration and oxygen isotope fractionation by the alternative oxidase. *J. Chem. Ecol* 22:801–805.

PONDER, F. Jr., and TADROS, S. H. 1985. Juglone concentration in soil beneath black walnut interplanted with nitrogen-fixing species. *J. Chem. Ecol* 11:937–942.

ROMAGNI, J. G., ALLEN, S. N., and DAYAN, F. E. 2000. Allelopathic effects of volatile cineoles on two weedy plant species. *J. Chem. Ecol* 26:303–313.

SHOUP, D., and AL-KHATIB, K. 2005. Fate of acifluorfen and lactofen in common waterhemp (*Amaranthus rudis*) resistant to protoporphyrinogen oxidase-inhibiting herbicides. *Weed Sci* 53:284–293.

SHULAEV, V., SILVERMAN, P., and ASKIN, I. 1997. Airborne signaling by methyl salicylate in plant pathogen resistance. *Nature* 385:718–721.

TANG, C.-S., and YOUNG, C.-C. 1982. Collection and identification of allelopathic compounds from the undisturbed root system of bigalta limpograss (*Hemarthria altissima*). *Plant Physiol* 69: 155–160.

TANRISEVER, N., FISCHER, N. H., and WILLIAMSON, G. B. 1988. Menthofurans from *Calamintha ashei*: Effects on *Schizachyrium scoparium* and *Lactuca sativa*. *Phytochemistry* 27:2523–2526.

TURLINGS, T. C., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

VAUGHN, S. F., and SPENCER, G. F. 1993. Volatile monoterpenes as potential parent structures for new herbicides. *Weed Sci* 41: 114–119.

WEIDENHAMER, J. D. 2005. Biomimetic measurement of allelochemical dynamics in the rhizosphere. *J. Chem. Ecol* 31:221–236.

WEIDENHAMER, J. D., MACIAS, F. A., FISCHER, N. H., and WILLIAMSON, G. B. 1993. Just how insoluble are monoterpenes? *J. Chem. Ecol* 19:1799–1807.

WEIDENHAMER, J. D., MENELAOU, M., MACIAS, F. A., FISCHER, N. H., ICHARDSON, D. R., and WILLIAMSON, G. B. 1994. Allelopathic potential of menthofuran monoterpenes from *Calamintha ashei*. *J. Chem. Ecol* 20:3345–3359.

WHITE, C. S. 1991. The role of monoterpenes in soil nitrogen cycling processes in ponderosa pine. *Biogeochemistry* 12:43–68.

WHITE, C. S. 1994. Monoterpenes: Their effects on ecosystem nutrient cycling. *J. Chem. Ecol* 20:1381–1406.

WILLIAMSON, G. B., FISCHER, N. H., RICHARDSON, D. R., and DE LA PEÑA, A. 1989. Chemical inhibition of fire-prone grasses by fire-sensitive shrub, *Conradina canescens*. *J. Chem. Ecol* 15: 1567–1577.

ZUNINO, M. P., and ZYGADLO, J. A. 2005. Changes in the composition of phospholipid fatty acids and sterols of maize root in response to monoterpenes. *J. Chem. Ecol* 31:1269–1283.

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Real-Time Analysis of Alarm Pheromone Emission by the Pea Aphid (*Acyrthosiphon Pisum*) Under Predation

Ezra G. Schwartzberg · Grit Kunert · Claudia Stephan · Anja David · Ursula S. R. Röse · Jonathan Gershenzon · Wilhelm Boland · Wolfgang W. Weisser

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Abstract Upon attack by predators or parasitoids, aphids emit volatile chemical alarm signals that warn other aphids of a potential risk of predation. Release rate of the major constituent of the alarm pheromone in pea aphids (*Acyrthosiphon pisum*), (*E*)-ß-farnesene (EBF), was measured for all nymphal and the adult stage as aphids were attacked individually by lacewing (*Chrysoperla carnae*) larvae. Volatilization of EBF from aphids under attack was quantified continuously for 60 min at 2-min intervals with a rapid gas chromatography technique (zNose™) to monitor headspace emissions. After an initial burst, EBF volatilization declined exponentially, and detectable amounts were still present after 30 min in most cases. Total emission of EBF averaged 16.33±1.54 ng and ranged from 1.18 to 48.85 ng. Emission was higher in nymphs as compared to adults. No differences between pea aphid life stages were detected for their speed of alarm signal emission in response to lacewing larvae attack. This is the first time that alarm pheromone emission from single aphids has been reported.

Keywords Aphid alarm pheromone · (*E*)-ß-farnesene · Pea aphid · *Acyrthosiphon pisum* · zNose

E. G. Schwartzberg · G. Kunert · A. David · U. S. R. Röse · J. Gershenzon · W. Boland
Max Planck Institute for Chemical Ecology,
Hans-Knöll-Straße 8,
07745 Jena, Germany

Present address:
E. G. Schwartzberg (✉)
Department of Entomology, Chemical Ecology Laboratory,
The Pennsylvania State University,
University Park, PA 16802, USA
e-mail: egs10@psu.edu

G. Kunert · C. Stephan · W. W. Weisser
Institute of Ecology, Friedrich-Schiller University,
Dornburger Str. 159,
07743 Jena, Germany

Present address:
U. S. R. Röse
ICG-III, Phytosphere, Research Center Juelich,
52425 Juelich, Germany

Introduction

Insects possess a wide range of chemical defenses that aid in protection against predation. Aphids are soft-bodied insects and are especially prone to attack by predators and parasitoids (Dixon 1998). Whereas aphids have means of protection against natural enemies through physical adaptations such as wax production (Smith 1999) and sclerotization among soldier forms of social aphids (Wool 2004), their primary protection from predators and parasitoids consists of escape responses mediated by the use of alarm pheromone signaling (Pickett et al. 1992; Dixon 1998). Aphids emit alarm pheromone by secreting a droplet of liquid from the ends of the siphunculi that contain chemical compounds. These compounds volatilize from the surface of the cornicle droplet and induce defense-related behavioral changes in conspecifics. Specifically, when aphids secrete a cornicle droplet in response to an attack by a predator or parasitoid, or in response to an artificial probe to simulate an attack, nearby aphids move away from the area and will often drop from their host plants (Losey and Denno 1998a; Braendle and Weisser 2001). Aphids have also been shown to mark predators with alarm pheromone by daubing them with cornicle droplets. This acts as a traveling signal, causing

aphids to disperse in response to an approaching predator (Mondor and Roitberg 2004). Recent results suggest that groups of aphids exposed to synthetic (*E*)-ß-farnesene (EBF), the main component of aphid alarm pheromone, (Bowers et al. 1972; Pickett and Griffiths 1980; Francis et al. 2005a), will be more likely to produce winged offspring (Kunert et al. 2005; Podjasek et al. 2005).

In addition to inducing defense-related behavioral changes in conspecifics, aphid alarm pheromone also induces behavioral changes in the predators and parasitoids that exploit them, acting as a kairomone used to home in on their aphid prey (Nakumuta 1991; Boo et al. 1998; Powell and Pickett 2003; Francis et al. 2004; Francis et al. 2005b).

Although the alarm pheromone, EBF, is used by many aphids of the family Aphididae, some species are more responsive to alarm pheromone than others (Losey and Denno 1998b), with variation in response to an alarm signal even among different clonal lines of the same species (Muller 1983; Braendle and Weisser 2001). In the pea aphid, *Acyrthosiphon pisum*, there is little variation in the amount of EBF emitted from different clonal lines in response to predation (Schwartzberg et al., unpublished); however, it has been shown that the concentration of EBF within cornicle droplets and the propensity to produce a cornicle droplet in response to an attack varies among instars (Mondor et al. 2000).

A number of studies have attempted to quantify the amounts of alarm pheromone produced by single aphids. Mondor et al. (2000) measured the EBF content within cornicle secretions of aphids disturbed by using a blunt probe. Schwartzberg et al. (unpublished) measured EBF in the headspace of aphid colonies attacked by predators, and the amount of EBF released by crushed aphids was measured by Francis et al. (2004) and Byers (2005). So far, however, no study has taken into consideration how natural predation may affect EBF emission from individual aphids, and how this may differ among aphid instars. Because the emission of EBF from a cornicle droplet is likely to be influenced by (1) the size and surface area of the cornicle droplet produced, (2) whether it is secreted as a single event, or continuously over a period of time, and (3) the concentration of EBF within the droplet, measuring the actual emission from single aphids is a much more direct and accurate method for comparing alarm signal among instars.

In this paper, we use a zNose™ (Electronic Sensor Technology, Newbury Park, CA, USA), which is an instrument for rapid gas chromatography that is capable of repeated quantitative sampling of headspace volatiles (Kunert et al. 2002; Tholl et al. 2006) to ask the following questions: (1) What is the temporal pattern of EBF volatization after attack by a predator? (2) Is there a time-lag between attack by the predator and EBF emission? (3) Do different instars differ in the amount of EBF emitted?

Materials and Methods

Pea aphids, *Acyrthosiphon pisum* (Hemiptera: Aphididae), used for alarm pheromone quantification were obtained from a naturally occurring pink pea aphid colony, "Jena Pink 1" (JP1), and were reared on 2-week-old dwarf broad bean plants, *Vicia faba* L. (The Sutton; Nickerson-Zwaan, UK) in 10-cm plastic pots with 16 h light: 8 h dark, 20°C, 75% relative humidity. Lacewing larvae, *Chrysoperla carnea* (Neuroptera: Chrysopidae), were obtained from a commercial supplier (Katz Biotech Services, Welzheim, Germany), and larvae were maintained on bean plants infested with pea aphids until they reached the second instar.

Aphid alarm pheromone emission was monitored from members of all instars, including the adult stage, by using a zNose™. Air was sampled from individual aphids and lacewing larvae within air entrainment vials (Fig. 1). The stainless steel inlet needle of the zNose™ was inserted through a small hole in the septum of a 4-ml glass vial. A clean air inlet was created by inserting a hypodermic syringe tip into the septum adjacent to the zNose™ inlet needle.

The zNose™ was programmed to sample air within the vial every 2 min with a flow rate of 30 ml min^{-1} for 20 s, drawing a total of 10 ml of air through the preconcentration trap during each 20 s sampling period. This quantity of air allowed the entrainment vial to be purged of most airborne volatiles prior to subsequent samples. The air was sampled repeatedly from the entrainment vial every 2 min for the duration of the bioassay.

For each replicate, a single aphid was introduced into a clean vial immediately after a baseline air entrainment. Once the aphid headspace was sampled, one second instar lacewing was introduced into the chamber with the aphid. Lacewing larvae did not attack aphids immediately so we were able to collect headspace volatile samples from aphid and lacewing larva before attack. Air within the vial was collected every 2 min from the introduction of the lacewing larva until 60 min after the beginning of lacewing attack (determined visually). Lacewing larvae feed by piercing their mouthparts into the soft bodies of their prey. The exoskeletons of prey are not consumed, and remain present even after feeding has finished. Therefore, it was not possible to discern exactly when the lacewing had stopped feeding. Because aphid alarm pheromone signals can exist beyond the life of an emitting aphid, the alarm signal was recorded even after the interior of the aphid was apparently fully consumed and the aphid was dead. We determined in preliminary tests that 60 min was a sufficient time period to measure all of the emitted alarm pheromone from an attacked aphid.

Air drawn from vials was collected on a Tenax™ absorbent trap during sampling. Trapped compounds were desorbed from the Tenax™ trap (300°C for 30 ms) and carried by helium (flow rate 3 ml min^{-1}) onto a GC column

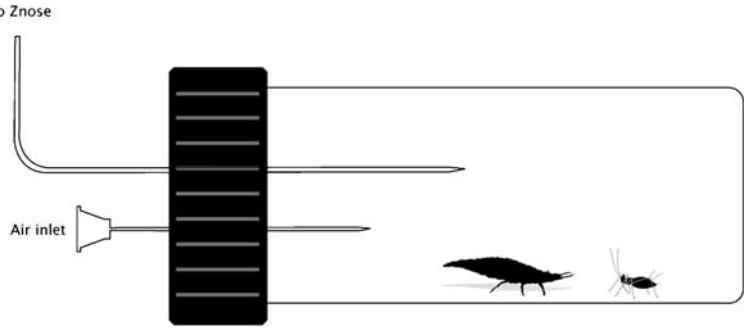

Fig. 1 Air entrainment setup showing 4 ml vial with inserted ZNose™ sampling needle and air inlet needle

(1 m, DB-5, film thickness 0.25 μm, ID 0.25 mm), which was programmed from 40 to 180°C at 5°C s^{-1}. As compounds exited the column, they were quantified as they pass over a temperature-controlled sensitive surface acoustic wave (SAW) quartz microbalance detector set at 40°C. The resonance frequency [Hz] of the SAW detector changes in a linear fashion as compounds from the column are attached to and heated off of the quartz detector. For quantification purposes, response factors were calculated by comparison with synthetic EBF (Bedoukian, Danbury, CT, USA) exposed to the zNose™ in known concentrations by using a heated desorber tube (glass, temperature controlled; 190°C; 3100 Vapor Calibrator; Electronic Sensor Technology, Newbury Park, CA, USA).

Alarm pheromone emission was quantified for nymphal stages L1 to L4 and for adult aphids (N=10 for each instar). The following variables were calculated from the data and used for the analysis: (1) total emission (calculated as the sum of all recordings after attack), (2) peak emission (the highest reading after attack), and (3) the lag time between initial attack and maximum emission (calculated as the time of attack subtracted from the time of maximum emission).

Because data were not normally distributed, we used a Kruskal–Wallis test for among-instar statistical comparisons.

Results

(*E*)-ß-farnesene was identified based on retention time comparison to a synthetic standard. There was only one peak detected in samples from JP1-aphids, and this peak was not present in control samples without aphids. Previous collections of volatiles from entire JP1-aphid colonies and analysis by GC-MS identified this peak as EBF (Schwarzberg et al., unpublished). The calibration curve for the SAW detector response to EBF was linear (y=0.230+0.000255x; y=ng of EBF, x=SAW detector response in hertz).

EBF volatization after an attack showed a typical pattern: after an initial burst it declined exponentially (Fig. 2). For the 46 cases where emission continued for more than 30 min,

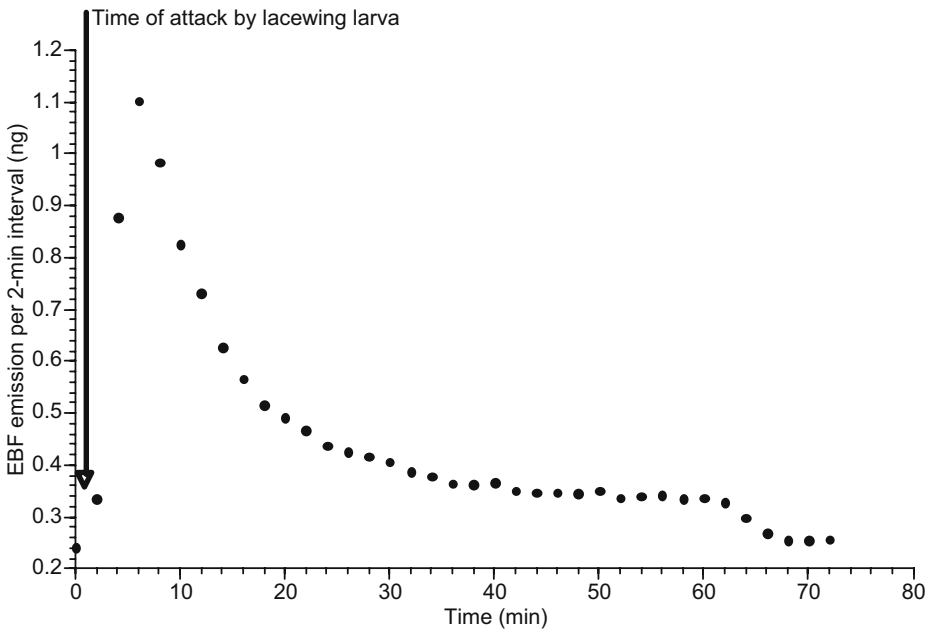

Fig. 2 Typical time course of (*E*)-ß-farnesene emission in the experiment. This example is from a first instar aphid (L1) attacked by a lacewing larva

amounts of over 0.3 ng per 2 min interval were measured in 30 replicates.

Total EBF emission over the entire 60 min collection period averaged 16.33±1.54 ng and ranged from 1.18 and 48.85 ng. Emission was different between first and second instar nymphs (L1 and L2) as compared to adults (χ^2= 13.34, *df*=4, *P*=0.01) with adult aphids emitting the least amount of EBF over the entire collection period (Fig. 3).

Peak EBF emission, measured as the maximum EBF emission for a 2 min interval within the total 60 min sampling period, had a mean of 1.98±0.32 ng per 2 min and ranged from 0.12 to 16.26 ng per 2 min. Mean peak emission varied among instars (χ^2=14.34, *df*=4, *P*=0.006, Fig. 4). While there was no significant difference in peak EBF emission among instars, adult peak emission was significantly lower.

The time lag between onset of attack by a lacewing larva and peak EBF emission was calculated to determine if different instars vary in their speed of alarm signal production. It is interesting to note that mean lag time was 10.7± 1.7 min and ranged from 2 to 50 min (*N*=48). Even if outliers (time-lag>13 min) were excluded, the mean was still 5.1± 0.4 min (*N*=36). Inspection of the 12 values with a time lag of 14 or more minutes revealed that in 10 cases (one for L1, five for L3, one for L4, and two for adults) there was no real burst of emission but rather a slow build up, associated with a low maximum emission value. In the other two cases (one L1, one L4), the observational determination of time of attack may have been wrong or there was a second burst of emission, possibly associated with a second attack by the predator. As a consequence of the variability, there was no statistical difference among instars (χ^2=3.98, *df*=4, *P*=0.409, Kruskal–Wallis, Fig. 5).

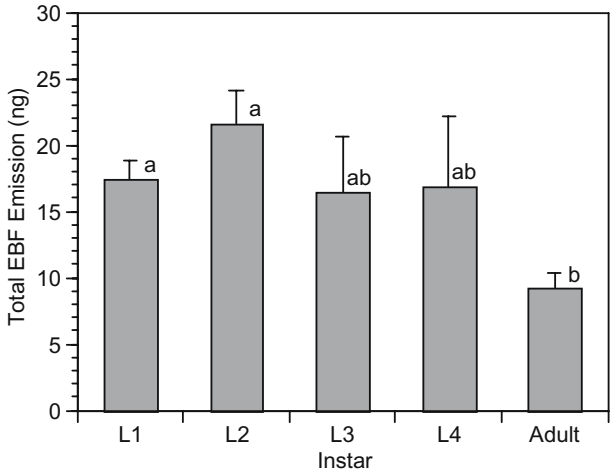

Fig. 3 Sum of (*E*)-ß-farnesene in nanogram±SE emitted over the entire 60-min collection period for immature instars (L1–L4) and adults. Significant differences in (*E*)-ß-farnesene production between instars are indicated by *different letters*; *P*≤0.05, Kruskal–Wallis test, *N*=10

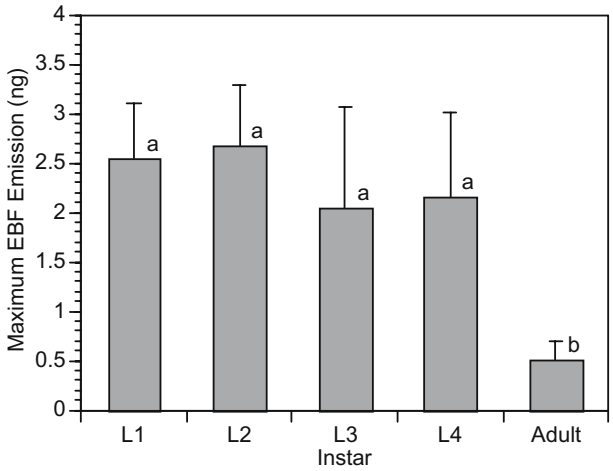

Fig. 4 Peak emission of (*E*)-ß-farnesene in nanogram±SE per 2 min sampling interval. Significant differences in (*E*)-ß-farnesene emission between instars are indicated by *different letters*; *P*≤0.05, Kruskal–Wallis test, *N*=10

Discussion

All life stages of pea aphids can be stimulated to produce a cornicle droplet when prodded, but adults do so less consistently (Mondor et al. 2000). This was also reflected in our results that show both total EBF emission and peak emission were lower in adults than in the younger instars. In addition to the differences among instars, our analysis showed that variability of EBF emission within instars was high and that detectable amounts of alarm pheromone were still present in the headspace of aphids for a considerable time after the initiation of attack.

The zNose™ was able to illustrate the temporal patterns of alarm pheromone emission from individual aphids. In addition to total emission over a 60-min time period, we were

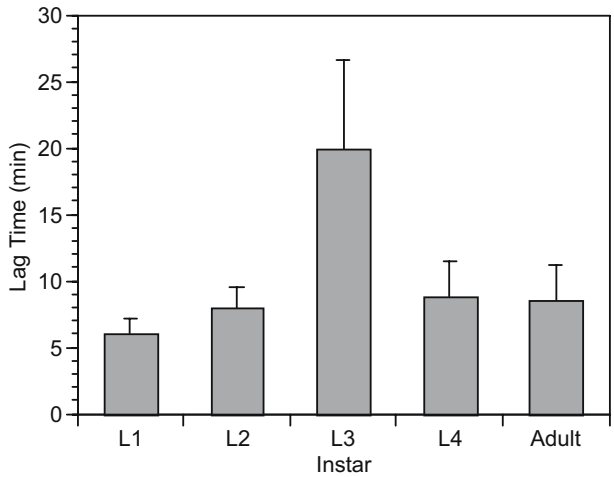

Fig. 5 Lag time between initial attack by lacewing larvae and peak emission of (*E*)-ß-farnesene in minutes±SE. There was no statistical difference in lag time among instars; *P*>0.05, Kruskal–Wallis test, *N*=10

able to record the initial peak of alarm signal production and latency of the alarm signal at 2 min intervals. The initial burst of alarm pheromone emission was relatively consistent among nymphal instars, but much lower in adults. The initial burst of alarm signal production is likely the most important, because aphids react within the first few seconds of perceiving the signal. Nevertheless, there was variability in peak emission from less than 1 ng up to 16 ng emitted in a single 2-min interval. Whereas we were able to determine visually when a lacewing larva grabbed an aphid, it was not possible to visually rate the strength of the attack and the time when the aphid died. The variability in peak emission may, therefore, reflect both the strength of initial attack and individual variability in cornicle secretion.

Aphid predators differ greatly in their handling time of prey, depending on their mode of feeding and the relative size of the predator compared to the prey. While we were not able to determine exactly the feeding time of the lacewing larvae in the vials, results from a separate experiment showed that feeding time of similar-sized lacewing larvae was 35.6± 4.8 min min after attack for adult pea aphids (N=12 larvae; Weisser, unpublished). In contrast, adult ladybirds consume an adult pea aphid within a few seconds. Handling times of ladybird larvae or hoverfly larvae are likely to be intermediate. Although most of the EBF that aphids produced during a lacewing larva attack volatilized during the first 15 min in our closed system, they continued to emit measurable quantities for 60 min. It is not known how EBF is synthesized, stored, and released by aphids during predation events, and it is possible that the larvae continually replenished their cornicle droplets while they were still not entirely eaten. Whereas our collection method was limited to a confined space with limited natural airflow in contrast to an open, natural system, where air currents may result in a faster rate of volatile emission from cornicle droplets, we believe that the EBF volatilization dynamics observed reflect natural dynamics. In experiments with adult ladybirds, a single flush of air after predation was in almost all cases enough to capture the entire EBF released (Hatano, personal communication). Regardless of whether natural dynamics may be slightly different, our design allowed for nonbiased comparison of signal strength and duration between nymphal and adult instars. It is interesting to note that these two temporally different signals were quantitatively similar among all nymphal instars.

Our results are consistent with those of other studies that attempt to measure alarm pheromone production from multiple life stages of aphids (Mondor et al. 2000; Byers 2005). In an elegant study, Mondor et al. (2000) determined the amounts of EBF in cornicle droplets after manual stimulation. Second to fourth instars contained on average between 11 and 13 ng, about five times higher than average peak emission in our study and slightly less than the 16 to 21 ng we found to be released over 60 min. In adults, Mondor et al. (2000) found on average 3–4 ng EBF in a cornicle droplet, about half of what we measured over the 60-min period, whereas EBF in adult cornicle droplets measured by Mondor et al. (2000) was four times as high as the mean peak emission in our study. The major difference between our and Mondor et al. (2000) results was for first instars, which emitted about 2 ng in their study, in contrast to 17 ng in ours. It is interesting to note that the 2 ng corresponded to what we measured as peak emission in our study. As mentioned above, it is conceivable that as the predator continues to feed on the aphid more EBF is released, either from a reservoir or by de novo synthesis, which would explain the higher amounts detected in summed headspace collections in our study. However, one thing to consider is that different predators feed differently, and this may have an effect on the duration and strength of the signal. For instance, a larger predator that completely consumes its prey may effectively quench the alarm signal of an aphid by consuming it.

Production of alarm pheromone, although altruistic in principle, is thought to be adaptive among groups of closely related individuals such as clones within aphid colonies because it allows related individuals to disperse and avoid predation or parasitization. However, alarm pheromone production comes at a cost (Mondor and Roitberg 2003; Byers 2005). This cost can be in terms of reproduction (Mondor and Roitberg 2003) or through the attraction of natural enemies (e.g., Francis et al. 2005a). Little is known about the emission dynamics of EBF in aphid colonies and how aphids may adjust alarm pheromone release to the actual ecological situation. An understanding of the temporal dynamics of EBF is necessary to further elucidate the role of aphid alarm pheromone in aphid–natural enemy interactions.

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References

Boo, K. S., Chung, I. B., Han K. S., Pickett, J. A., and Wadhams, L. J. 1998. Response of the lacewing *Chrysopa cognata* to pheromones of its aphid prey. *J. Chem. Ecol.* 24:631–643.

Bowers, W. S., Webb, R. E., Nault, L. R., and Dutky S. R. 1972. Aphid alarm pheromone: isolation, identification, synthesis. *Science* 177:1121–1122.

Braendle, C., and Weisser, W. W. 2001. Variation in escape behavior of red and green clones of the pea aphid. *J. Insect Behav.* 14:497–509.

BYERS, J. A. 2005. A cost of alarm pheromone production in cotton aphids, *Aphis gossypii*. *Naturwissenschaften* 92:69–72.

DIXON, A. F. G. 1998. Aphid Ecology. Chapman & Hall, London.

FRANCIS, F., LOGNAY, G., and HAUBRUGE, E. 2004. Olfactory responses to aphid and host plant volatile releases: (E)-beta-farnesene, an effective kairomone for the predator *Adalia bipunctata*. *J. Chem. Ecol.* 30:741–755.

FRANCIS, F., VANDERMOTEN, S., VERHEGGEN, F., LOGNAY, G., and HAUBRUGE, E. 2005a. Is (E)-beta-farnesene the only volatile terpenoid in aphids? *J. Appl. Entomol.* 129:6–11.

FRANCIS, F. D., MARTIN, T., LOGNAY, G., and HAUBRUGE, E. 2005b. Role of (E)-beta-farnesene in systematic aphid prey location by *Episyrphus balteatus* larvae (Diptera : Syrphidae). *Eur. J. Entomol.* 102:431–436.

KUNERT, G., OTTO, S., ROSE, U. S. R., GERSHENZON, J., and WEISSER, W. W. 2005. Alarm pheromone mediates production of winged dispersal morphs in aphids. *Ecol. Lett.* 8:596–603.

KUNERT, M., BIEDERMANN, A., KOCH, T., and BOLAND, W. 2002. Ultrafast sampling and analysis of plant volatiles by a hand-held miniaturised GC with pre-concentration unit: kinetic and quantitative aspects of plant volatile production. *J. Sep. Sci.* 25:677–684.

LOSEY, J. E. and DENNO, R. F. 1998a. The escape response of pea aphids to foliar-foraging predators: factors affecting dropping behaviour. *Ecol. Entomol.* 23:53–61.

LOSEY, J. E. and DENNO, R. F. 1998b. Interspecific variation in the escape responses of aphids: effect on risk of predation from foliar-foraging and ground-foraging predators. *Oecologia* 115: 245–252.

MONDOR, E. B., and ROITBERG, B. D. 2003. Age-dependent fitness costs of alarm signaling in aphids. *Can. J. Zool.* 81:757–762.

MONDOR, E. B., and ROITBERG, B. D. 2004. Inclusive fitmess benefits of scent-marking predators. *Proc. R. Soc. Lond. B* 271:341–343.

MONDOR, E. B., BAIRD, D. S., SLESSOR, K. N., and ROITBERG, B. D. 2000. Ontogeny of alarm pheromone secretion in pea aphid, *Acyrthosiphon pisum*. *J. Chem. Ecol.* 26:2875–2882.

MULLER, F. P. 1983. Differential alarm pheromone responses between strains of the aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 34:347–348.

NAKUMUTA, K. 1991. Aphid alarm pheromone component, (E)-beta-farnesene, and local search by a predatory lady beetle, *Coccinella septempunctata bruckii* Mulsant (Coleoptera: Coccinellidae). *Appl. Entomol. Zool.* 26:1–7.

PICKETT, J. A., and GRIFFITHS, D. C. 1980. Composition of aphid alarm pheromones. *J. Chem. Ecol.* 6:349–360.

PICKETT, J. A., WADHAMS, L. J., WOODCOCK, C. M., and HARDIE J. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

PODJASEK, J. O., BOSNJAK, L. M., BROOKER, D. J., and MONDOR, E. B. 2005. Alarm pheromone induces a transgenerational wing polyphenism in the pea aphid, *Acyrthosiphon pisum*. *Can. J. Zool.* 83:1138–1141.

POWELL, W. and PICKETT, J. A. 2003. Manipulation of parasitoids for aphid pest management: progress and prospects. *Pest Manag. Sci.* 59:149–155.

SMITH, R. G. 1999. Wax glands, wax production and the functional significance of wax use in three aphid species (Homoptera: Aphididae). *J. Nat. Hist.* 33:513–530.

THOLL, D., BOLAND, W., HANSEL, A., LORETO, F., ROSE, U.S.R., and SCHNITZLER, J. 2006. Practical approaches to plant volatile analysis. *Plant J.* 45:540–560.

WOOL, D. 2004. Galling aphids: specialization, biological complexity, and variation. *Annu. Rev. Entomol.* 49:175–192.

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An Antiaphrodisiac in *Heliconius melpomene* Butterflies

Stefan Schulz · Catalina Estrada · Selma Yildizhan · Michael Boppré · Lawrence E. Gilbert

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Abstract Gilbert (1976) suggested that male-contributed odors of mated females of *Heliconius erato* could enforce monogamy. We investigated the pheromone system of a relative, *Heliconius melpomene*, using chemical analysis, behavioral experiments, and feeding experiments with labeled biosynthetic pheromone precursors. The abdominal scent glands of males contained a complex odor bouquet, consisting of the volatile compound (*E*)-β-ocimene together with some trace components and a less volatile matrix made up predominately of esters of common C_{16}- and C_{18}-fatty acids with the alcohols ethanol, 2-propanol, 1-butanol, isobutanol, 1-hexanol, and (*Z*)-3-hexenol. This bouquet is formed during the first days after eclosion, and transferred during copulation to the females. Virgin female scent glands do not contain these compounds. The transfer of ocimene and the esters was shown by analysis of butterflies of both sexes before and after copulation. Additional proof was obtained by males fed with labeled D-$^{13}C_6$– glucose. They produced ^{13}C-labeled ocimene and transferred it to females during copulation. Behavioral tests with ocimene applied to unmated females showed its repellency to males. The esters did not show such activity, but they moderated the evaporation rate of ocimene. Our investigation showed that β-ocimene is an antiaphrodisiac pheromone of *H. melpomene*.

Keywords Pheromones · *Heliconius* · Antiaphrodisiacs · Sperm competition · Ocimene · Fatty acid esters · Labeled pheromone · Pheromone biosynthesis

S. Schulz (✉) · S. Yildizhan
Institut für Organische Chemie,
Technische Universität Braunschweig,
Hagenring 30,
38106 Braunschweig, Germany
e-mail: stefan.schulz@tu-bs.de

C. Estrada · L. E. Gilbert
Section of Integrative Biology, The University of Texas,
Austin, TX 78712, USA

M. Boppré
Forstzoologisches Institut, Albert-Ludwigs-Universität,
79085 Freiburg i.Br., Germany

Introduction

Male insects may use morphological, behavioral, or physiological adaptations that reduce the probability of female remating (Simmons 2001; Wedell 2005). One of these adaptations consists of male pheromones, transferred during mating, that make mated females repellent to subsequent males (Happ 1969; Gilbert 1976; Kukuk 1985; Andersson et al. 2000). These pheromones, known as antiaphrodisiacs (Happ 1969), confer direct selective advantage for the donor male by reducing sperm competition. However, there are also indirect advantages for other males and the females themselves, as courtship behavior and male harassment may result in reduced longevity and increased predation risk (Thornhill and Alcock 1983; Cook et al. 1994; Clutton-Block and Langley 1997; Bateman et al. 2006). Although antiaphrodisiac pheromones have been found in a wide range of insects, such as mealworm beetles, sweat bees, fruit flies, and butterflies (Happ 1969; Gilbert 1976; Kukuk 1985; Scott 1986), the chemical structures have been identified only for few species (Scott 1986; Andersson et al. 2000, 2003).

In butterflies, antiaphrodisiac pheromones were first found in the genus *Heliconius* (Lepidoptera: Nymphalidae). Gilbert (1976) suggested that the strong odor of *H. erato* mated females could inhibit further mating. He concluded

that the odor would repel courting males, as males waiting on female pupae rapidly left when exposed to the abdomen of mated females. All species of *Heliconius* and related genera, when handled, release strong odors that can be detected by humans (Eltringham 1925). These are produced in glands located in the last abdominal segments on both sexes. In females, it is a large yellow gland, which, when extruded, exposes a pair of club-shaped structures that probably serve to distribute the volatiles. Male glands, on the other hand, are located inside the two highly chitinized claspers that grasp the female abdomen during copulation (Eltringham 1925; Emsley 1963; Simonsen 2006).

In addition to their possible role in sexual communication, odors from abdominal glands may serve as warning signals about the toxicity of these butterflies (Eltringham 1925). Fritz Müller described the odors as strong and nauseous, and suggested they are also distasteful to enemies because they are released only after handling the butterflies (Eltringham 1925). The major volatile released from the abdominal scent glands of males and females of the heliconiine *Agraulis vanillae* is 6-methyl-5-hepten-2-one (Ross et al. 2001). The authors proposed that this ketone has a protective function, as it is a known defensive allomone of ants and cockroaches (Tomalsky et al. 1987; Ross et al. 2001). Their conclusions were based on observations of bird predation, but their experiments did not discriminate between protection due to gland constituents and those resulting from the wing warning coloration and toxicity (Nahrstedt and Davis 1983, 1985; Engler-Chaouat and Gilbert 2007). The idea of a male antiaphrodisiac, already reported in pierid butterflies and *Heliconius* (Gilbert 1976; Andersson et al. 2000, 2003), was not discussed by Ross et al. (2001).

Although *Heliconius* butterflies have been extensively studied and their characteristic odors described subjectively more than one century ago, few attempts have been made to elucidate their function and chemical composition (Eltringham 1925; Gilbert 1976; Miyakado et al. 1989; Ross et al. 2001). Here, we report work on the chemical composition of abdominal scent glands of *H. melpomene* and their role as an antiaphrodisiac pheromone. We show that the chemicals from abdominal scent glands are synthesized by the males only, are transferred to the females during copulation, and repel other courting males from mated females.

Methods and Materials

Chemicals Ocimene was prepared according to the method of Matsushita and Negishi (1982), resulting in a 7:3 mixture of (*Z*)- and (*E*)-ocimene. The amount of the (*E*)-isomer was increased to a 4:6 *Z*/*E* ratio by argentation-chromatography with AgNO3-treated silica (Williams and Mander 2001). We were unable to obtain a higher ratio, as no convenient and reproducible synthetic route to pure (*E*)-ocimene currently exists.

The ester mixture was prepared in a combinatorial way by using a mixture of fatty acids (ratios, see Table 1). The acids were converted into their acid chlorides by reaction with oxalyl chloride (Drutu et al. 2001). The acid chloride mixture was treated with an equimolar amount of an alcohol mixture (ratios, see Table 1) according to standard methods (Franklin et al. 2003). This ensured that every acid present in the mixture was esterified with each alcohol, resulting in a mixture of 25 esters. The analysis of the purified reaction product revealed that this ester mixture (hereafter called esters) was similar in composition and relative abundance of each component to the ester bouquet produced by male *H. melpomene*.

Analytical Procedures Chemical analyses were carried out on individual butterflies from a colony of *H. melpomene rosina* held in a greenhouse at Freiburg (Germany). The culture, originating from butterflies collected in Corcovado, Osa Peninsula, Costa Rica, was reared for about five generations with *Passiflora caerulea* as host plant and *Lantana camara* as source of pollen. The butterflies also had access to sucrose solution supplemented with pollen. Additional samples were obtained by a colony of *H. melpomene rosina* held at the University of Texas at Austin, originating from individuals brought from Costa Rica. They were reared in greenhouses at about 32°C and high humidity (>80%). Butterflies had access to *P. oerstedi* (larval host plant), sugar, and honey water, and sources of

Table 1 Relative proportion of acids and alcohols used in the synthesis of the ester mixture used in bioassays

Acid	Percent	Alcohol	Percent
Palmitic	5	2-propanol	8
Stearic	5	Isobutanol	17
Oleic	68	1-Butanol	18
Linoleic	13	1-Hexanol	52
Linolenic	9	(*Z*)-3-Hexenol	5

This mixture has similar composition and relative abundance to that produced by male *H. melpomene* butterflies.

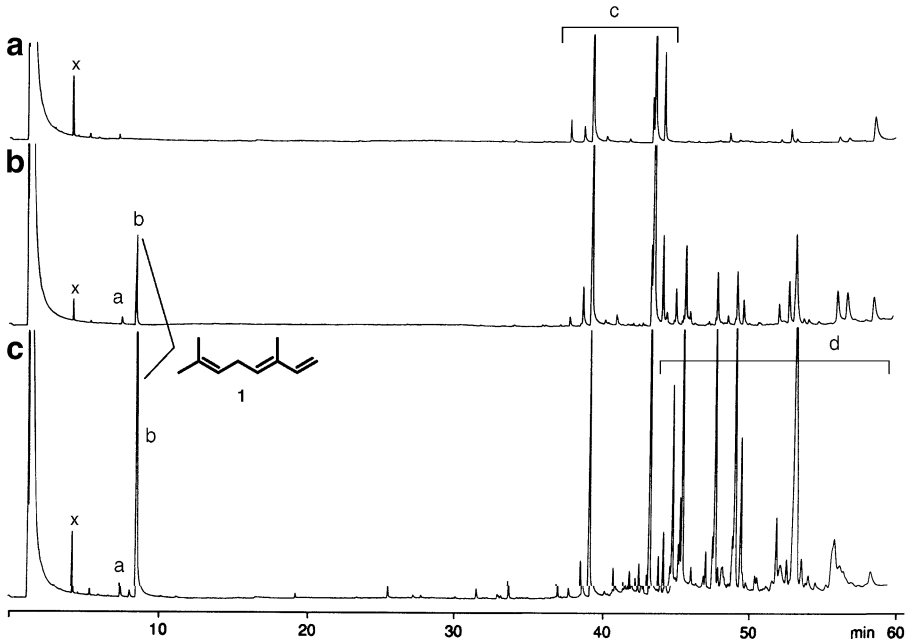


Fig. 1 Gas chromatogram of an extract of the scent glands of virgin females (**a**), mated females (**b**), and males (**c**) of *Heliconius melpomene*. *a*: (*Z*)-β-ocimene, *b*: (*E*)-β-ocimene, *c*: fatty acids, *d*: esters, *x*: internal standard (nonane)

pollen from *Psiguria* sp., *Psychotria poeppigiana* and *L. camara* flowers.

Butterflies were analyzed individually. The extracts were prepared either immediately after emergence from pupae or 5 d later. Some 5-d-old males were allowed to mate, and their glands were analyzed either directly after copulation or 3 d later.

Claspers of males and the last abdominal segment of females were dissected from bodies of freshly killed butterflies and placed individually in vials with approximately 100 µl of pentane. The lower tips of the abdomens were also cut and stored in vials with pentane. Analyses of the latter served to identify compounds found in tissues surrounding scent glands. Samples were kept at −70°C until analyzed.

Gas chromatography and mass spectrometry (GC-MS) of pentane extracts were performed with a Hewlett-Packard model 5973 mass selective detector connected to a Hewlett-Packard model 6890 gas chromatograph with a BPX5-fused silica capillary column (SGE, 30 m×0.25 mm, 0.25-µm film thickness). Injection was performed in splitless mode (250°C injector temperature) with helium as the carrier gas (constant flow of 1 ml/min). The temperature program started at 50°C, was held for 1 min, and then rose to 320°C with a heating rate of 5°C/min. All compounds were identified by comparison of the mass spectra and retention times with those of authentic reference samples in the different compound classes as well as by analysis of mass spectral fragmentation patterns.

The quantification of individual components was difficult because of many overlapping peaks, therefore peak areas of several compound classes were combined, and the sum of this area determined against an internal standard (hecadecanenitril). Results in Table 3 show individual variation of the scent gland composition. These data and results of the analysis of additional individuals were combined to examine the proportion of compounds transferred during copulation (Fig. 3).

Odors released by mated females or females of treatment 3 (see below) were analyzed by using headspace Solid

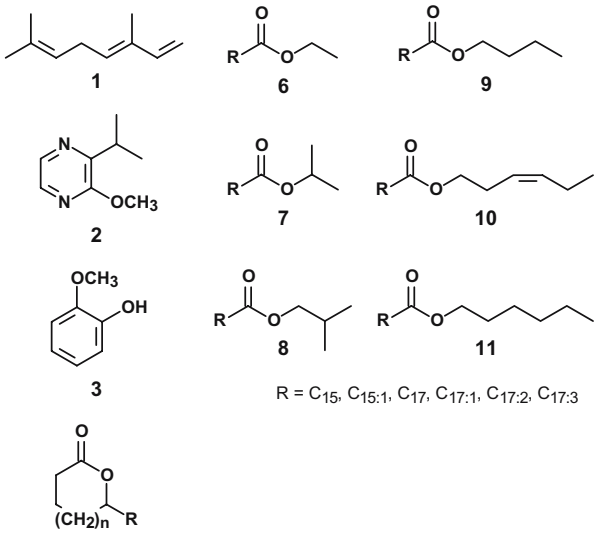


Fig. 2 Representative compounds identified in scent glands of *H. melpomene*. (*E*)-β-Ocimene (**1**), 3-isopropyl-2-methoxypyrazine (**2**), guaiacol (**3**), macrolides (**4** and **5**), fatty acid esters (**6** to **11**)

Table 2 Compounds identified in scent glands of *Heliconius melpomene*[a]

Compound	males	mated females	unmated females
Alcohols			
Hexanol		+	
(*Z*)-3-Hexenol	+		
Terpenes			
(*E*)-β-Ocimene (**1**)	+++	++	
(*Z*)-β-Ocimene	+	+	
Alloocimene	+		
Myrcene	+		
β-Cyclocitral	+		
(*E,E*)-α-Farnesene	+		
Squalene	+	+	
Aromatic compounds			
Guaiacol (**3**)	+	+	+
Methyl salicylate	+		+
3-Isopropyl-2-methoxypyrazine (**2**)	+		
3-*sec*-Butyl-2-methoxypyrazine	+	+	
3-Isobutyl-2-methoxypyrazine	+	+	
Macrolides			
11-Dodecanolide	+		
12-Dodecanolide	+		
14-Tetradecanolide	+		
15-Hexadecanolide	+	+	
16-Hexadecanolide	+		
17-Octadecanolide	+		
18-Octadecanolide	+		
18-Octadecenolide	+		
18-Octadecadienolide	+		
20-Icosanolide	+		
Esters of fatty acids			
Ethyl hexadecanoate	+	+	
Ethyl hexadecenoate	+	+	
Ethyl octadecanoate	+	+	
Ethyl octadecenoate	+	+	
Ethyl octadecadienoate	+	+	
Ethyl octadecatrienoate	+	+	
Isopropyl hexadecanoate	+	+	
Isopropyl octadecanoate	+	+	
Isopropyl octadecenoate	++	+	
Isopropyl octadecadienoate	+	+	
Isopropyl octadecatrienoate	+	+	
Butyl hexadecanoate	+	+	
Butyl octadecanoate	+	+	
Butyl octadecenoate	++	+	
Butyl octadecadienoate	+	+	
Butyl octadecatrienoate	+	+	
Isobutyl hexadecanoate	+	+	
Isobutyl octadecanoate	+	+	
Isobutyl octadecenoate	++	+	
Isobutyl octadecadienoate	+	+	
Isobutyl octadecatrienoate	+	+	
Hexyl hexadecanoate	++	+	
Hexyl octadecanoate	+	+	
Hexyl octadecenoate	+++	+	
Hexyl octadecadienoate	+	+	
Hexyl octadecatrienoate	+	+	
(*Z*)-3-Hexenyl hexadecanoate	++	+	

Table 2 (continued)

Compound	males	mated females	unmated females
(*Z*)-3-Hexenyl octadecanoate	+	+	
(*Z*)-3-Hexenyl octadecenoate	+++	+	
(*Z*)-3-Hexenyl octadecadienoate	+	+	
(*Z*)-3-Hexenyl octadecatrienoate	+	+	
Alkanes			
Hexadecane	+		
Heptadecane	+		
Octadecane	+		
Nonadecane	+		
Icosane	+		
Henicosane	+++	++	
Henicosene	++	+	
Docosane	+		
Tricosane	+	+	
Tricosene	+	+	
11-Methyltricosane	+	+	
Pentacosane	+	+	
11-Methylpentacosane	+	+	
13-Methylpentacosane	+	+	
Hexacosane	+	+	
13-Methylhexacosane	+	+	
Heptacosane	+	+	
13-Methylheptacosane	+	+	
Nonacosane	+	+	
11-Methylnonacosane	+	+	
13-Methylnonacosane	+	+	
15-Methylnonacosane	+	+	
11,15-Dimethylnonacosane	+	+	
13,17-Dimethylhentriacontane	+	+	
13,19-Dimethyltritriacontane	+	+	
Ketones			
2-Henicosanone	+	+	
2-Tricosanone	+	+	
Fatty acids			
Nonanoic acid		+	
Decanoic acid		+	
Dodecanoic acid	+	+	+
Tetradecanoic acid	+	+	+
Pentadecanoic acid	+	+	+
Pentadecenoic acid		+	+
Hexadecanoic acid	+++	+++	+++
Hexadecenoic acid	+	++	+
Heptadecanoic acid	+	+	
Heptadecenoic acid	+	+	
Octadecanoic acid	++	++	++
Octadecenoic acid	+++	+++	+++
Octadecadienoic acid	+++	++	++
Octadecatrienoic acid	+++	++	++
Nonadecenoic acid	+	+	
Nonadecadienoic acid	+	+	
Eicosanoic acid	+	+	+
Docosanoic acid	+	+	+
Tetracosanoic acid	+	+	
Dialkyltetrahydrofurans			
5-Nonyl-2-octadecyltetrahydrofuran	+		
2-Eicosyl-5-heptyltetrahydrofuran	+		

Table 2 (continued)

Compound	males	mated females	unmated females
2-Nonadecyl-5-nonyltetrahydrofuran	+		
2-Eicosyl-5-nonyltetrahydrofuran	+		
Steroids			
Cholesterol	+	+	

[a] +++: >20% of largest peak area; ++: <20% >3% of largest peak area; +: <3% of largest peak area.

Phase Microextraction (SPME) with a 65-μm polydimethylsiloxane/divinylbenzene (PDMS/DVB) fiber (Supelco) for 5 min. Analysis was performed with a Finnigan Q-GC gas chromatograph with a DB5-MS-fused silica capillary column (13 m×0.32 mm, 0.25-μm film thickness) coupled to a GCQ Polaris mass spectrometer. Splitless injection at 250°C was used with a temperature program rising from 60°C to 220°C, with heating rate of 10°C/min. Carrier gas was helium at a pressure of 4 psi.

Feeding Experiments Feeding experiments were carried out at the University of Texas at Austin. Freshly eclosed males were fed daily with isotopically labeled compounds of any of three potential biosynthetic precursors of ocimene:

Table 3 Quantitative composition of the scent gland of selected *Heliconius melpomene* individuals (μg/individual)[a]

Butterfly	1	18-i3	18-i4	18-4	16-6	18-6	C-21	C-25	Squ
Males, freshly emerged									
334A								+	+
334B								+	+
334C			+	+		0.07	+	+	0.09
334D			0.02	0.02	+	0.14	0.11	0.03	0.21
334E						0.12		+	0.11
Males, 5 days unmated									
331	18.35	0.06	0.12	0.14	0.09	1.04	0.35	0.05	0.06
331A	0.30	0.13	0.18	0.24	0.14	1.37	1.15	0.15	0.20
331B	23.97	0.09	0.19	0.22	+	0.81	0.33	0.05	+
331C	16.18	+	0.03	0.02	+	0.20	0.13	+	+
331D	11.87	0.04	0.08	0.12	+	0.48	0.27	0.08	0.03
Males, shortly after mating									
335	17.41	0.06	0.18	0.22	0.13	0.95	0.45	0.08	0.13
335A	9.58	0.06	0.12	0.13	0.06	1.07	0.53	0.13	0.10
Males, 3 days after mating									
336A	35.27	0.27	0.67	0.50	0.08	2.35	0.71	0.13	0.69
336B	63.61	0.75	1.64	2.17	0.32	3.37	1.51	0.37	0.51
336C	12.76	0.10	0.34	0.37	0.05	1.27	0.28	0.07	0.38
Females, freshly hatched									
332A								+	+
332B								+	0.73
333								+	+
Females, 5 days unmated									
337A								+	+
337B								0.07	0.27
337C								+	1.13
Females, directly after mating									
338	0.02					0.04	+	+	0.37
338A	+		0.01	0.02		0.09	0.01	0.05	0.04
Females, 3 days after mating									
339A	+	+	+	+	+	0.08	+	+	0.15
339B	1.66	0.01	0.03	0.02	0.01	0.22	0.05	0.04	0.04

a18-i3: Isopropyl esters of different saturated and unsaturated C_{18}-acids; 18-i4: respective isobutyl esters; 18-4: respective butyl esters; 16-6: hexyl and (*Z*)-3-hexenyl esters of different saturated and unsaturated C_{16}-acids; 18-6: respective esters of different saturated and unsaturated C_{18}-acids; C-21: Henicosane; C-25: Pentacosane; Squ: Squalene; +: trace component (<0.01 μg).

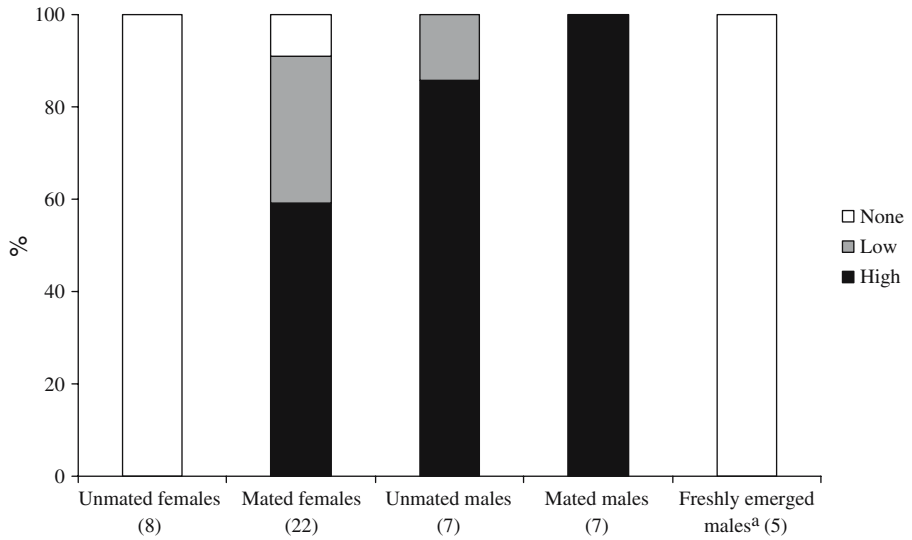


Fig. 3 Amounts of **1** and all esters in abdominal scent glands from individuals of *H. melpomene*. High: >1 μg of **1** and all esters; low: <1 μg of **1** and all esters; none: no detected. [a]Three males contained trace amounts of esters, but no **1**

25 mg of 99% D-$^{13}C_6$-glucose, 5 mg of 99% $^{13}C_2$-sodium acetate, and 10 mg of 4,4,6,6,6-D_5-mevalolactone (Dickschat et al. 2005), all in 5 ml of honey water. Each group of males was enclosed in a 2×2×2 m insectary with access to sugar water and one of the feeding mixtures added to an artificial flower. Males were also fed by gently holding the butterfly and placing drops of solution directly onto the proboscis with a syringe until satiation (ca. 25 μl). This was done daily during 10 d or more, until they mated to a virgin female. After mating, terminal abdominal segments of both sexes containing putative pheromone glands were dissected and analyzed by GC-MS as described in the analytical procedures.

Behavioral Bioassays The experiment were also conducted in greenhouses at the University of Texas at Austin. Abdomens of 1- to 5-d-old virgin females were painted with 2 μl of one of three chemical mixtures: (1) analytical grade hexane used as control; (2) a 1:1 solution of the synthetic ester mixture (Table 1) and hexane (1 mg/individual of ester mixture); and (3) a 0.1:1:1 solution of 6:4 (*E*/*Z*)-β-ocimene, ester mixture, and hexane (100 μg/individual of β-ocimene).

Males, randomly chosen from a population held in a separate greenhouse and never exposed to females, were tested individually for their behavior in the presence of treated virgin females. Only males more than 3 d old were used in the experiments. Each test consisted of two phases. The male was first exposed to a virgin female for 10 min, during which time the number and duration of courtship bouts were recorded. Once the male grasped the female abdomen with his claspers, the couple was immediately separated, and the male was allowed to rest for 10 min. The immediate separation after males initiated copulation assured that there was no transfer of spermatophore or scents from males. The aim of this phase was to verify the sexual receptivity of both butterflies; therefore, only males that succeeded on grasping female abdomens were used in the second phase of the test. In the second phase, the same female was transferred back to the male insectary after applying on the top of her abdomen 2 μl of one of the test solutions. Here, number and duration of courtship bouts were measured for 10 min or until the male grasped the female's abdomen again. A total of 30 test were carried out, 10 for each treatment of virgin females. Females were used once and 4 out of 26 males were used in more than one test. Whenever males were reused, tests were done at least 2 d apart, and different chemical mixtures were used in the experimental phase.

We tested whether the proportion of matings in experiments done with females painted with test mixtures that contained possible repellents was lower than those with control females by using *Fisher's exact test* (Sokal and Rohlf 1969). Mean courtship events and courtship times between the two phases of tests were compared with a nonparametric *Wilcoxon Matched Pairs Test*. We also compared these variables between experiments with females painted either control or test mixtures using nonparametric *Mann–Whitney U Tests* (STATISTICA for Windows 1999).

Results

Chemical Analyses The GC-MS analyses of abdominal gland extracts of males showed a bouquet made up of more than 100 components belonging to different chemical classes (Figs. 1 and 2, Table 2). The extracts of mated

females showed a similar chemical composition, albeit in lower concentrations (Fig. 1, Table 2), whereas those of unmated female glands exhibited a markedly reduced composition (Fig. 1, Table 2). It is clear from the total ion chromatogram (TIC, Fig. 1) that the male blend consisted of early and late eluting parts. The early eluting compounds are made up of several volatile components clustered in the TIC mostly around the major constituent, (*E*)-β-ocimene (**1**). Minor amounts of the terpenes (*Z*)-β-ocimene, allo-ocimene, β-cyclocitral, (*E*,*E*)-α-farnesene, and traces of the alcohols hexanol and (*Z*)-3-hexenol were detected. Additional trace compounds include three pyrazines (e.g., **2**), as well as guaiacol (**3**).

The later eluting (and thus higher boiling) fraction was dominated by a complex ester mixture (Table 2) of common C_{16}- and C_{18}-fatty acids with the alcohols tabulated in Table 1 (**6**–**11**). A range of macrolides derived from a bishomolog series of C_{12}- to C_{20}-acids, oxidized at the ω or ω-1 position, were also present in trace amounts (**4** and **5**). Along with esters, this fraction also contained typical cuticular components of insects as unbranched and methyl-branched alkanes, together with small amounts of 2,5-dialkyltetrahydrofurans (Schulz et al. 1998). The fatty acid content was variable (ranging from major to minor constituents of the bouquet). However, analyses of abdominal samples without the scent glands showed that fatty acids, alkanes, cholesterol, squalene, and dialkyltetrahydrofurans are not specific to the scent glands, so their presence probably reflects more the quality of the sample preparation than the physiological state of an individual. Consequently, we excluded these compounds from the bioassays. The composition of the male scent glands was remarkably similar among individual butterflies raised at the different locations and on different host plants, showing only slight variations in the trace components and the proportions of esters and alkanes.

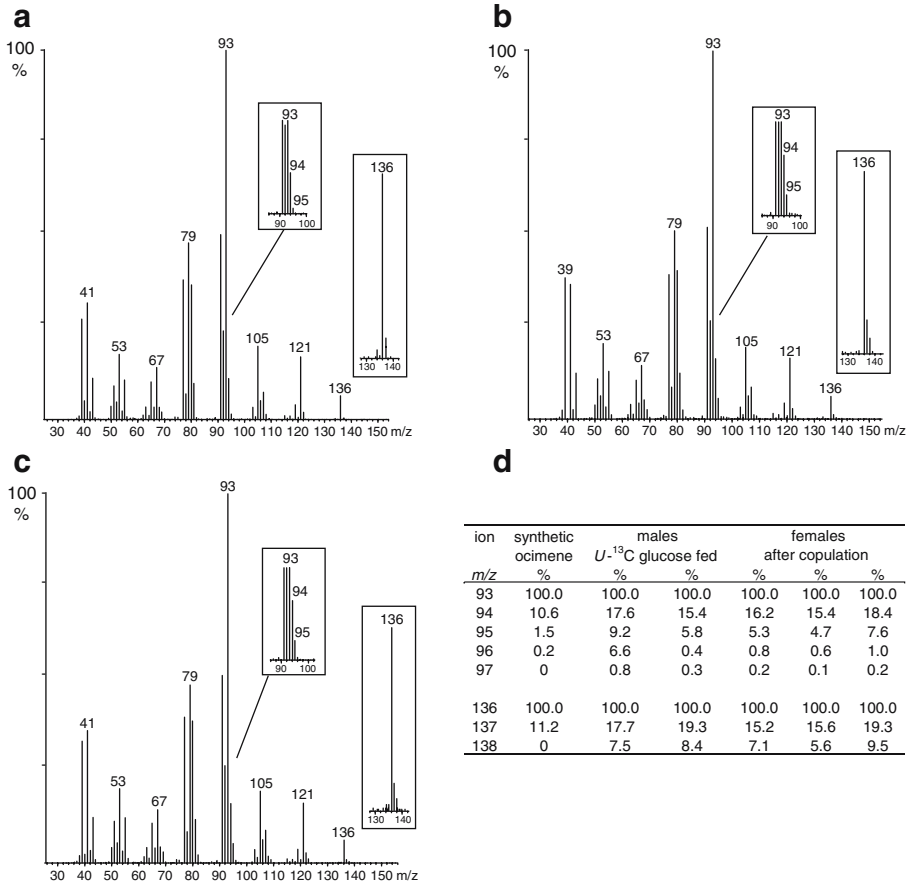
An obvious difference between the two parts of the blend is volatility. The calculated vapor pressure of **1**, the most prominent volatile component, is 1.56 Torr, whereas a major late eluting compound, hexyl octadecenoate, has a value of 4.94×10^{-8} Torr. One of the earliest eluting, and thus more volatile esters, ethyl hexadecanoate, has a value of 7.63×10^{-5} Torr (Scifinder database, ACS 2007). We, therefore, designate the ester part of the mixture as a matrix. Furthermore, because of their vapor pressures, macrolides, alkanes, tetrahydrofuranes, ketones, fatty acids, and cholesterol were all considered to belong to the matrix.

Abdominal glands from individuals of different age were also analyzed (Table 3). Freshly emerged males and females lacked volatile compounds, except for small amounts of guaiacol (**3**) in their scent glands. The only other compounds identified were the common C_{16}- and C_{18}-fatty acids together with hydrocarbons, cholesterol, and squalene, which are not gland-specific compounds. Five days after eclosion, males had developed the whole bouquet of compounds with **1** reaching up to 75 μg/individual. In males, bouquets before and after mating did not change significantly. Unmated females only produced **3**, but had a bouquet similar to that of males after mating. Even 3 d after mating, bouquet components were detectable. A quantitative analysis of a limited number of individual butterflies is found in Table 3. Occurrence of **1** and the esters in individuals of different physiological state is shown in Fig. 3.

Feeding Experiments with Labeled Percursors Deuterated mevalolactone, often incorporated by plants and bacteria into terpenes, was incorporated into **1** in males, but at a low rate (0.5%). ^{13}C-labeled acetate also gave low incorporation into **1**, but proved to be toxic, and surviving males did not mate. The best results were obtained by using uniformly labeled U-^{13}C-glucose. Glucose can be converted by butterflies into doubly labeled acetate via the citric acid cycle. This acetate can enter the mevalonate pathway of terpene synthesis. Together with two additional unlabeled acetate units, present in excess in experimental butterflies, it is transformed into isopentenyl pyrophosphate building blocks that contain one or two ^{13}C-atoms. This labeling pattern is expected because one carbon from the three acetate units is lost during this process, being either a ^{12}C- or ^{13}C-atom (Cane 1999). Indeed, **1** labeled with one or two ^{13}C-atoms was observed in the experiments. The possibility of glucose entering into terpene biosynthesis by the novel desoxyxylulose pathway (Eisenreich et al. 2004) was tested, but there was no evidence that this occurred.

The incorporation rate was determined by the relative abundance of the ions *m*/*z* 136/137/138 and *m*/*z* 93/94/95 in the mass spectrum of **1**, taking into account the natural abundance of ^{13}C (Fig. 4). The obtained values of 6 to 10% showed that males indeed biosynthesized **1** *de novo*. Females contained labeled **1** after mating with U-^{13}C-glucose fed males. Labeled esters, hydrocarbons, and tetrahydrofurans were also found in those females. These findings confirm that not only **1**, but also the esters and hydrocarbons, are transferred from males to females during mating.

Laboratory Bioassays In preliminary tests, β-ocimene showed a repellent effect on males, but its effect vanished quickly. We, therefore, choose to test this compound in combination with an artificial mixture of esters, similar in proportion to those found in the abdominal scent glands. Our assumption was that the esters might serve as a matrix, reducing the rate of evaporation of β-ocimene, so that the signal would stay longer on the females. We used the synthetic ester mixture because the esters are the most important compound class of the low boiling constituents of



ion	synthetic ocimene	males		females after copulation		
		U-^{13}C glucose fed				
m/z	%	%	%	%	%	%
93	100.0	100.0	100.0	100.0	100.0	100.0
94	10.6	17.6	15.4	16.2	15.4	18.4
95	1.5	9.2	5.8	5.3	4.7	7.6
96	0.2	6.6	0.4	0.8	0.6	1.0
97	0	0.8	0.3	0.2	0.1	0.2
136	100.0	100.0	100.0	100.0	100.0	100.0
137	11.2	17.7	19.3	15.2	15.6	19.3
138	0	7.5	8.4	7.1	5.6	9.5

Fig. 4 Mass spectra of **a**: synthetic (*E*)-β-ocimene (**1**); **b**: male *H. melpomene* after feeding U-^{13}C-glucose; **c**: females after copulation with males fed with U-^{13}C-glucose. **d**: Ion abundances relative to the largest ion in each series

the secretion. The other compounds were present only in low concentration or were part of the cuticule, as stated above. The odor emitted from females painted with the β-ocimene/ester solution was similar to the odor sampled from recently mated females when compared by GC-MS.

Males did not significantly change their behaviors toward females painted with hexane (control) as neither the mean number of courtship bouts (Z=1.069, P=0.285, Wilcoxon matched pair test, N=10) nor the mean courtship duration (Z=0.815, P=0.451, Wilcoxon matched pair test, N=10)

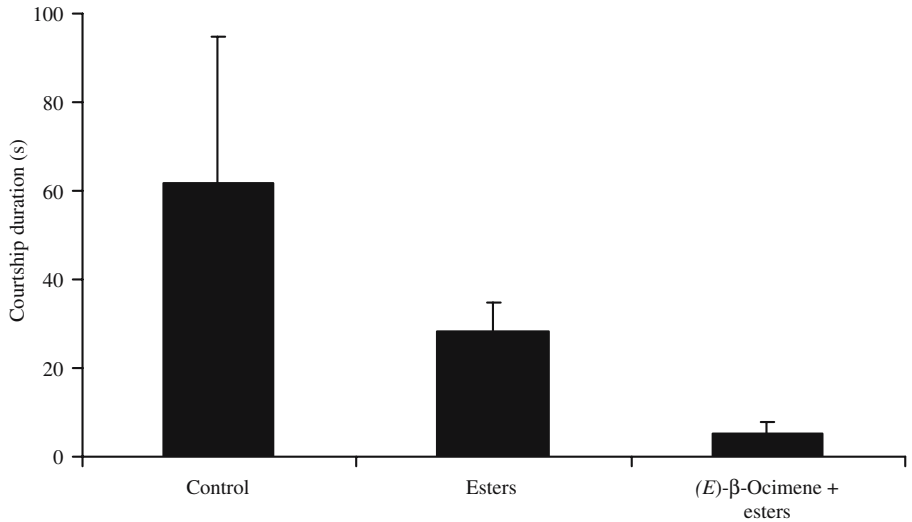


Fig. 5 Mean courtship duration in experiments where female abdomens were painted with 2 μl of control (hexane) or test mixtures to assess their effect on male sexual behavior. Test mixtures contained 1 mg of esters or a combination of 100 μg of 1:1 (*E*/*Z*)-β-ocimene plus esters

differed in the two experimental phases. Moreover, in 8 out of 10 tests males initiated copulation by grasping female abdomens after hexane was added to their abdomens.

Similarly, the ester mixture alone did not make females less attractive to courting males, as courtships were as long as those directed to control females (Mann–Whitney Test, $P=0.705$, $N=10$, Fig. 5). Furthermore, 70% of the couples engaged in copulation after painting females with esters, a percentage comparable to the one observed in control experiments (Fisher's exact test, $P=1$, $N=10$). Male behavior toward control or ester-painted females was similar to that observed in the first experimental phase. In all cases, males quickly approached and courted females, and in more than 90% of these tests, they grasped female abdomens within the first 2 min of the experiment.

In contrast to esters, β-ocimene strongly reduced female attractiveness. Although males exposed to females with β-ocimene plus esters quickly and repeatedly approached them, they courted them only for a few seconds and then left. The courtship duration was in average significantly shorter than in control experiments (Mann–Whitney Test, $P=0.002$, $N=10$, Fig. 5). In addition, only one of the 10 couples engaged in copulation in the second experimental phase, a lower proportion compared to that in control tests (*Fisher's exact test*, $P=0.006$, $N=10$).

Discussion

Our results show that male *H. melpomene* produce a complex odor profile that consists of volatile components as **1**, and a matrix of low-boiling-point components dominated by esters and hydrocarbons. This mixture is transferred to females during copulation, and the volatile component β-ocimene (**1**) makes females unattractive to courting males. As both ocimene isomers occur naturally and our synthetic sample also contained both, we cannot determine their relative strengths as repellents at this time. However, the prevalence of the (*E*)-isomer suggests this isomer to be active. The semivolatile ester matrix does not repel males, but in our experiments it reduced the evaporation rate of the ocimene, thus suggesting that this could be, at least in part, its function in female glands. Because the abdominal scent glands in this butterfly produce a bouquet that contains many different compounds, the possibility that other constituents of the scent gland also have antiaphrodisiac activity cannot be excluded. Such components might also provide other kinds of information to conspecifics, as in insects, even trace compounds of extracts can be important cues (e.g., Danci et al. 2006).

Interestingly, (*E*)-β-ocimene is one of the most prominent plant semiochemicals. It is commonly released by leaves in response to insect feeding or by flowers to attract pollinators (Pare and Tumlinson 1999; Andersson et al. 2002). Although the presence of this volatile has not been investigated in natural host plants or preferred pollen sources of *H. melpomene* (Boggs et al. 1981; Estrada and Jiggins 2002), **1** has been found in larval and adult host plants that are common in disturbed areas or that are frequently used to keep these butterflies in captivity, e.g. *L. camara* and *P. caerulea* (Piel et al. 1998; Andersson et al. 2002; Andersson and Dobson 2003b). Therefore, even if our experiments with labeled precursors show that *H. melpomene* has the capacity to synthesize **1** *de novo*, the additional contribution of **1** obtained from flowers or larval host plants cannot be ruled out.

Andersson and Dobson (2003a) showed that male and female *H. melpomene* antennae detect **1** and, in combination with other flower volatiles and color cues, was attractive to both sexes of butterflies. Taking into account the acute vision of *Heliconius* (Swihart 1972; Stavenga 2002; Zaccardi et al. 2006), the attractive or repellent activity of **1** might be explained by context specificity. In combination with other flower volatiles and visual flower cues, ocimene might be attractive, whereas with other conspecific gland constituents and visual signals, **1** could be repellent. This model points to further functions of the other male bouquet components besides the matrix effect discussed above. It also supports the observation that the antiaphrodisiac acts only over a short range.

Male behavior of *H. melpomene* toward mated females was similar to that described for pierid butterflies (Andersson et al. 2000) and *Drosophila melanogaster* (Scott 1986). Males approach mated females as often as unmated ones, clearly attracted by visual cues (Jiggins et al. 2004). When males come into close range to the mated females, the latter take mate-refusal posture by exposing their scent glands and release previously transferred compounds. Males immediately leave, so they spend less time courting mated than virgin females. In *Heliconius*, wing color patterns of females and males are identical, and males are visually attracted to both sexes. Males also release β-ocimene from their genitalia (claspers, unpublished data), which might serve for recognition of males and terminate male–male encounters. Therefore, it does appear that the antiaphrodisiac(s) of *H. melpomene* make mated females unattractive because they smell like males, as suggested earlier in *Heliconius* (Gilbert 1976) and found in *Pieris brassicae* and *D. melanogaster* (Scott 1986; Andersson et al. 2003).

Although antiaphrodisiac pheromones have been found in other groups of butterflies (Andersson et al. 2003), only the females of the Heliconiinae expose abdominal scent glands when they are courted or disturbed (Emsley 1963). This suggests that these odor mixtures might serve both as

antiaphrodisiac and protection. In fact, defense mechanism and sexual communication are tightly linked in unpalatable butterflies and moths (Boppré 1978, 1984; Weller et al. 1999; Conner et al. 2000; Schulz et al. 2004). The Lepidopteran scent glands studied so far show that the bouquets contain many different compounds (Miyakado et al. 1989; Ross et al. 2001; Schulz et al. 2007). Hence, it is possible that such odors provide conspecifics and predators with information. For example, the odor bouquet of *H. melpomene* contains three pyrazines, e.g., 3-isopropyl-2-methoxypyrazine (**2**), which are general warning odors of chemically defended insects and deter rats and birds (Kaye et al. 1989; Moore et al. 1990; Lindström et al. 2001). Unpalatable insects often use combinations of warning signals that target different sensory modalities (Lindström et al. 2001; Jetz et al. 2001). There is no evidence, other than the response to rough handling, that *Heliconius* females expose glands during attacks by predators. However, components in Heliconiinae odors, together with visual signals from the yellow glands, could alert, remind, and discourage predators.

Our findings that semiochemicals with evidence of antiaphrodisiac function are transferred from male to female *H. melpomene* support the observations published earlier by Gilbert (1976) for *H. erato*. Furthermore, we have similar results for other *Heliconius* and two other Heliconiinae in the genus *Argynnis* (Schulz, Yildizhan, Boppré, Estrada, Gilbert, unpublished; Schulz et al. 2007). Although the composition of these bouquets varies widely, male secretions of most Heliconiinae consists of more volatile compounds and a complex matrix of semivolatile components. This suggests a similar function of the scent glands in other Heliconiinae.

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References

ANDERSSON, S. and DOBSON, H. E. M. 2003a. Antennal responses to floral scents in the butterfly *Heliconius melpomene*. *J. Chem. Ecol.* 29:2319–2330.

ANDERSSON, S. and DOBSON, H. E. M. 2003b. Behavioral foraging responses by the butterfly *Heliconius melpomene* to *Lantana camara* floral scent. *J. Chem. Ecol.* 29:2303–2318.

ANDERSSON, J., BORG-KARLSON, A. K., and WIKLUND, C. 2000. Sexual cooperation and conflict in butterflies: A male-transferred anti-aphrodisiac reduces harassment of recently mated females. *Proc. R. Soc. Lond. B* 267:1271–1275.

ANDERSSON, S., NILSSON, L. A., GROTH, I., and BERGSTRÖM, G. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Botan. J. Linn. Soc.* 140:129–153.

ANDERSSON, J., BORG-KARLSON, A. K., and WIKLUND, C. 2003. Antiaphrodisiacs in pierid butterflies: A theme with variation! *J. Chem. Ecol.* 29:1489–1499.

BATEMAN, P. W., FERGUSON, J. W. H., and YETMAN, C. A. 2006. Courtship and copulation, but not ejaculates, reduce the longevity of female field crickets (*Gryllus bimaculatus*). *J. Zool.* 268:341–346.

BOGGS, C. L., SMILEY, J. T., and GILBERT, L. E. 1981. Patterns of pollen exploitation by *Heliconius* butterflies. *Oecologia* 48:284–289.

BOPPRÉ, M. 1978. Chemical communication, plant relationships, and mimicry in the evolution of danaid butterflies. *Entomol. Exp. Appl.* 24:264–277.

BOPPRÉ, M. 1984. Chemically mediated interactions between butterflies, pp. 259–275, in R. I. Vane-Wright and P. R. Ackery (eds.). The Biology of Butterflies. GB-London: Academic Press, reprinted edition 1989 by Princeton University Press.

CANE, D. E. 1999. Isoprenoid biosynthesis: Overview, pp. 1–13, in D. Barton, K. Nakanishi, O. Meth-Cohn, D. E. Cane (eds.). Comprehensive Natural Products Chemistry Vol. 2. Elsevier, Amsterdam.

CLUTTON-BLOCK, T. and LANGLEY, P. 1997. Persistent courtship reduces male and female longevity in captive tsetse flies *Glossina morsitans morsitans* Westwood (Diptera: Glossinidae). *Behav. Ecol.* 8:392–395.

CONNER, W. E., BOADA, R., SCHROEDER, F. C., GONZALEZ, A., MEINWALD, J., and EISNER, T. 2000. Chemical defense: Bestowal of a nuptial alkaloidal garment by a male moth on its mate. *Proc. Natl. Acad. Sci. USA* 97:14406–14411.

COOK, S. E., VERNON, J. G., BATESON, M., and GUILFORD, T. 1994. Mate choice in the polymorphic African swallowtail butterfly, *Papilio dardanus*: male-like female may avoid sexual harassment. *Anim. Behav.* 47:389–397.

DANCI, A., GRIES, R., SCHAEFER, P. W., and GRIES, G. 2006. Evidence for four-component close-range sex pheromone in the parasitic wasp *Glyptapanteles flavicoxis*. *J. Chem. Ecol.* 32:1539–1554.

DICKSCHAT, J. S., BODE, H. B., MAHMUD, T., MÜLLER, R., and SCHULZ, S. 2005. A novel type of geosmin biosynthesis in myxobacteria. *J. Org. Chem.* 70:5174–5182.

DRUTU, I., KRYGOWSKI, E. S., and WOOD, J. L. 2001. Reactive enols in synthesis 2. Synthesis of (+)-latifolic acid and (+)-latifoline. *J. Org. Chem.* 66:7025–7029.

EISENREICH, W., BACHER, A., ARIGONI, D., and ROHDICH, F. 2004. Biosynthesis of isoprenoids via the non-mevalonate pathway. *Cell. Mol. Life Sci.* 61:1401–1426.

ELTRINGHAM, M. A. 1925. On the abdominal glands in *Heliconius* (Lepidoptera). *Trans. Entomol. Soc. Lond.* 269–275.

EMSLEY, M. G. 1963. A morphological study of image Heliconiinae (Lep.: Nymphalidae) with a consideration of the evolutionary relationships within the group. *Zoologica* 48:85–131.

ENGLER-CHAOUAT, H. S. and GILBERT, L. E. 2007. De novo synthesis vs. sequestration: Negatively correlated metabolic traits and the evolution of host plant specialization in cyanogenic butterflies. *J. Chem. Ecol.* 33:25–42.

ESTRADA, C. and JIGGINS, C. D. 2002. Patterns of pollen feeding and habitat preference among *Heliconius* species. *Ecol. Entomol.* 27:448–456.

FRANKLIN, C. L., LI, H., and MARTIN, S. F. 2003. Design, Synthesis, and Evaluation of water-soluble phospholipid analogues as

inhibitors of phospholipase C from *Bacillus cereus*. *J. Org. Chem*. 68:7298–7307.

GILBERT, L. E. 1976. Postmating female odor in *Heliconius* butterflies: A male-contributed antiaphrodisiac? *Science* 193:419–420.

HAPP, G. 1969. Multiple sex pheromones of the mealworm beetle, *Tenebrio molitor* L. *Nature* 222:180–181.

JETZ, W., ROWE, C., and GUILFORD, T. 2001. Non-warning odors trigger innate color aversions—as long as they are novel. *Behav. Ecol.* 12:134–139.

JIGGINS, C. D., ESTRADA, C., and RODRIGUES, A. 2004. Mimicry and the evolution of premating isolation in *Heliconius melpomene* Linnaeus. *J. Evol. Biol.* 17:680–691.

KAYE, H., MACKINTOSCH, N. J., ROTHSCHILD, M., and MOORE, B. P. 1989. Odour of pyrazine potentiates an association between environmental cues and unpalatable taste. *Anim. Behav.* 37:1–6.

KUKUK, P. 1985. Evidence for an antiaphrodisiac in the sweat bee *Lasioglossum* (*Dialictus*) *zephyrum*. *Science* 227:656–657.

LINDSTRÖM, L., ROWE, C., and GUILFORD, T. 2001. Pyrazine odour makes visually conspicuous prey aversive. *Proc. R. Soc. Lond B.* 268:159–162.

MATSUSHITA, H. and NEGISHI, E. 1982. Palladium-catalyzed reactions of allylic electrophiles with organometallic reagents. A regioselective 1,4-elimination and a regio- and stereoselective reduction of allylic derivatives. *J. Org. Chem.* 47:4161–4165.

MIYAKADO, M., MEINWALD, J., and GILBERT, L. E. 1989. (*R*)-(*Z,E*)-9,11-Octadecadien-13-olide: An intriguing lactone from *Heliconius pachinus* (Lepidoptera). *Experientia* 45:1006–1008.

MOORE, B. P., BROWN, W. V., and ROTHSCHILD, M. 1990. Methylalkylpyrazines in aposematic insects, their hostplants and mimics. *Chemoecology* 1:43–51.

NAHRSTEDT, A. and DAVIS, R. H. 1983. Occurrence, variation and biosynthesis of the cyanogenic glucosides linamarin and lotaustralin in species of the Heliconiini (Insecta: Lepidoptera). *Comp. Biochem. Physiol.* 75B:65–73.

NAHRSTEDT, A. and DAVIS, R. H. 1985. Biosynthesis and quantitative relationships of the cyanogenic glucosides, linamarin and lotaustralin, in genera of the Heliconiini (Insecta: Lepidoptera). *Comp. Biochem. Physiol.* 82B:745–749.

PARE, P. W. and TUMLINSON, J. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol.* 121:325–332.

PIEL, J., DONATH, J., BANDEMER, K., and BOLAND, W. 1998. Mevalonate-independent biosynthesis of terpenoid volatiles in plants: induced and constitutive emission of volatiles. *Angew. Chem. Int. Ed.* 37:2478–2481.

ROSS, G. N., FALES, H. M., LLOYD, H. A., JONES, T., SOKOLOSKI, E. A., MARSHALL-BATTY, K., and BLUM, M. S. 2001. Novel chemistry of abdominal defensive glands of nymphalid butterfly *Agraulis vanillae*. *J. Chem. Ecol.* 27:1219–1228.

SCHULZ, S., BECCALONI, G., NISHIDA, R., ROISIN, Y., VANE-WRIGHT, R. I., and MCNEIL, J. N. 1998. 2,5-Dialkyltetrahydrofurans, common components of the cuticular lipids of Lepidoptera. *Z. Naturforsch.* 53c:107–116.

SCHULZ, S., BECCALONI, G., BROWN, K. S., BOPPRÉ, M., FREITAS, A. V. L., OCKENFELS, P., and TRIGO, J. R. 2004. Semiochemicals derived from pyrrolizidine alkaloids in male ithomiine butterflies (Lepidoptera: Nymphalidae: Ithomiinae). *Biochem. Syst. Ecol.* 32:699–713.

SCHULZ, S., YILDIZHAN, S., STRITZKE, K., ESTRADA, C., and GILBERT, L. E. 2007. Macrolides from the scent glands of the tropical butterflies *Heliconius cydno* and *Heliconius pachinus*. *Org. Biomol. Chem*. 5:3434–3441.

SCOTT, D. 1986. Sexual mimicry regulates the attractiveness of mated *Drosophila melanogaster* females. *Proc. Natl. Acad. Sci. USA* 83:8429–8433.

SIMMONS, L. W. 2001. Sperm Competition and Its Evolutionary Consequences in the Insects. Princeton University Press, Princeton and Oxford.

SIMONSEN, T. J. 2006. Glands, muscles and genitalia. Morphological and phylogenetic implications of histological characters in the male genitalia of fritillary butterflies (Lepidoptera: Nymphalidae: Argynnini). *Zool. Scripta* 35:231–241.

SOKAL, R. R. and ROHLF, J. 1969. Biometry. W. H. Freeman and Company, San Francisco.

STAVENGA, D. G. 2002. Reflections on colourful ommatidia of butterfly eyes. *J. Exp. Biol.* 205:1077–1085.

SWIHART, C. A. 1972. The neural basis of color vision in the butterfly, Heliconius erato. *J. Insect Physiol.* 18:1015–1025.

THORNHILL, R. and ALCOCK, J. 1983. The Evolution of Insect Mating Systems. Harvard University Press, Cambridge.

TOMALSKY, M. D., BLUM, M. S., JONES, T. H., FALES, H. M., HOWARD, D. F., and PASSERA, L. 1987. Chemistry and function of exocrine glands of the ants *Tapinoma melanocephalum* and *T. erraticum*. *J. Chem. Ecol.* 13:253–263.

WEDELL, N. 2005. Female receptivity in butterflies and moths. *J. Exp. Biol.* 208:3433–3440.

WELLER, S. J., JACOBSON, N. L., and CONNER, W. E. 1999. The evolution of chemical defenses and mating systems in tiger moths (Lepidoptera: Arctiidae). *Biol. J. Linn. Soc.* 68:557–578.

WILLIAMS, C. M. and MANDER, L. N. 2001. Chromatography with silver nitrate. *Tetrahedron* 57:425–447.

ZACCARDI, G., KELBER, A., SISON-MANGUS, M. P., and BRISCOE, A. D. 2006. Color discrimination in the red range with only one long-wavelength sensitive opsin. *J. Exp. Biol.* 209:1944–1955.

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Ethyl 4-Methyl Heptanoate: A Male-Produced Pheromone of *Nicrophorus vespilloides*

Wolf Haberer · Thomas Schmitt · Klaus Peschke · Peter Schreier · Josef K. Müller

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Abstract Sexually mature male beetles of the genus *Nicrophorus* (Coleoptera: Silphidae) exhibit a conspicuous behavior, recognized as pheromone-releasing activity. Laboratory and field studies demonstrated that females are attracted to males that exhibit this behavior, both on or off reproductive resources. Here, we report the results of a study in which volatile chemicals released by calling *Nicrophorus vespilloides* were collected by solid-phase microextraction and analyzed by using coupled gas chromatography–mass spectrometry. These analyses revealed that ethyl 4-methyl heptanoate and (*E*)-geranylacetone are emitted by males that engage in the behavior. In the field, traps baited with racemic ethyl 4-methyl heptanoate caught roughly equal numbers of male and female *N. vespilloides*. Some male and female *Nicrophorus vespillo* and male *Nicrophorus humator* were also caught in traps baited with this compound. Traps baited with (*E*)-geranylacetone did not catch significant numbers of beetles.

Keywords *Nicrophorus* · Silphidae · Burying beetle · Male attractant pheromone · Volatile · Ethyl 4-methyl heptanoate · (*E*)-geranylacetone · Coleoptera · Silphidae

W. Haberer (✉) · T. Schmitt · K. Peschke · J. K. Müller
Department of Evolutionary Biology and Ecology,
Faculty of Biology, University of Freiburg,
79104 Freiburg, Germany
e-mail: wolf.haberer@biologie.uni-freiburg.de

P. Schreier
Department of Food Chemistry, University of Würzburg,
Würzburg, Germany

Introduction

In the past few decades, the chemical signals and defenses produced by Coleoptera have been the subject of numerous studies that focus on occurrence, biosynthesis, and biological significance (Francke and Dettner 2005). However, some coleopteran families have received relatively little investigation. In particular, there have been few studies on the chemical ecology of the Silphidae. In *Necrodes surinamensis*, several terpenes (necrodols) with a repellent function have been identified (Eisner and Meinwald 1982; Eisner et al. 1986; Roach et al. 1990). *Silpha americana* (Meinwald et al. 1985) and *Silpha novaboracensis* (Meinwald et al. 1987) utilize steroids that act as defensive chemicals. A recent study analyzed the composition of anal secretions in *Nicrophorus marginatus* (Woodard 2006).

Beetles of the genus *Nicrophorus* (Coleoptera, Silphidae) search for small vertebrate carcasses that are suitable as a reproductive resource. Once a suitable carcass has been found, the beetles drive away intra- and interspecific competitors and bury the carrion, thus hiding it from other potential competitors. Although fights over the carcass often result between competing *Nicrophorus* beetles, various breeding associations may also form on the carcass, from monogamous pairs to polygynandrous groups (reviewed in Eggert and Müller 1997).

Males of most, if not all, *Nicrophorus* species engage in pheromone emission during a species-specific time of day (Pukowski 1933; Eggert and Müller 1989a, 1997; Scott 1998). Males select elevated sites and assume a headstand-like posture, pointing the head down and raising the tip of the abdomen. Males differ from females morphologically in that they have an additional distal abdominal segment, presumably the site of the pheromone gland (Mosebach 1936). Since Pukowski's (1933) detailed study of burying

beetles provided the first accurate description of this behavior, numerous studies have attempted to elucidate its biological implications. Pukowski (1933) called the behavior "sterzeln," stating that *Nicrophorus* males exhibit sterzeln only when they have discovered a reproductive resource on which no female is present. Later observations revealed that male pheromone emission is not contingent on the presence of reproductive resources, at least in some species (Müller and Eggert 1987; Eggert and Müller 1989a; Beeler et al. 1999), with pheromone emission in the absence of carrion being interpreted as an alternative mate-finding behavior (Eggert 1992).

The time of day and the extent to which pheromone emission occurs is known for several *Nicrophorus* species (Müller and Eggert 1987). Furthermore, Müller and Eggert (1987) have demonstrated that individuals of congeneric species may also be attracted to pheromone-emitting males, although the ecological significance of such interspecific attraction is unclear. Beeler et al. (2002) proposed that females discriminate among potential mates by assessing pheromone signals, which would require that the signals contain information about the calling individual beyond sex and species. In this study, we report the identification of the male-produced pheromone of *Nicrophorus vespilloides*, the most abundant *Nicrophorus* species in Central Europe.

Methods and Materials

Beetles Nicrophorus vespilloides males used for headspace analysis were first- or second-generation offspring of beetles caught in pitfall traps baited with pieces of pigs' lung in a field near Freiburg, Germany (48°02′14″N, 7°50′52″E). The traps used were identical to those used in the experiment that tested the attractiveness of synthetic chemicals in the field (Fig. 1). Individual beetles were kept in transparent plastic boxes (100×100×65 mm) filled with moist peat under a 16:8 light/dark photoperiod. Some male beetles were provided with a small mouse carcass, whereas others were given decapitated mealworms.

Headspace Sampling Headspace chemicals were obtained from 15 individual males, in separate plastic boxes, by using solid-phase microextraction fibers (SPME, coating polydimethylsiloxane/divinylbenzene, Supelco, Bellefonte, PA, USA) and analyzed by gas chromatography–mass spectrometry (GC-MS). GC-MS analysis was performed on a Hewlett Packard (Palo Alto, CA, USA) 6890-5973 system equipped with a DB-1 column (30 m×0.25 mm ID; *df*=0.25 μm; J & W, Folsom, CA, USA). Conditions were as follows: injector temperature 250°C, splitless mode, oven temperature 50–250°C at 10°C min^{-1}. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 230°C.

For collection of volatile chemicals, small holes were bored into the lids of boxes such that fibers could be placed close to the male during pheromone emission. Sampling took place from just before to the end of a beetle assuming the headstand posture typical of "sterzeln." Pheromone emission typically occurred between 1 hr before and 3 hr after lights off. After lights off, sampling continued under red light. We sampled the headspace of males with or without access to a carcass.

Chemicals Synthesis of racemic ethyl 4-methyl heptanoate was carried out by the method of Joung et al. (1998). The product was purified by liquid chromatography. (*E*)-geranylacetone was purchased from Fluka (Deisenhofen, Germany).

Field Experiment The field experiment was carried out in the oak-dominated mixed forest near Freiburg, southwestern Germany, from which our laboratory colonies originated. Five pitfall traps (Fig. 1) were arranged in a circle equidistant (ca. 50 m) from each other. A dispenser consisted of a 1.5 ml glass vial filled with silica gel (Merck, Darmstadt, Germany), 0.7 ml distilled water for wetting, and a 50-μl solution of the test chemicals. Vials were secured inside a tea ball with a small amount of liquid plaster. Each trap was baited with a tea ball placed above the center of the trap opening (Fig. 1). The vial remained closed until the bait was placed above a trap, at which point

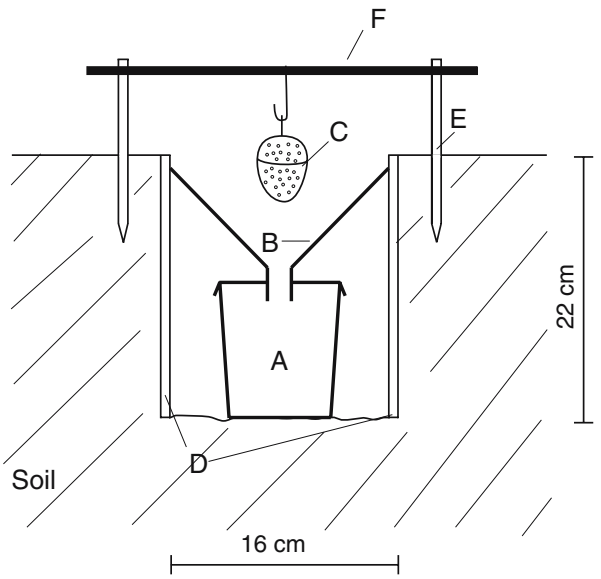

Fig. 1 Cross section of pitfall trap used in the field experiment. *A* container for trapped beetles, polyethylene. *B* funnel, polyethylene. *C* bait unit, consisting of a tea ball with dispenser inside. *D* PVC tube. *E* nail, stainless steel. *F* crossbar, aluminum

Table 1 Numbers of *N. vespilloides* beetles captured in five baited traps during 10 d of trapping

No.	Bait	D	Sum	Median	Range	
1	Ethyl 4-methyl heptanoate	9	23	2	0–5	a
2	Water (control A)	1	1	–	0–1	b
3	Pentane/dichloromethane (control B)	2	2	–	0–1	b
4	(*E*)-Geranylacetone	2	3	–	0–2	b
5	Ethyl 4-methyl heptanoate/(*E*)-geranylacetone	9	37	2	0–12	a

Different letters in the last column indicate differences between numbers of captured beetles (Wilcoxon–Wilcox post hoc test, $P<0.05$)
D = Days (out of ten) on which beetles were trapped with the respective bait.

the cap was removed. The following treatments were tested: bait 1, ethyl 4-methyl heptanoate (34 nmol); bait 2 (control A), silica gel with distilled water; bait 3 (control B), same as control A but with 50 µl of *n*-pentane/dichloromethane (9:1); bait 4, (*E*)-geranylacetone (2.6 nmol); and bait 5, ethyl 4-methyl heptanoate and (*E*)-geranylacetone (34 and 2.6 nmol, respectively). The ratio of methyl heptanoate to geranylacetone in bait 5 was similar to that observed in the SPME analyses.

Fresh baits were placed above the traps an hour before sunset and left for 24 hr, after which the baits and the trapping containers were removed. Each trap was left without bait for 1 d before adding a new bait and trapping container. The position of each treatment was moved to the next trap in a clockwise direction. This procedure was repeated until the dispensers had rotated through all the traps twice; i.e., a total of 10 d of trapping, alternating with a total of 10 d without baits. The field trial was carried out between June 26 and July 14, 2006.

Results

Gas Chromatography–Mass Spectrometry SPME GC-MS analysis of the SPME samples revealed two volatile compounds that were detectable only when *Nicrophorus* males assumed the typical "sterzeln" posture. Based on mass spectra and retention times of the two compounds, and comparison with those of the synthetic versions, we concluded that the compounds were ethyl 4-methyl heptanoate {m/z (relative abundance): 172 [M+] (<1), 143 (6), 129 (13), 127 (28), 115(15), 109 (26), 101 (100), 88 (88), 73 (30); RT 7.99 min} and (*E*)-geranylacetone {m/z (relative abundance): 194 [M+] (2), 176 (2), 161 (3), 151 (28), 136 (24), 125 (12), 107 (22), 93 (13), 69 (64), 43 (100); RT 11.91 min}, in a ratio of approximately 1:13. No differences were detected in the composition of pheromone blends of males with and without carcasses.

Attractiveness of Compounds In the field, the number of *N. vespilloides* captured (Table 1) differed significantly among bait types (Friedman test, $P<0.0001$), as did the number of successful vs. unsuccessful trapping days ($\chi^2=26.3$, $df=4$, $P<0.001$). Traps baited with either ethyl 4-methyl heptanoate or a mixture of ethyl 4-methyl heptanoate and geranylacetone caught the greatest number of beetles (Wilcoxon–Wilcox-test, $P<0.05$); both of these treatments caught *N. vespilloides* on all but one of the trapping days (Table 1). The catches in traps baited with either of these two treatments did not differ (Wilcoxon–Wilcox-test, $P>0.1$). Traps with treatments that lacked ethyl 4-methyl heptanoate (i.e., the two controls and geranylacetone) caught few beetles. Comparison of the daily catches of *N. vespilloides* showed similar numbers of males and females trapped (Wilcoxon MPSR, $P>0.3$, Table 2). Most of the beetles caught in the trial (80.5%) were *N. vespilloides*, but some *Nicrophorus vespillo* (17.1% of total) and *Nicrophorus humator* (2.4%) were also caught in traps that contained ethyl 4-methyl heptanoate (Table 2). There was no effect of

Table 2 Numbers of male and female *Nicrophorus* spp. captured in traps baited with different test stimuli

No.	Bait	*N. vespilloides*		*N. vespillo*		*N. humator*	
		m	f	m	f	m	f
1	Ethyl 4-methyl heptanoate	9	14	6	2	1	
2	Water (control A)		1				
3	Pentane/dichloromethane (control B)	2					
4	(*E*)-Geranylacetone	1	2				
5	Ethyl 4-methyl heptanoate/(*E*)-geranylacetone	18	19	4	2	1	
Sum		30	36	10	4	2	–

m = males, f = females

trap position on the number of beetles trapped (Friedman test, $P>0.25$).

Discussion

The data from both the headspace analysis and the field experiment suggest that ethyl 4-methyl heptanoate is a male-produced pheromone of *N. vespilloides*. The role of geranylacetone, also released by male *N. vespilloides*, remains unclear; catches with this compound alone were not different from those of the controls. Furthermore, addition of this compound to ethyl 4-methyl heptanoate did not yield increased catches compared to those with ethyl 4-methyl heptanoate alone. Interestingly, ethyl 4-methyl heptanoate has also been found as a minor component of volatile emissions from the beetle *Oryctes rhinoceros* (Coleoptera: Scarabaeidae), a widespread coconut pest in Asia. The principal pheromone component of *O. rhinoceros* is, however, ethyl 4-methyl octanoate (Hallett et al. 1995). Behavioral data suggest that the beetles produce a mix of both enantiomers of ethyl 4-methyl octanoate (Hallett et al. 1995). We did not determine the stereochemistry of ethyl 4-methyl heptanoate in *N. vespilloides*, but will do so in future studies.

Following the definition of Wertheim et al. (2005), ethyl 4-methyl heptanoate should be classified as an aggregation pheromone, rather than as a sex pheromone, of *N. vespilloides* because beetles of both sexes are attracted to this compound. Interestingly, in the field, males appear to attract primarily females (Müller and Eggert 1987). Males of the rove beetle *Aleochara curtula* also release a pheromone that attracts both sexes (Peschke et al. 1999).

Pheromone emission without carrion has been interpreted as an alternative to the primary mate-finding tactic of searching for carcasses (Eggert 1992; Beeler et al. 1999; Müller et al. 2007). According to Eggert (1992), pheromone emission benefits males irrespective of the presence of a reproductive resource. Males that have access to a suitable carcass can attempt copulation once a female arrives, and males without such a resource can secure matings with attracted females. In a field study of *N. vespilloides*, males that had buried a carcass prior to pheromone emission attracted equal numbers of females as males that had not (Eggert and Müller 1989b). Responding to the pheromone also benefits females by allowing them access to a carcass and/or a mate. Benefits for males that are attracted to males are less clear, especially if the pheromone-emitting male does not have a carcass. One possibility is that the attracted male could mate with surplus females that the pheromone emitter attracts. If the calling male has a carcass, then presumably the attracted male would fight the caller for the resource.

In the field trial, two other *Nicrophorus* species were attracted to ethyl 4-methyl heptanoate, consistent with earlier findings that calling male *Nicrophorus* attract members of other congeneric species (Müller and Eggert 1987). There is some evidence (Haberer, Schmitt, and Müller, unpublished) that the attracted *Nicrophorus* species use similar (i.e., 4-methyl branched heptanoate and octanoate) esters for their own sex pheromones. Müller and Eggert (1987) suggested that the response of larger congeners to the pheromones of a smaller species might be adaptive. If the pheromone-emitting male has access to a carcass, members of larger species would be able to displace the original owner and utilize the resource for their own reproduction. The two additional species, *N. vespillo* and *N. humator*, that were attracted to ethyl 4-methyl heptanoate are both larger than and competitively superior to *N. vespilloides* (Pukowski 1933; Otronen 1988). Future investigations of other *Nicrophorus* pheromones will aid our understanding of cross-attraction within the genus.

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References

BEELER, A. E., RAUTER, C. M., and MOORE, A. J. 1999. Pheromonally mediated mate attraction by males of the burying beetle *Nicrophorus orbicollis*: Alternative calling tactics conditional on both intrinsic and extrinsic factors. *Behav. Ecol.* 10:578–584.

BEELER, A. E., RAUTER, C. M., and MOORE, A. J. 2002. Mate discrimination by females in the burying beetle *Nicrophorus orbicollis*: The influence of male size on attractiveness to females. *Ecol. Entomol.* 27:1–6.

EGGERT, A.-K. 1992. Alternative male mate-finding tactics in burying beetles. *Behav. Ecol.* 3:243–254.

EGGERT, A.-K., and MÜLLER, J. K. 1989a. Pheromone-mediated attraction in burying beetles. *Ecol. Entomol.* 14:235–238.

EGGERT, A.-K., and MÜLLER, J. K. 1989b. Mating success of pheromone-emitting *Necrophorus* males: Do attracted females discriminate against resource owners? *Behaviour* 110:248–257.

EGGERT, A.-K., and MÜLLER, J. K. 1997. Biparental care and social evolution in burying beetles: Lessons from the larder, pp. 216–236, in: J. C. Choe and B. J. Crespi (eds.). Social Behavior in Insects and Arachnids. Cambridge University Press, Cambridge.

EISNER, T., and MEINWALD, J. 1982. Defensive spray mechanism of a silphid beetle (*Necrodes surinamensis*). *Psyche* 89:357–368.

EISNER, T., DEYRUP, M., JACOBS, R., and MEINWALD, J. 1986. Necrodols: Anti-insectan terpenes from defensive secretion of carrion beetle (*Necrodes surinamensis*). *J. Chem. Ecol.* 12:1407–1415.

FRANCKE, W., and DETTNER, K. 2005. Chemical signalling in beetles. Topics in current chemistry, pp. 85–166. Springer-Verlag, Berlin.

HALLETT, R. H., PEREZ, A. L., GRIES, G., GRIES, R., PIERCE, H. D., Jr., YUE, J., OEHLSCHLAGER, A. C., GONZALEZ, L. M., and BORDEN, J. H. 1995. Aggregation pheromone of coconut rhinoceros beetle, *Oryctes rhinoceros* (L.) (Coleoptera: Scarabaeidae). *J. Chem. Ecol.* 21:1549–1570.

JOUNG, M. J., AHN, J. H., LEE, D. W., and YOON, N. M. 1998. Coupling reaction of alkenes with α-bromo carboxylic acid

derivatives using nickel boride and borohydride exchange resin in methanol. *J. Org. Chem.* 63:2755–2757.

MEINWALD, J., ROACH, B., HICKS, K., ALSOP, D., and EISNER, T. 1985. Defensive steroids from a carrion beetle (*Silpha americana*). *Experientia* 41:516–519.

MEINWALD, J., ROACH, B., and EISNER, T. 1987. Defensive steroids from a carrion beetle (*Silpha novaboracensis*). *J. Chem. Ecol.* 13:35–38.

MOSEBACH, E. 1936. Aus dem Leben des Totengräbers (*Necrophorus*). *Nat. Volk* 66:222–231.

MÜLLER, J. K., and EGGERT, A.-K. 1987. Effects of carrion-independent pheromone emission by male burying beetles (Silphidae: *Necrophorus*). *Ethology* 76:297–304.

MÜLLER, J. K., BRAUNISCH, V., HWANG, W., and EGGERT, A.-K. 2007. Alternative tactics and individual reproductive success in natural associations of the burying beetle, *Nicrophorus vespilloides*. *Behav. Ecol.* 18:196–203.

OTRONEN, M. 1988. The effect of body size on the outcome of fights in burying beetles (*Nicrophorus*). *Ann. Zool. Fenn.* 25:191–201.

PESCHKE, K., FRIEDRICH, P., KAISER, U., FRANKE, S., and FRANCKE, W. 1999. Isopropyl (Z9)-hexadecenoate as a male attractant pheromone from the sternal gland of the rove beetle Aleochara curtula (Coleoptera: Staphylinidae). *Chemoecology* 9:47–54.

PUKOWSKI, E. 1933. Ökologische Untersuchungen an *Necrophorus* F. *Z. Morphol. Okol. Tiere* 27:518–586.

ROACH, B., EISNER, T., and MEINWALD, J. 1990. Defense mechanisms of arthropods. 83. Alpha- and beta-necrodol, novel terpenes from a carrion beetle (*Necrodes surinamensis*, Silphidae, Coleoptera). *J. Org. Chem.* 55:4047–4051.

SCOTT, M. P. 1998. The ecology and behavior of burying beetles. *Annu. Rev. Entomol.* 43:595–618.

WERTHEIM, B., VAN BAALEN, E. J. A., DICKE, M., and VET, L. E. M. 2005. Pheromone-mediated aggregation in nonsocial arthropods: An evolutionary ecological perspective. *Annu. Rev. Entomol.* 50:321–346.

WOODARD, C. B. S. 2006. Odor masking of a vertebrate carcass by a burying beetle (*Nicrophorus marginatus*). Master's thesis, Texas Tech University.

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RAPID COMMUNICATION

4-Methylquinazoline is a Minor Component of the Male Sex Pheromone in *Nasonia vitripennis*

Joachim Ruther · Sven Steiner · Leif-Alexander Garbe

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Abstract We identified 4-methylquinazoline (4-MeQ) as a minor component of the male sex pheromone of the parasitoid *Nasonia vitripennis*. Like the major components (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolide (HDL), 4-MeQ is synthesized in the abdomen of males. At doses of 6 or 1 ng, 4-MeQ synergized the response of virgin females to the HDL-diastereomers in a still-air olfactometer, but was not attractive as a single component. 4-MeQ is also responsible for the characteristic medicinal odor of *N. vitripennis* males.

Keywords *Nasonia vitripennis* · Parasitoid · Pteromalidae · Sex pheromone · 4-methylquinazoline

Introduction

There is increasing evidence that with parasitic wasps, for some species, females attract mates with volatile sex pheromones, whereas in other species, males release the attractive chemicals (Cônsoli et al. 2002; Ruther et al. 2007). Males of the jewel wasp *Nasonia vitripennis*, a pupal parasitoid of cyclorraphous flies, release from their abdomen a mixture of (4*R*,5*R*)- and (4*R*,5*S*)-5-hydroxy-4-decanolides (HDL) that is attractive to virgin but not mated females (Ruther et al. 2007). Sample containers used for the storage of live *N. vitripennis* males and abdomen extracts from males possess a characteristic medicinal odor. However, the HDL-diastereomers smell fruity, reminiscent of peaches, suggesting that *N. vitripennis* males release another volatile chemical responsible for the characteristic odor. Here, we report identification of 4-methylquinazoline (4-MeQ) as a minor component of the male sex attractant of *N. vitripennis*, which synergizes the response of virgin females to HDL and which is responsible for the characteristic odor of *N. vitripennis* males as sensed by the human nose.

J. Ruther (✉) · S. Steiner
Freie Universität Berlin, Institut für Biologie,
Haderslebener Straße 9, 12163 Berlin, Germany
e-mail: ruther@zedat.fu-berlin.de

L.-A. Garbe
Technische Universität Berlin, Institut für Biotechnologie,
Seestraße 13, 13353 Berlin, Germany

Methods and Materials

Insects N. vitripennis were reared on puparia of the green bottle fly *Lucilia caesar* as described elsewhere (Ruther et al. 2007). Three-day-old unmated male and female wasps were used for the experiments.

Preparation of Abdomen Extracts The abdomens of individual freeze-killed males were dissected under a stereomicroscope with a scalpel and transferred to 100-µl vial inserts filled with 20 µl dichloromethane that contained 20 ng/µl methyl undecanoate (Sigma-Aldrich) as an internal standard (N = 10). Each abdomen was extracted for 30 min.

Chemical Analysis Extracts (1 µl) were analyzed by gas chromatography-mass spectrometry (GC-MS) on a Fisons 8060 GC coupled to a Fisons MD800 quadrupole MS (EI, 70eV). The GC was equipped with (a) a nonpolar 30 m × 0.32 mm i.d. DB-5MS column, film thickness 0.25 µm or (b) a polar 30 m × 0.32 mm i.d. DB-WAX column, film

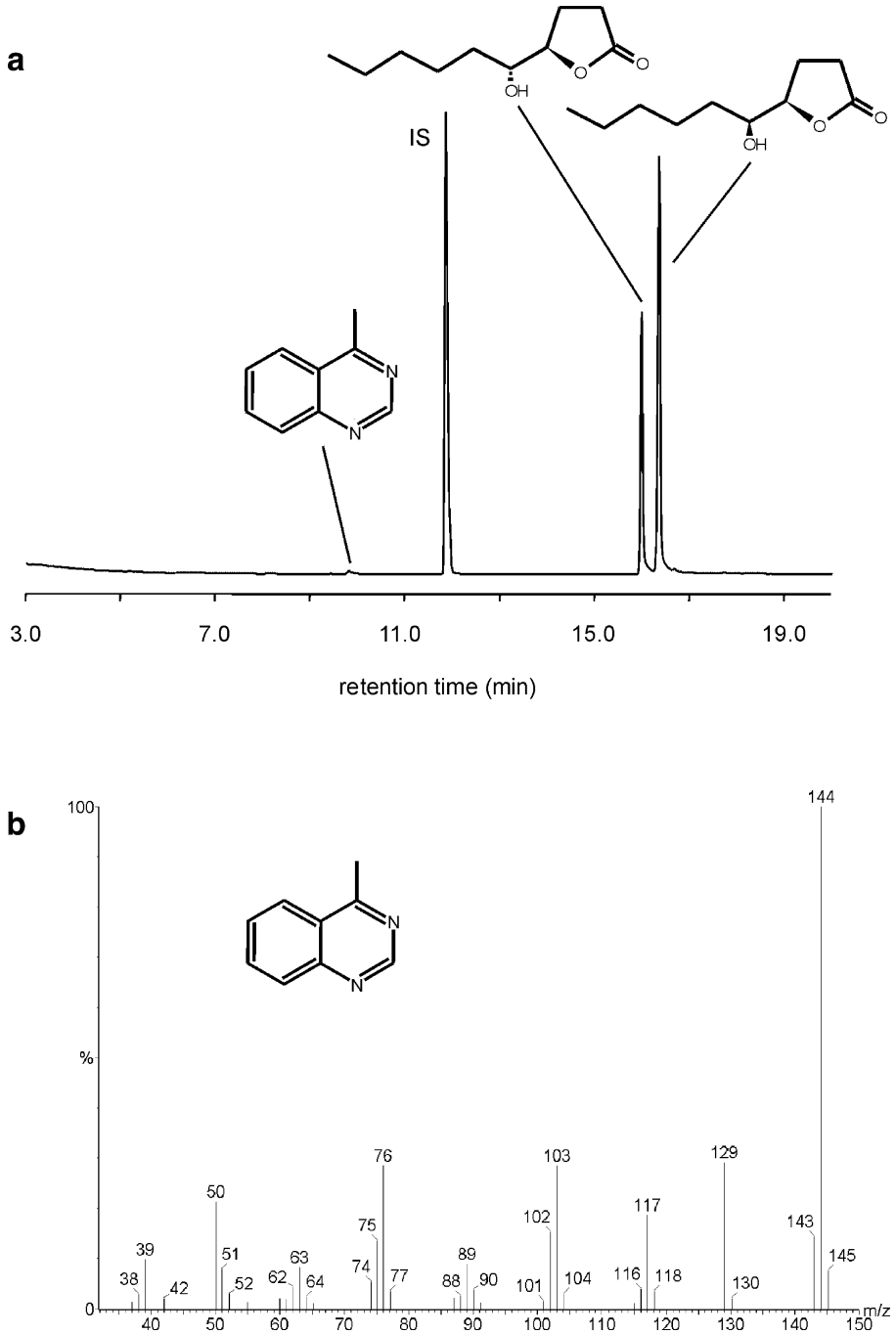

Fig. 1 **a** Total ion current chromatogram (nonpolar DB-5MS stationary phase) of a dichloromethane extract of the abdomen of a 3-d-old *N. vitripennis* male. Elution profile of higher boiling cuticular hydrocarbons not shown. (IS=20 ng of methyl undecanoate as internal standard). **b** Mass spectrum (EI, 70 eV) of the minor compound identified as 4-methylquinazoline

thickness 0.50 μm (J&W Scientific, Folsom, CA, USA) with helium as carrier gas (2 ml/min). Initial oven temperature was 80°C for both columns, increased at 5°C/min to 280°C (DB-5MS) and 240°C (DB-WAX), respectively. The maximum temperature was held for 30 min. 4-MeQ was identified by comparison of its mass spectrum and retention indices (in relation to co-injected *n*-alkanes) on nonpolar and polar stationary phases with those of a synthetic reference compound synthesized by reaction of 2-aminobenzonitrile with methylmagnesium iodide and subsequent treatment with ethyl formate as described by Bergmann et al. (1986). (4*R*,5*R*)- and (4*R*,5*S*)-HDL were identified as described previously by GC-MS and enantioselective GC (Ruther et al. 2007) with reference chemicals synthesized by published methods (Garbe and Tressl 2003). Volatiles were quantified by relating peak areas to the internal standard.

Table 1 Residence time (mean±SE) of virgin *N. vitripennis* females in test and control zones of a linear still-air olfactometer during a 5-min observation period

Test stimulus	Control	Number (*N*)	Residence time (sec)		
			Test zone	Control zone	*P*-level
40 ng HDL[a]	Solvent	21	193±17	58±18	<0.001
6 ng 4-MeQ	Solvent	21	116±22	108±22	0.842
6 ng 4-MeQ + 40 ng HDL[a]	40 ng HDL[a]	21	169±16	81±14	0.006
1 ng 4-MeQ + 40 ng HDL[a]	40 ng HDL[a]	21	193±21	71±20	0.006

[a] (4*R*,5*R*)- and (4*R*,5*S*)-HDL in equal amounts

Bioassays Behavioral responses of *N. vitripennis* females were investigated in a linear still-air olfactometer that consisted of an angular clear acrylic tube (14 × 1 × 1 cm) divided into three zones, i.e., 4-cm test and control zones at both ends of the tube and a 6-cm neutral zone in the center. The tube had an open base, and the ends were sealed with perforated Parafilm. Centrally, the olfactometer had a cylindrical entry adapter (5 mm length × 1 cm i.d.) to which 1 ml microcentrifuge tubes with the parasitoids could be connected. Test and control solutions of synthetic volatiles were applied to filter paper disks (5 mm diameter), and the solvent was allowed to evaporate for 120 sec. Subsequently, the disks were placed at the end of the test and control zones for 60 sec, enabling the formation of an odor gradient, and the parasitoid females were allowed to enter the olfactometer tube. Residence times of females in test and control zones were recorded for 5 min (N = 21 per test). The following treatments were tested: (1) 40 ng of a 1:1 mixture of (4*R*,5*R*)- and (4*R*,5*S*)-HDL vs solvent control, (2) 6 ng 4-MeQ vs solvent control, (3) 6 ng 4-MeQ + 40 ng HDL vs HDL only, and (4) 1 ng 4-MeQ + 40 ng HDL vs HDL only. Mean residence times in test and control zones were compared by a *t*-test for dependent data by using Statistica 4.5 scientific software (StatSoft, Hamburg, Germany).

Results and Discussion

GC-MS analysis of the extracts from male abdomens revealed the presence of (4*R*,5*R*)-HDL and (4*R*,5*S*)-HDL as major components (100 ± 13 and 190 ± 23 ng per abdomen, mean ± SE) (Fig. 1a). These values are higher than previously reported for whole body extracts (Ruther et al. 2007) probably because of a more effective penetration of solvent into the dissected abdomen. In addition to HDL, there was a minor component in male abdomen extracts with retention indices of 1,337 (DB-5MS) and 2,111 (DB-WAX), respectively. The mass spectrum of this compound showed *m/z* 144 as both molecular ion and base peak, thus suggesting an aromatic compound (Fig. 1b). Losses of a methyl radical (*m/z* 129, M-15), hydrogen cyanide (*m/z* 117, M-27), and methyl cyanide (*m/z* 103, M-41) together with the even-numbered molecular ion (nitrogen rule) pointed to a methylated nitrogen-containing heterocycle with an even number of nitrogen atoms. The mass spectrum and retention indices on both nonpolar and polar stationary phases matched exactly those of synthetic 4-MeQ, which also had the characteristic medicinal odor of *N. vitripennis* males even at very low doses (1 ng). 4-MeQ occurred in abdomen extracts in amounts between 1 and 6 ng (mean value 3 ± 0.5 ng) but was not detectable in extracts from the head or thorax (data not shown). When applied alone to filter paper disks at a dose of 6 ng, 4-MeQ did not influence the behavior of female wasps in the olfactometer (Table 1). However, when applied at doses of 6 or 1 ng together with 40 ng of a 1:1 mixture of (4*R*,5*R*)- and (4*R*,5*S*)-HDL, females significantly preferred the combined odor over HDL alone. We conclude from these results that 4-MeQ is a minor component of the male sex attractant used by *N. vitripennis* males to attract receptive females. In addition, the sexual communication system of *N. vitripennis* comprises a still unknown aphrodisiac released by males from an oral gland that elicits receptivity in females (van den Assem et al. 1980) and a female contact pheromone that consists of cuticular hydrocarbons (Steiner et al. 2006). Quinazoline alkaloids are widespread in nature with 4-MeQ occurring in some *Pseudomonas* bacteria (D'yakonov and Teleszhenetskaya 1997) and in the feces of *Triatoma* bugs (Cruz-López and Morgan 1995). Furthermore, the compound is a putative semiochemical of ferrets (Zhang et al. 2005) and has been shown to interfere with the host-finding ability of the salmon louse *Lepeophtheirus salmonis* (Bailey et al. 2006). The structural differences between the primary pheromone components of *N. vitripennis* (lactones) and the synergist (nitrogen containing heterocycle) are striking and point to different biosynthetic pathways. These are the subject of ongoing research including investigation of endosymbiotic microorganisms as a possible source of 4-MeQ in *N. vitripennis*.

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References

BAILEY, R. J. E., BIRKETT, M. A., INGVARSDOTTIR, A., MORDUE, A. J., MORDUE, W., O'SHEA, B., PICKETT, J. A., and WADHAMS, L. J. 2006. The role of semiochemicals in host location and non-host avoidance by salmon louse (*Lepeophtheirus salmonis*) copepodids. *Can. J. Fish. Aqu. Sci.* 63:448–456.

BERGMANN, J., BRYNOLF, A., ELMAN, B., and VUORINEN, E. 1986. Synthesis of quinazolines. *Tetrahedron* 42:3697–3706.

CÔNSOLI, F. L., WILLIAMS, H. J., VINSON, S. B., MATTHEWS, R. W., and COOPERBAND, M. F. 2002. trans-Bergamotenes-male pheromone of the ectoparasitoid *Melittobia digitata*. *J. Chem. Ecol* 28:1675–1689.

CRUZ-LÓPEZ, L. and MORGAN, D. 1995. Chemical investigation of aggregation behavior of *Triatoma* bugs (Hemiptera: Reduviidae). *J. Chem. Ecol* 21:2069–2078.

D'YAKONOV, A. L. and TELEZHENETSKAYA, M. V. 1997. Quinazoline alkaloids in nature. *Chem. Nat. Prod* 33:221–267.

GARBE, L. A. and TRESSL, R. 2003. Metabolism of deuterated isomeric 6,7-dihydroxydodecanoic acids in *Saccharomyces cerevisiae*—diastereo- and enantioselective formation and characterization of 5-hydroxydecano-4-lactone (= 4,5-dihydro-5-(1-hydroxyhexyl)furan-2(3H)-one) isomers. *Helv. Chim. Acta* 86:2349–2363.

RUTHER, J., STAHL, L. M., STEINER, S., GARBE, L. A., and TOLASCH, T. 2007. A male sex pheromone in a parasitic wasp and control of the behavioral response by the female's mating status. *J. Exp. Biol* 210:2163–2169.

STEINER, S., HERMANN, N., and RUTHER, J. 2006. Characterization of a female-produced courtship pheromone in the parasitoid *Nasonia vitripennis*. *J. Chem. Ecol* 32:1687–1702.

VAN DEN ASSEM, J., JACHMANN, F., and SIMBOLOTTI, P. 1980. Courtship behaviour of *Nasonia vitripennis* (Hym., Pteromalidae): Some qualitative, experimental evidence for the role of pheromones. *Behaviour* 75:301–307.

ZHANG, J. X., SOINI, H. A., BRUCE, K. E., WIESLER, D., WOODLEY, S. K., BAUM, M. J., and NOVOTNY, M. V. 2005. Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30:727–737.

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Identification of the Sex Pheromone of *Sesamia cretica* Lederer

Arman Avand-Faghih · Brigitte Frérot

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Abstract By using solid phase micro-extraction and gas chromatography–mass spectrometry analyses, a sex pheromone blend for the stem borer, *Sesamia cretica* Lederer (Lepidoptera, Noctuidae), was identified as consisting of (*Z*)-9-tetradecen-1-ol (80%), (*Z*)-9-tetradecen-1-yl acetate (10%), and (*Z*)-11-hexadecen-1-ol (10%). The first two components had previously been discovered as attractants for *S. cretica* in field tests, but had not been identified in the female's sex pheromone gland. A field-trapping trial showed that the three-component blend gave the highest catches of male *S. cretica*. This blend, in a sticky trap, was used to monitor a population of *S. cretica* in Iran, allowing the seasonal flight activity of this insect to be compared with that of a sympatric population of *S. nonagrioides*. The role of pheromones in the reproductive isolation of these species is discussed.

Keywords *Sesamia cretica* · Stem borer · Sex pheromone · SPME · (*Z*)-11-hexadecen-1-ol · Trapping · Sympatric species · Lepidoptera · Noctuidae

Introduction

The stem borer, *Sesamia cretica* Lederer (Lepidoptera, Noctuidae, Amphipyrinae), is one of the main insect pests of sorghum, maize, sugarcane, and cereal crops in the Mediterranean area (Anglade 1972). In certain regions, the species occurs sympatrically with other lepidopterans, including *Sesamia nonagrioides* (Lef), *Sesamia inferens* (Walk), *Ostrinia nubilalis* (Hüb), *Chilo agamemnon* (Bleszynski), and *Chilo partellus* (Swinhoe). *S. cretica* can be difficult to control on crops because the larvae develop inside the stem of host plants. Insecticidal control of this pest is, thus, most effective when the insecticide is applied when the eggs are on the leaves of the host plant and before the first instars bore into the plant stem.

Synthetic sex pheromones of moths have proven to be useful tools in integrated pest management (Cardé and Minks 1996). As baits in traps, they are used for monitoring moth flight activity, and hence, for timing control methods, such as insecticide sprays or the release of natural enemies. A synthetic sex pheromone could greatly aid control of *S. cretica* on agricultural crops by allowing precise timing of insecticidal treatments. During attractant field-screening experiments in Sudan, Arsura et al. (1977) found that *S. cretica* males were attracted to a 75:25 mixture of (*Z*)-9-tetradecen-1-ol (Z9-14:OH) and (*Z*)-9-tetradecen-1-yl acetate. Subsequently, this formulation was tested in various Mediterranean countries but failed to catch significant numbers of male moths (P. Moyal and G. Fédière, personal communication). In this paper, we report the results of the chemical analysis of the pheromone gland of female *S. cretica* and the field testing of an attractive three-component blend for males.

Methods and Materials

Insect Collection Insects were collected from maize in Iran (Mazandaran province) and in Egypt (near Cairo). After

A. Avand-Faghih
Iranian Research Institute of Plant Protection,
P.O. Box 1454, Tehran 19395, Iran
e-mail: a_avandfaghih@yahoo.fr

B. Frérot (✉)
INRA, UMR PISC,1272, Route de St-Cyr,
78026 Versailles cedex, France
e-mail: frerot@versailles.inra.fr

emergence, females were placed in individual plastic containers and maintained at 24°C in 50% R.H. and with a reverse-phase L16:D8 photoperiod.

Pheromone Collection and Identification Sex pheromone was collected from 2-3-d-old females exhibiting the characteristic calling posture. The pheromone gland of each female was extruded by gentle pressure on the abdomen and kept in this position with forceps while the adsorbent part of a solid-phase microextraction (SPME) fiber (CW/DVB, 65 μm, Supelco Inc., Bellefonte, PA, USA) was gently rubbed on the gland surface according to the method of Frérot et al. (1997). The fiber was then desorbed in the heated injector (245°C) of a Varian 3400 gas chromatograph (GC) coupled with a Varian Saturn II ion trap (mass spectrometry, MS). Mass spectra were recorded in electronic ionization mode over a mass range of 40–300 amu. A 30 m×0.32 id, 0.5-μm film thickness, RTX-5MS column (Restek, Bellefonte, PA, USA) was used for the mass spectral analyses. The oven temperature was programmed from 50°C (held for 1 min) to 300°C at 8°C min^{-1} with helium as carrier gas. GC analyses were also conducted on a polar RTX-Wax column, (30 m×0.32 id, 0.5-μm film thickness, Restek) with the oven temperature programmed from 50°C (held for 1 min) to 100°C at 15°C min^{-1}, held for 1 min, and increased to 245°C at 5°C min^{-1}. The carrier gas was helium. Pheromone components were identified by comparison of mass spectra, and retention times on both columns, with the same parameters of authentic compounds.

Field Trapping Field trapping was carried out in a maize farm at Safi-Abad Agriculture and Natural Resources Research Center, near Dezful (Khouzestan Province) in Southwest Iran. The first experiment was performed between the 10th August and 31st November 2003 and the second from 12th September to 14th November 2004. Sticky Delta traps (NPP, France) were used in the experiments. All traps were deployed at the border of the field on wooden stakes at the height of the maize canopy. Traps were checked twice a week (every 3 or 4 days) and the trap sticky cards renewed each week.

Each chemical was dissolved in hexane (2 μg/μl) and the appropriate amount deposited onto a red rubber septum (Sigma-Aldrich). Septa were replaced every 4 wk of the trials. The different mixtures (1, 2, 3) tested in the *S. cretica* field experiment are given in Table 1. Traps for *S. nonagrioides* were also set up in the maize farm so as to compare the phenology of the two *Sesamia* species. The pheromone blend used for *S. nonagrioides* was: (*Z*)-11-hexadecen-1-yl acetate (Z11-16:Ac), (*Z*)-11-hexadecenal (Z11-16: Al), and (*Z*)-11-hexadecen-1-ol (Z11-16:OH) (850:100:50 μg; Ameline and Frérot 2001). All compounds tested were available in the laboratory and were >99% isomerically pure as determined by GC analysis.

Field trials were conducted as complete randomized block designs with four replicates of each treatment. Traps were placed approximately 30 m apart in each block, with blocks at least 100 m apart. In field trial 1, mixture 1 (the three component blend) was formulated based on our initial GC analyses, whereas mixture 2 was similar to the two-component blend reported by Arsura et al. (1977). In the second field trial, mixture 1 was again tested along with mixture 3, which lacked Z9-14:Ac. Blank traps, without any bait, were used as a control in both field trials.

Statistical Analysis Numbers of male *S. cretica* caught were transformed by $\ln(X+1)$ and subjected to analysis of variance (ANOVA; generalized linear model procedure). The mean values for different treatments were compared by using Tukey–Kramer tests. Statistical differences are reported at $P<0.05$.

Results

Pheromone Identification SPME collections were performed from glands of five females originating from Egypt and

Table 1 Composition of mixtures tested for *Sesamia cretica* trapping and the mean numbers (±SE) of males captured in each traps

Mixtures	Composition of Synthetic Attractant (μg) per Cap			Mean Capture per Trap ([a] ±SE[b])	
	Z9-14:OH	Z9-14:Ac	Z11-16:OH	1st assay (2003)	2nd assay (2004)
Mixture 1	80	10	10	73±14.2a	84.5±9.3a
Mixture 2	80	20	–	5±1.7b	–
Mixture 3	80	–	10	–	14.25±5.2b
Control	–	–	–	0±0c	1±0.4c

[a] Four replicates

[b] Means within a column followed by different letters are significantly different (Tukey–Kramer test, $P<0.001$). Results of ANOVA are as follows: first assay (2003): $P<0.001$, $df=2$, $F=110.89$, second assay (2004): $P<0.001$, $df.=2$, $F=121.38$.

glands of nine females from Iran. GC–MS analysis revealed the presence of a monounsaturated tetradecenol [*m/z*= 41:77%, 55:88%, 81:100%, 95:57%, 194 (M^+ −18):7%]; a monounsaturated tetradecenyl acetate [*m/z*=43:55%, 55:58%, 67:86%, 81:100%, 95:57%, 194 (M^+ −60):16%], and a monounsaturated hexadecenol [m/z=41:57%, 55:75%, 81:100%, 95:63%, 222 (M^+ −18):7%]. On the polar, RTX-Wax column, *Z* and *E* isomers, as well as various positional isomers, were separated clearly. Comparison of retention times of the unknown compounds with those of various synthetic standards led to the identification of Z9-14:OH, Z9-14:Ac, and Z11-16:OH in a ratio of roughly 80:10:10. The compounds and their relative ratio did not differ significantly between the Egyptian and Iranian populations.

Field Trapping Three different mixtures of the chemicals identified in female *S. cretica* were tested in field trials in 2003 and 2004. Across both trials, traps baited with mixture 1, containing the three components, caught significantly more males than traps baited with mixture 2, lacking Z11-16:OH, or mixture 3, lacking Z9-14:Ac. Traps baited with mixtures 2 and 3, however, caught significantly more insects than the control traps (Table 1).

Traps baited with synthetic *S. nonagrioides* pheromone were also used in the second trial in order to compare the phenologies of the two insects. Reasonably high numbers of male *S. cretica* were caught throughout the entire duration (approximately 2 mo.) of the field trial. However, *S. nonagrioides* males were caught in substantial numbers only during the latter half of the trial (Fig. 1). No males of either species were caught in traps baited with pheromone of the other species.

Discussion

We identified three chemicals, Z11-14:OH, Z11-14:Ac and Z11-16:OH, in the sex pheromone gland of female *S. cretica*. Two of these (Z11-14:OH and Z11-14:Ac) had previously been reported to be attractants for *S. cretica* males (Arsura et al. 1977). Our field trials showed that the most attractive blend tested was the one containing all three compounds; removal of either Z9-14:Ac or Z11-16:OH from the blend resulted in a significant decrease in catch of male *S. cretica*. Mixtures of 14-carbon and 16-carbon compounds have been reported as comprising pheromones of other *Sesamia* species, e.g., *Sesamia calamistis* (Zagatti et al. 1988).

Our comparison of the flight phenologies of *S. cretica* and *S. nonagrioides* in the second field trial showed that in the area of study, the two congeneric species have different but overlapping flight periods. Although these two species are found in the same geographical location and have overlapping flight periods, their reproductive isolation is probably maintained by their distinct pheromone systems, as evidenced by the lack of cross-attraction of the respective pheromone blends, i.e., traps baited with the *S. nonagrioides* blend did not catch *S. cretica* males and traps baited with the *S. cretica* blend did not catch *S. nonagrioides* males. The chemical compositions of the respective sex pheromones of the two species are distinct. Although they have the component, Z11-16:OH, in common, the pheromone of *S. nonagrioides* consists entirely of 16-carbon compounds (Z11-16:Ac and Z11-16:Al in addition to Z11-16:OH) (Sreng et al. 1985), whereas the pheromone of *S. cretica* contains two 14-carbon compounds.

To farmers, the two species are difficult to differentiate because of their similar appearance and similar damage they cause. Monitoring with the respective pheromones will

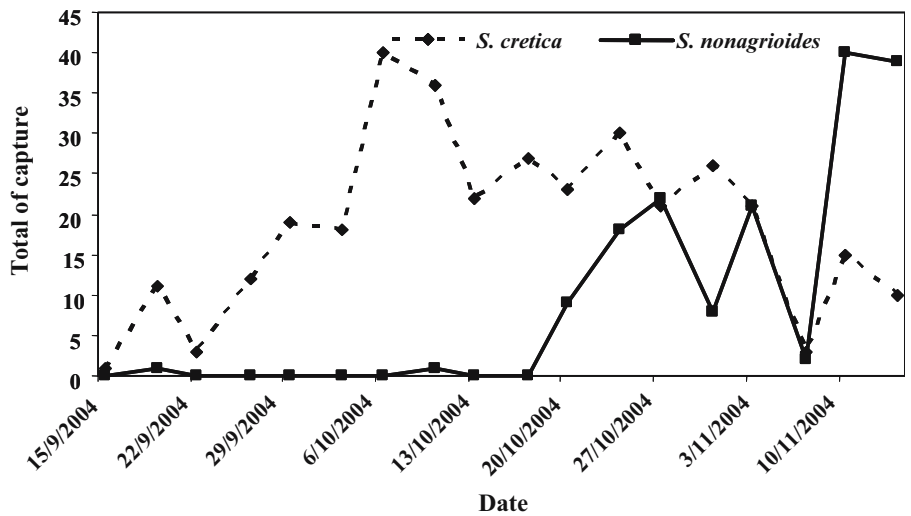

Fig. 1 Weekly catches of male *Sesamia cretica* (mixture 1) and *Sesamia nonagrioides* in sticky Traps baited with their respective sex pheromones, from September to November 2004 in Safi-Abad, Dezful, Khuzestan Province, Iran

allow easy detection and identification of which of the species is present, and therefore attacking the crop, in different regions.

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References

AMELINE, A., and FRÉROT, B. 2001. Pheromone blends and trap designs can affect catches of *Sesamia nonagrioides* Lef. (Lep., Noctuidae) males in maize fields. *J. Appl. Entomol.* 125:15–18.

ANGLADE, P. 1972. Les Sesamia, pp. 1398–1401, in A. S. Balachowsky (ed.). Entomologie Appliquée à l'Agriculture, Lépidoptères, Tome IIMasson, Paris.

ARSURA, E., CAPIZZI, A., PICCARDI, P., and SPINELLI, P. 1977. (*Z*)-9-tetradecen-1-ol and (*Z*)-9-tetradecenyl acetate: a potent attractant system for male *Sesamia cretica* Led. (Lep., Noctuidae). *Experientia* 33:1423–1424.

CARDÉ, R. T., and MINKS, A. K. 1996. Insect Pheromone Research: New Directions. Chapman and Hall, New York684 pp.

FRÉROT, B., MALOSSE, C., and CAIN, A. H. 1997. Solid-phase microextraction (SPME): a new tool in pheromone identification in Lepidoptera. *J. High Resolut. Chromatogr.* 20:340–342.

SRENG, I., MAUME, B., and FRÉROT, B. 1985. Analyse de la séréction phéromonale produite par les femelles vierges de *Sesamia nonagrioides* (Lef.) (Lépidoptère, Noctuidae). *C. R. Acad. Sci. Paris* 301(III), 9:439–442.

ZAGATTI, P., HAMADOUS, A., LETTERE, M., BORDAT, D., GALLOIS, M., and MALOSSE, C. 1988. Phéromone sexuelle de *Sesamia calamistis* (Hampson) (Lepidoptera: Noctuidae). *C. R. Acad. Sci. Paris* 307:III837–840.

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New Sex Attractant Composition for the Click Beetle *Agriotes proximus*: Similarity to the Pheromone of *Agriotes lineatus*

Miklós Tóth · Lorenzo Furlan · Amália Xavier · József Vuts · Teodora Toshova · Mitko Subchev · István Szarukán · Venyamin Yatsynin

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Abstract While testing traps baited with a blend of geranyl octanoate and geranyl butanoate (pheromone components previously identified for *Agriotes lineatus*, Coleoptera, Elateridae) in Portugal and Bulgaria, large numbers of the closely related *Agriotes proximus* were captured. In the literature, two different compounds, (*E*,*E*)-farnesyl acetate and neryl isovalerate had previously been identified as pheromone components of *A. proximus*. Subsequent field tests, conducted in several European countries, revealed that *A. proximus* was weakly attracted to geranyl butanoate on its own, while *A. lineatus* was weakly attracted to geranyl octanoate on its own. However, the largest catches for both species were observed with a blend of both compounds. No *A. proximus* was caught in traps baited with the blend of (*E*,*E*)-farnesyl acetate and neryl isovalerate at any of the test sites. In electroantennographic studies, antennae of male *A. proximus* and *A. lineatus* both gave greater responses to geranyl butanoate than to geranyl octanoate, suggesting that the perception of these two compounds was similar for both species. A 1:1 blend of geranyl octanoate and geranyl butanoate can be used as a bait in traps for the detection and monitoring of both *A. lineatus* and *A. proximus* in many European countries.

Keywords Geranyl butanoate · Geranyl octanoate · *Agriotes proximus* · *A. lineatus* Coleoptera · Elateridae · Sex attractant · Trapping

M. Tóth (✉) · J. Vuts
Plant Protection Institute, Hungarian Academy of Sciences,
Pf. 102, Budapest 1525, Hungary
e-mail: h2371tot@ella.hu

L. Furlan
Department of Agronomy, Entomology, Padova University,
Agripolis, Via Romea 16,
Legnaro 35020, Italy

A. Xavier
DRAN,
Rua da Restauração 336,
4050-501 Porto, Portugal

T. Toshova · M. Subchev
Institute of Zoology, Bulgarian Academy of Science,
blvd Car Osvoboditel 1,
1000 Sofia, Bulgaria

I. Szarukán
Agricultural University,
POB 58, Debrecen 4001, Hungary

V. Yatsynin
Krasnodarskiy NIISKh im. P.P. Lukyanenko,
Krasnodar 12, 350012, Russia

Introduction

Pheromone gland extracts of *Agriotes lineatus* L. (Coleoptera, Elateridae) contain large amounts of geranyl octanoate (Borg-Karlson et al. 1988; Kudryavtsev et al. 1993; Siirde et al. 1993) and much smaller amounts of the related geranyl butanoate (Yatsynin et al. 1991, 1996; Tóth et al. 2003). A blend of 10:1 geranyl octanoate/geranyl butanoate was found to be optimal for field trapping *A. lineatus* (Tóth et al. 2003). In the course of a Europe-wide testing of click beetle pheromone baits, traps baited with the above composition captured *A. lineatus* in many European countries. However, in Portugal and Bulgaria, traps with this bait also caught beetles of the closely related species, *Agriotes proximus* Schwarz (Subchev et al. 2005, 2006; Tóth and Furlan 2005). Catches of *A. proximus* were

unexpected because two different compounds, (*E*,*E*)-farnesyl acetate and neryl isovalerate, had previously been identified as pheromone components of a Russian population of *A. proximus* (Yatsynin et al. 1980, 1996).

The objective of the work here was to conduct a study on the field and electrophysiological responses of *A. proximus* and *A. lineatus* to the above compounds, with a practical aim of defining a sex pheromone bait for trapping *A. proximus* populations across Europe.

Materials and Methods

Field-Trapping Tests

Synthetic geranyl butanoate and geranyl octanoate were purchased from Bedoukian (Danbury, CT, USA) and were >99% pure as determined by gas chromatography (GC). (*E*,*E*)-farnesyl acetate was obtained from Sigma-Aldrich Kft, (Budapest, Hungary) and was certified by the supplier to be >95% geometrically pure. Neryl isovalerate was synthesized as described in Tóth et al. (2003) and was >95% pure as determined by GC. The compounds were formulated in polyethylene dispensers (for details, see Tóth et al. 2003). Yf click beetle traps (Furlan et al. 2004; produced by RO-SA Micromecanica, San Doną di Piave, Venice, Veneto, Italy) were used in field tests.

Field tests were conducted at several sites in Hungary, Portugal, Bulgaria, and the European part of Russia. For details of single tests, refer to the description of a particular experiment. Traps were arranged in a randomized complete block design. Traps within blocks were separated by 8–10 m, with blocks at least 30 m apart.

Capture data were transformed to $(x+0.5)^{1/2}$ and were analyzed by analysis of variance (ANOVA). If the ANOVA was significant ($P<0.05$), then treatment means were separated by a Games–Howell test (Games and Howell 1976; Jaccard et al. 1984). All statistical procedures were conducted with the software packages StatView® v4.01 and SuperANOVA® v1.11 (Abacus Concepts, Berkeley, CA, USA).

Experiment 1 This compared the attractancy of blends of geranyl octanoate and geranyl butanoate, single compounds, and unbaited controls. Parallel tests (with five replicate blocks each) were conducted at: Vairao, Portugal, 30 March–20 July, 2004; Hajdúböszörmény, Hungary, 6 May–21 June, 2005; Krasnodar, Russia, 25 April–27 June, 2005; Brenitsa, Bulgaria, 12 May–26 July, 2005.

Experiment 2 This compared a range of dosages of a 1:1 blend of geranyl octanoate and geranyl butanoate and of geranyl butanoate on its own and was conducted at Koshava, Bulgaria, 11 May–20 June, 2005, with five replicate blocks.

Electroantennography

For recording electroantennograms (EAGs), a stainless-steel tube (Teflon-coated inside) with a constant humidified airflow of ca. 0.7 l/min was used. An antenna, freshly amputated at the base, from a live adult male click beetle was mounted between two glass capillaries that contained 0.1 M KCl solution, with the mounted antenna ca. 3 mm from the end of the steel tube. One electrode was grounded, while the other was connected to a high-impedance DC amplifier (AM-02, Syntech, Hilversum, The Netherlands). Test compounds (10 μg each) were administered in hexane to a 10×10 mm piece of filter paper inside a Pasteur pipette. A stimulus consisted of pushing 1 ml of air through the Pasteur pipette into the airstream flowing over the antenna. Response amplitudes were normalized against the means of responses to (*E*,*E*)-farnesyl butanoate (that elicited medium–high responses from antennae of both species), which was administered before and after the test compounds. Stimuli were delivered at ca. 20–30 s intervals. Experimental insects were collected from the field in Portugal (*A. proximus*) or in Hungary (*A. lineatus*). Thirty male specimens of each species were tested.

Results

Field-Trapping Tests

In experiment 1, in Portugal, traps baited with binary blends caught significantly more *A. proximus* than traps baited with single compounds (Fig. 1a). Traps containing only geranyl butanoate caught significantly more *A. proximus* than traps with geranyl octanoate alone. Traps with a 100:1 blend of (*E*,*E*)-farnesyl acetate/neryl isovalerate (the optimal ratio for *A. proximus* determined by Yatsynin et al. 1980) did not catch any *A. proximus*. In Bulgaria, few *A. proximus* were caught (Fig. 1b). Ratios of 10:1 and 1:1 (octanoate/butanoate) caught significantly more than the single components or traps with (*E*,*E*)-farnesyl acetate plus neryl isovalerate (Fig. 1b). Catches of the other treatments did not differ from each other.

In both tests, randomly selected specimens from the traps were inspected and all found to be males. No *A. lineatus* was caught in the Portuguese and Bulgarian trials.

Catches of *A. lineatus* in Hungary and Russia (Krasnodar region) showed a similar tendency, with catches to the

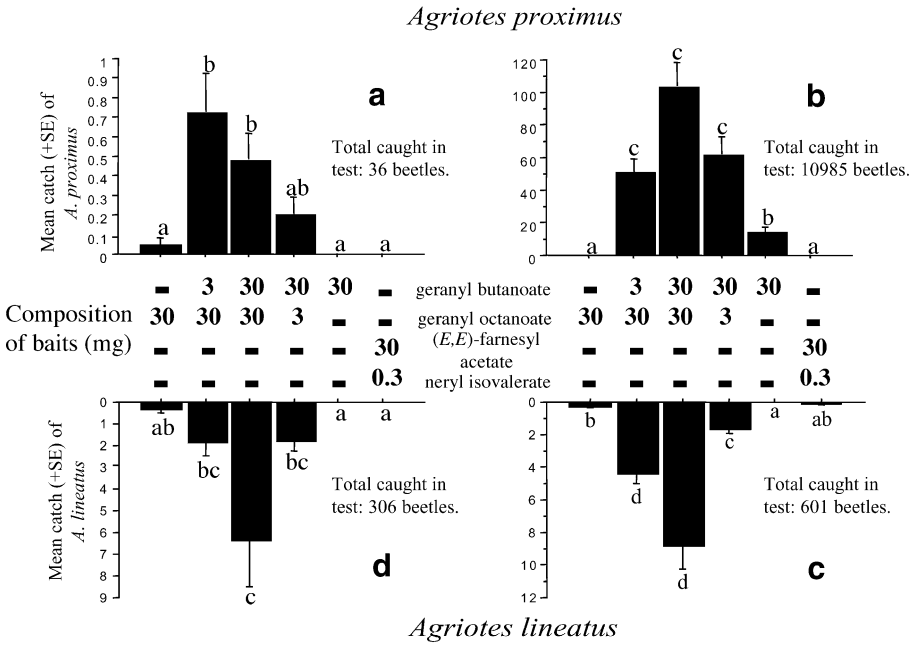

Fig. 1 Catches of *A. proximus* and *A. lineatus* in traps baited with mixtures of geranyl octanoate and geranyl butanoate, a 100:1 blend of (*E*,*E*)-farnesyl acetate and neryl isovalerate, and unbaited control traps (experiment 1). **a** Portugal, **b** Bulgaria, **c** Russia, **d** Hungary (for details of each test, please refer to "Materials and Methods"). Columns with same letter within one diagram not different at $P=5\%$ by ANOVA, Games–Howell

binary blends generally being greater than catches to the single compounds (Fig. 1c, d). At both sites, some specimens were caught in traps baited only with geranyl octanoate. Only at the Russian site was the number of *A. lineatus* caught in traps baited with geranyl octanoate significantly greater than the number caught in traps baited with geranyl butanoate (Fig. 1c). No *A. lineatus* was caught in traps baited with the (*E*,*E*)-farnesyl acetate and neryl isovalerate blend. No *A. proximus* was caught in the Hungarian and Russian trials.

Other click beetle species were captured during these tests. In Hungary and Russia, traps baited with only geranyl butanoate caught 3.3±0.9 and 3.6±0.9 (mean+SE) *Agriotes sputator* L., respectively, significantly higher than in other treatments which caught few or zero *A. sputator*. In Bulgaria, traps baited with (*E*,*E*)-farnesyl acetate and neryl isovalerate caught 134.8±22.3 (mean±SE) of *Agriotes ustulatus* Schwarz.

In a trial testing the effect of different dosages on *A. proximus* catch, binary blends of geranyl butanoate and geranyl octanoate caught significantly more *A. proximus* than geranyl butanoate alone at all dosages tested (Table 1). There was no significant difference in the catches of beetles among dosages of the binary blend tested. The only other click beetle species caught in the test was *A. sputator*; catches to the 100 and 30 mg geranyl butanoate treatments were significantly greater than to the corresponding binary blends that contained the same amount of geranyl butanoate (Table 1). Traps baited with the binary mixtures caught similar numbers of *A. sputator* as the unbaited control.

Electroantennograms

Antennae of male *A. proximus* exhibited greater EAG responses to geranyl butanoate than to geranyl octanoate at a dose of 0.001 μg and higher (Fig. 2). *A. lineatus* antennae also exhibited higher EAG responses to geranyl butanoate than to geranyl octanoate, but the difference was significant only at doses of 0.01 μg and greater (Fig. 2).

Even at a 10 μg dose, (*E*,*E*)-farnesyl acetate and neryl isovalerate elicited very low EAG responses from the antennae of both species (data not shown).

Table 1 Mean captures of *Agriotes* spp. in traps baited with different dosages of geranyl butanoate and its 1:1 mixture with geranyl octanote (experiment 2)

Bait composition (mg)		Mean number of beetles caught (per trap per inspection)	
Geranyl butanoate	Geranyl octanote	*A. proximus*	*A. sputator*
100	100	9.73b	0.53abc
30	30	8.46b	0.40ab
10	10	5.42b	0.60abcd
100	–	0.17a	2.15d
30	–	0.83a	1.80cd
10	–	0.25a	0.85bcd
–	–	0.00a	0.05a
Total number caught in test		297	126

Means with same letter not significantly different at $P=5\%$ by ANOVA, Games–Howell. Traps were inspected weekly.

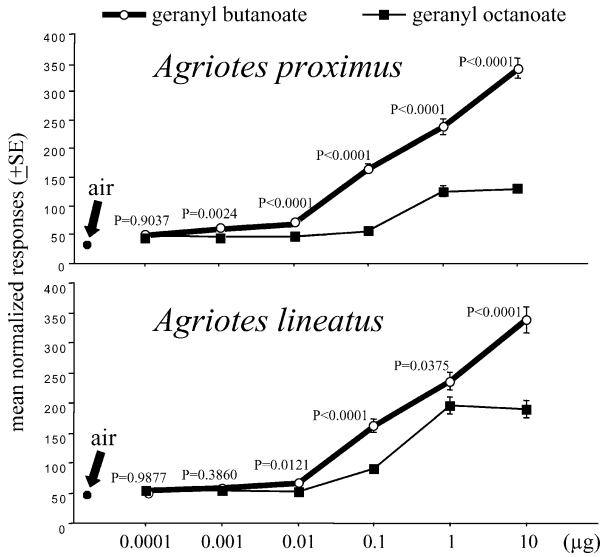

Fig. 2 Normalized EAG responses of male *A. proximus* and *A. lineatus* to a dosage series of geranyl butanoate and geranyl octanoate. Responses were normalized against the response to (*E*,*E*)-farnesyl butanoate. *P* values derive from Student's *t* test comparing mean responses to the two compounds at the given dose level

Discussion

Our field-trapping data show that a mixture of geranyl butanoate and geranyl octanoate attracted males of *A. proximus* at sites in southwestern and southeastern Europe, thus confirming our preliminary findings from Portugal (Tóth and Furlan 2005) and Bulgaria (Subchev et al. 2005, 2006). *A proximus* responded to a wide range of ratios (1:10 to 10:1) of the binary blend. Based on this work, practical application trials with geranyl butanoate and geranyl octanote blends were conducted and were successful for detecting the appearance and the flight dynamics of *A. proximus* in both Portugal (Tóth et al., unpublished) and Bulgaria (Subchev et al. 2005, 2006).

The inability of the previously described pheromone blend of (*E*,*E*)-farnesyl acetate and neryl isovalerate (Yatsynin et al. 1980, 1996) to attract *A. proximus* in our studies was surprising. Further studies, particularly looking at the production of pheromone components by *A. proximus* females from different locations (notably Portugal and Bulgaria), are needed to elucidate the reasons for this lack of response.

It is interesting to note that a similar inter-populational difference has been noted for *A. lineatus*. While geranyl octanoate is the main pheromone component used by populations in Eastern Europe (Russia), a mixture of (*E*,*E*)-farnesyl acetate and neryl isovalerate has been reported to attract *A. lineatus* populations in Western Ukraine (Kudryavtsev et al. 1993; Siirde et al. 1993). However, populations of *A. lineatus* respond to blends with geranyl octanoate as the main component all over Europe, even in areas (Hungary, Romania, and Bulgaria) neighboring Western Ukraine (Tóth and Furlan 2005; Furlan et al. 2007). *A. lineatus* populations in Canada (introduced from Europe in the past) also respond to the geranyl octanoate and geranyl butanoate blend (Vernon and Tóth 2007).

In the field trials that tested different ratios of geranyl butanoate and geranyl octanoate, *A. lineatus* showed similar responses to those of *A. proximus*. That the greatest catches of *A. lineatus* were obtained to a blend relatively high in geranyl butanoate (ratio of 1:1) was unexpected, since, in chemical studies, this compound was detected only in trace amounts in female pheromone glands (Siirde et al. 1993; Yatsynin et al. 1996; Tóth et al. 2003). One possible explanation is that compounds extracted by direct solvent extraction of pheromone glands (Siirde et al. 1993; Yatsynin et al. 1996; Tóth et al. 2003) do not reflect the ratio of components emitted into the air by a calling female. The analysis of volatiles emitted by *A. lineatus* and *A. proximus* females is underway in our laboratory in order to test this hypothesis.

In our study, *A. proximus* showed a low response to geranyl butanoate alone, whereas *A. lineatus* did not appear to respond to this compound. Furthermore, *A. lineatus* has been reported as being attracted (albeit in low numbers) to geranyl octanoate alone (Kudryavtsev et al. 1993; Siirde et al. 1993; Tóth et al. 2003). This suggested to us that there may be a difference between the two species in their respective sensitivity to these compounds. However, our EAG studies showed no difference in antennal responses: geranyl butanoate evoked higher EAG antennal responses than geranyl octanoate for both species.

The similarity in both field and antennal responses between the two species raises the question of whether *A. proximus* and *A. lineatus* are distinct species. Taxonomically, the two species are considered clearly separated, primarily because of one morphological difference: the ratio of the length and the width of the prothorax. However, there are no data that demonstrate separation at a biological level. Genetic or classical biological studies are needed to address this issue.

For pest-management purposes, a 1:1 blend of geranyl octanoate and geranyl butanoate can be used as bait in traps for the detection and monitoring of both *A. lineatus* and *A. proximus* in most parts of Europe. Although in most cases, insect species produce and respond to unique pheromone blends, there is precedent for similarity in pheromone composition between closely related species of click beetles. *Agriotes sordidus* Illiger and *Agriotes rufipalpis* Brullé both use geranyl hexanoate in their sex pheromones (Tóth et al. 2002, 2003; Furlan et al. 2004). For both pairs of *A. sordidus–A. rufipalpis* and *A. proximus–A. lineatus*, we have yet to find both species of a pair at the same site

(Tóth and Furlan 2005; Furlan et al. 2007). However, when traps baited with geranyl butanoate and geranyl octanoate are used for monitoring *A. lineatus* or *A. proximus* across Europe, the possibility of the presence of both species at a site should be considered.

Catches of other click beetle species in this study corresponded well with their respective sex-pheromone compositions. The main pheromone component of *A. sputator* is geranyl butanoate (Yatsynin et al. 1986, 1996; Siirde et al. 1993; Tóth et al. 2003). Lower catches of *A. sputator* in traps baited with blends that contain geranyl octanoate, compared to traps baited with geranyl butanoate alone, suggest that geranyl octanoate may act as a behavioral antagonist to *A. sputator*. Catches of *A. ustulatus* in traps baited with the blend of (*E*,*E*)-farnesyl acetate and neryl isovalerate are explained by the presence of the farnesyl compound, which is the main pheromone component of this species (Kudryavtsev et al. 1993; Siirde et al. 1993; Yatsynin et al. 1996; Tóth et al. 2003).

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References

Borg-Karlson, A. K., Agren, L., Dobson, H., B, and Bergström, BG. 1988. Identification and electroantennographic activity of sex-specific geranyl esters in an abdominal gland of female *Agriotes obscurus* (L.) and *A. lineatus* (L.) (Coleoptera: Elateridae). *Experientia* 44:531–534.

Furlan, L., Garofalo, N., and Tóth, M. 2004. Biologia comparata di *Agriotes sordidus* Illiger nel Nord e Centro-Sud d'Italia. *Informatore Fitopatologico* 10:49–54.

Furlan, L., Tóth, M., and Cooperators. 2007. Occurrence of click beetle pest spp. (Coleoptera, Elateridae) in Europe as detected by pheromone traps: survey results of 1998–2006. *IOBC/WPRS Bull.* 30:19–25.

Games, P. A., and Howell, J. F. 1976. Pairwise multiple comparison procedures with unequal n's and/or variances: a Monte Carlo study. *J. Edu. Stat* 1:113–125.

Jaccard, J., Becker, M. A., and Wood, G. 1984. Pairwise multiple comparison procedures: a review. *Psychol. Bull* 96:589–596.

Kudryavtsev, I., Siirde, K., Lääts, K., Ismailov, V., and Pristavko, V. 1993. Determination of distribution of harmful click beetle species (Coleoptera, Elateridae) by synthetic sex pheromones. *J. Chem. Ecol* 19:1607–1611.

Siirde, K., Lääts, K., Erm, A., Kogerman, A., Kudryavtsev, I., Ismailov, V., and Pristavko, V. 1993. Structure-activity relationships of synthetic pheromone components in sex communication of click beetles (Coleoptera, Elateridae). *J. Chem. Ecol* 19:1597–1606.

Subchev, M., Toshova, T., Furlan, L., and Tóth, M. 2005. Click beetles (Coleoptera: Elateridae) and their seasonal swarming as established by pheromone traps in different plant habitats in Bulgaria: 2. Maize. *Acta Zool. Bulg* 57:321–332.

Subchev, M., Toshova, T., and Furlan, L., Tóth, M. 2006. Click beetles (Coleoptera: Elateridae) and their seasonal swarming as established by pheromone traps in different plant habitats in Bulgaria: 3. Potato. *Acta Zool. Bulg* 58:361–370.

Tóth, M., Furlan, L., Szarukán, I., and Ujváry, I. 2002. Geranyl hexanoate attracting male click beetles *Agriotes rufipalpis* Brullé and *Agriotes sordidus* Illiger (Col., Elateridae). *Z. Angew. Ent* 126:312–314.

Tóth, M., Furlan, L., Yatsynin, V. G., Ujváry, I., Szarukán, I., Imrei, Z., Tolasch, T., Francke, W., and Jossi, W. 2003. Identification of pheromones and optimization of bait composition for click beetle pests in Central and Western Europe (Coleoptera: Elateridae). *Pest Manag. Sci* 59:1–9.

Tóth, M., and Furlan, L. 2005. Pheromone composition of European click beetle pests (Coleoptera, Elateridae): common components—selective lures. *IOBC/WPRS Bull* 28:133–142.

Vernon, R. S., and Tóth, M. 2007. Evaluation of pheromones and a new trap for monitoring *Agriotes lineatus* and *Agriotes obscurus* in the Fraser Valley of British Columbia. *J. Chem. Ecol* 33:345–351.

Yatsynin, V. G., Oleschenko, I. N., ubanova, E. V., and Ismailov, V. Y. 1980. Identification of active components of the sex pheromones of click beetles *Agriotes gurgistanus, A. litigiosus* and *A. lineatus*. (in Russian). *Khim. Sel'sk. Khoz.* Edition Moskva, Khimiya, pp. 33–35.

Yatsynin, V. G., Karpenko, N. N., and Orlov, V. N. 1986. Sex pheromone of the click beetle *Agriotes sputator* L. (Coleoptera: Elateridae) (in Russian). *Khim Komm Zhivot*, Edition Moskva, Nauka pp. 53–57.

Yatsynin, V. G., ubanova, E. V., Orlov, V. N., Lebedeva, K. V., and Bocharova, N. I. 1991. Pheromones of the click beetles *Agriotes tadzhikistanicus*, *A. lineatus*, *A. meticulosus*, *A. caspicus* (Coleoptera, Elateridae) (in Russian). *Prob. Khim. Komm. Zhiv.*, Edition Moskva, Nauka pp. 101–106.

Yatsynin, V. G., Rubanova, E. V., and Okhrimenko, N. V. 1996. Identification of female-produced sex pheromones and their geographical differences in pheromone gland extract composition from click beetles (Col., Elateridae). *J. Appl. Ent* 120:463–466.

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Production and Bioactivity of Common Lichen Metabolites as Exemplified by *Heterodea muelleri* (Hampe) Nyl.

Armin Hager · Georg Brunauer · Roman Türk · Elfie Stocker-Wörgötter

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Abstract Based on results of a former study in 2005, this investigation aimed at quantifying UV- and cold temperature stress-induced changes within the secondary metabolite production of the cultured mycobiont of the lichen *Heterodea muelleri* (Hampe) Nyl. The chemical profiles of the mycobiont cultures and the lichen thallus were determined by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) analyses. The voucher specimen of *H. muelleri* produced diffractaic acid as a major polyketide and barbatic acid as a satellite compound, whereas the untreated mycobiont did not contain any detectable secondary metabolites. While UV-C stress caused a general increase in substance formation, cold temperature stress resulted in a strong activation of barbatic acid biosynthesis. A further series of experiments focused on the effect of diffractaic and barbatic acids on the growth of the symbiotic photobiont *Trebouxia jamesii*; usnic acid was similarly tested. Pure substances were obtained from mycobiont cultures by performing preparative TLC. A determined quantity of algae was incubated on BBM plates that contained three different concentrations of the pure lichen metabolites. The growth of the photobionts was monitored over a period of 1 mo to evaluate the impact of each substance on the cultured algae. While diffractaic and usnic acid had no noticeable effect, barbatic acid strongly inhibited algal growth and resulted in cell death.

A. Hager (✉) · G. Brunauer · R. Türk · E. Stocker-Wörgötter
Department of Organismic Biology, University of Salzburg,
Hellbrunner Strasse 34,
5020 Salzburg, Austria
e-mail: armin.hager@sbg.ac.at

Keywords *Heterodea* · *Trebouxia* · Lichen culture · Growth inhibition · Barbatic acid · Diffractaic acid · Usnic acid

Introduction

Lichen secondary metabolites, especially polymalonyl-derived polyketides, have been found to exhibit manifold biological activities in various screenings (Huneck 1999, 2001; Dayan and Romagni 2001; Müller 2001). Although the biological activities of lichen substances are well known, the biological and ecological functions of these compounds for the lichen symbiosis and the lichen itself are poorly understood. Although more than 800 lichen substances are known and their structures have been elucidated (Huneck 2001), many others remain to be characterized. In general, lichen secondary metabolites can be grouped into three classes according to their biosynthetic origin; polymalonate, mevalonate, and shikimate derivatives (Asahina and Shibata 1954; Culberson 1969, 1970). Lichens, like other ascomycetous fungi, form chemically heterogenous polyketides that comprise aliphatic and aromatic structural motifs. The orcinol and β-orcinol derivatives (depsides, depsidones, dibenzofurans) are especially interesting as they presumably play a role in the establishment of the lichen symbiosis and probably also in the interaction between the symbionts and with their environment (Armaleo 1995). A few of the structurally simple depsides have been found in non-lichenized fungi, such as lecanoric acid in the genus *Pyricularia* (Umezawa et al. 1974), a plant pathogenic fungus. In former studies, this group of organisms has been considered as the possible origin of the establishment of fungal symbioses that result in the lichen symbiosis (Ahmadjian 1970; Gargas et al. 1995). More

recent and extensive studies by Lutzoni et al. (2004) and James et al. (2006) have superseded the former speculations, indicating that many pathogens are derived from lichen-forming fungi.

The over-riding interest in these compounds has been in respect to their potential source as prospective pharmacophores (González-Tejero et al. 1995; Huneck 1999), but it is difficult to produce and isolate large quantities. To obtain rare compounds in large quantities without endangering the natural populations of the source organisms, optimized cultures of lichen fungi are desirable. It is, therefore, relevant to investigate what the ecological implications are in producing particular substances and what factors trigger the fungus to express a certain chemical profile (Culberson and Armaleo 1992; Armaleo 1995; Hamada and Miyagawa 1995).

The secondary metabolites of *Heterodea muelleri* (Hampe) Nyl. were previously analyzed by high-performance liquid chromatography (HPLC) and thin layer chromatography (TLC) and described by Hager and Stocker-Wörgötter (2005). Under particular stress conditions (e.g., exposure to UV light), cultured mycobionts produced the typical depsides, diffractaic and barbatic acids. In another screening experiment, Nishitoba et al. (1987) showed that diffractaic and barbatic acids strongly inhibited the growth of lettuce seedlings, and Takahagi et al. (2006) reported an inhibition of photosystem II in tobacco cells by barbatic acid.

Inspired by these results, we investigated whether these metabolites have any effect on the photobionts of *H. muelleri*. For this reason, we tested whether lichen substances protect the lichen photobiont against environmental stress and control its photosynthesis and growth. The major aim was to test the effects of typical lichen metabolites, diffractaic and barbatic acids, formed both by natural thalli and by cultured mycobionts on the growth of the cultured algal symbionts. A further experiment studied the effects of usnic acid extracted from *Heterodea beaugleholei*, the closest relative of *H. muelleri*.

Methods and Materials

Voucher Specimens The voucher specimens, fruticose thalli of *H. muelleri*, were collected from the hills of Canberra (Mt. Ainslie, S 35°20′57″, E 149°07′32″) in the Australian Capital Territory (Fig. 1a). The collected material is kept and preserved in the Herbarium of the University of Salzburg (Herbarium SZU L 23035).

Culture Method Single spore isolation was performed according to Ahmadjian (1973). Apothecia were cut off the thallus and transferred to 10 ml of water in a beaker (for 10 min.). After removal of the water film with filter paper, the apothecia were fixed to the top cover of a petri dish with petroleum jelly. Spores were discharged onto a sterile S2% medium in a petri dish [Sabouraud-2%–Glucose–Agar (Fluka 84086)]. After 2 d, several groups of spores were present on the surface of the agar plate. The cover that contained the apothecia was then removed to avoid further contamination. Germinated, single spore isolates were transferred to tubes containing S2%. Mycelia that developed from these spores (incubated for 4–6 mo) were gently homogenized to obtain subcultures.

Culture Media for Photobionts Algal symbionts were isolated and subcultured on Bold's basal medium(BBM; Deason and Bold 1960). For the growth experiment, petri dishes with BBM+b (BBM, as a solid medium impregnated by barbatic acid), BBM+d (BBM with diffractaic acid), and BBM+u (BBM with usnic acid) were used.

Culture Conditions Cultures were maintained in a culture chamber under a changing 12 hr/27°C:12 hr/24°C L:D regime, and a light intensity of 50–100 μmol m^{-2} s^{-1} (=standard conditions).

Stress Parameters (1) Well-developed subcultures (spore isolates, 4 mo old) of *H. muelleri* were subjected to a low temperature stress of 4°C for 6 wk. A control was kept

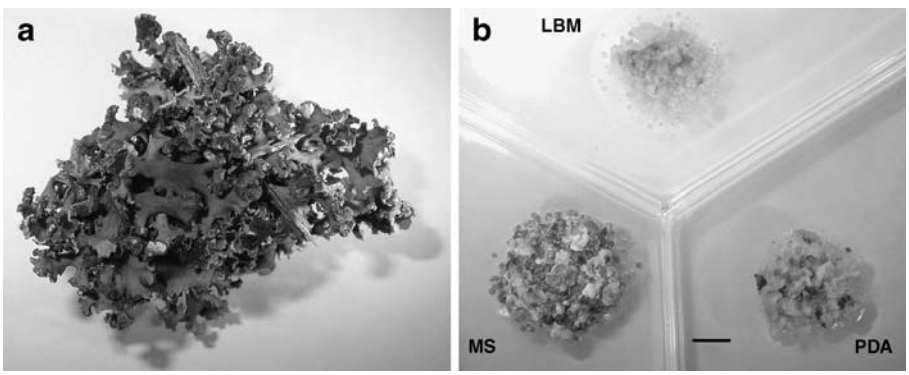

Fig. 1 **a** *Heterodea muelleri* thallus from Mt. Ainslie (Canberra, Australia). **b** Nutrient media growth experiment; mycobiont cultures on three different media after 5 mo (showing strong pigmentation on MS); *bar*=5 mm

under standard conditions. Methanol extracts of the freeze–dried cultures were analyzed by HPLC. (2) Four-month-old subcultures of the lichen fungus were exposed to UV-C (254 nm; 300 μW cm^{-2}) for 1 hr each day at room temperature (under a sterile hood with deactivated airflow to avoid dryness) over a period of 2 wk. Cultures were maintained under standard conditions (as described above) for the remaining time. A control culture was kept as a reference. Methanol extracts of freeze–dried cultures were analyzed by HPLC.

Preparation of Specimens, DNA and Chemical Analyses

Preparation Circular plugs (ca 1 cm in diameter) were cut from each agar plate overgrown by the mycobiont, and the origin of each subculture was recorded. To prevent contamination, the plugs for chemical testing were prepared in a hood under sterile conditions. Samples were vacuum freeze–dried at −42°C for 24 hr by using an ice condenser connected to a vacuum pump. The dried discs were transferred to glass tubes and extracted with methanol for 4 hr. Extracts were then transferred to HPLC vials, and an aliquot of 20 μl from every sample was injected.

DNA Extraction Total DNA was extracted from cultured mycobionts and for the comparisons from the voucher specimens (Armaleo and Clerc 1995).

DNA Amplification and Purification The ITS-regions, the 5.8 region, and the flanking parts of the small and large subunits (SSU 18S and LSU 28S) of the rDNA were amplified by using a GeneAmp polymerase chain reaction (PCR) system thermal cycler. Primers for the PCR were ITS1F (Gardes and Bruns 1993), ITS2, and ITS4 (White et al. 1990) designed for the lichen fungus. The PCR mix contained 1.25 U of Dynazyme Taq polymerase (Finnzymes), 0.2 mM of each of the 4 dNTPs, 0.5 μM of each primer, and 10–50 ng genomic DNA. The PCR products were cleaned by using a Quiaquick PCR product purification kit. Sequences were run on an ABI 310 automated sequencer. For the alignments of sequences, a Pile-up Programme was used, and the adjustments were done manually. For the photobionts, special *Trebouxia* primers (ITS1T and ITS4T, Kroken and Taylor 2000) were used.

Chromatographic Analysis Lichen thalli (100 mg dry weight) and vacuum-dried mycobionts (removed from the medium; 100 mg dry weight) were extracted for 4 hr with methanol at room temperature for HPLC. HPLC analysis was performed following Stocker-Wörgötter and Elix (2002) by using a Hitachi/Merck HPLC system that included two pumps, a Photodiode array detector (190–800 nm wavelength range), a Beckman 5C18 column (4.6× 250 mm, 5 μm), and a computer system with an integration package based on Windows NT.

Extraction of Pure Metabolites To obtain pure barbatic and diffractaic acids (Fig. 2a,b), a preparative TLC was performed: the yield of ten mycobiont subcultures was collected and vacuum freeze–dried for 12 hr. Dried cultured material (ca 5–10 g) was extracted with acetone for 4 hr at room temperature. The mixture was transferred to a silica plate (as one continuous line, 160×5 mm). As the two substances are closely related, the separation was achieved by using solvent B (120 ml hexane, 90 ml methyl tertiary-butyl ether, 230 ml formic acid) and on TLC plates (20× 20 cm). The substance bands were scraped from the silica plate and redissolved in acetone. The supernatant that contained the pure lichen compound was carefully separated from the silica gel, and the remaining acetone was evaporated in a hood. Finally, the purity of the substance was checked by HPLC.

Algal Growth Experiments and Effect of Biologically Active Metabolites Growth experiments with *Trebouxia jamesii* (photobiont) were done on BBM agar plates over a period

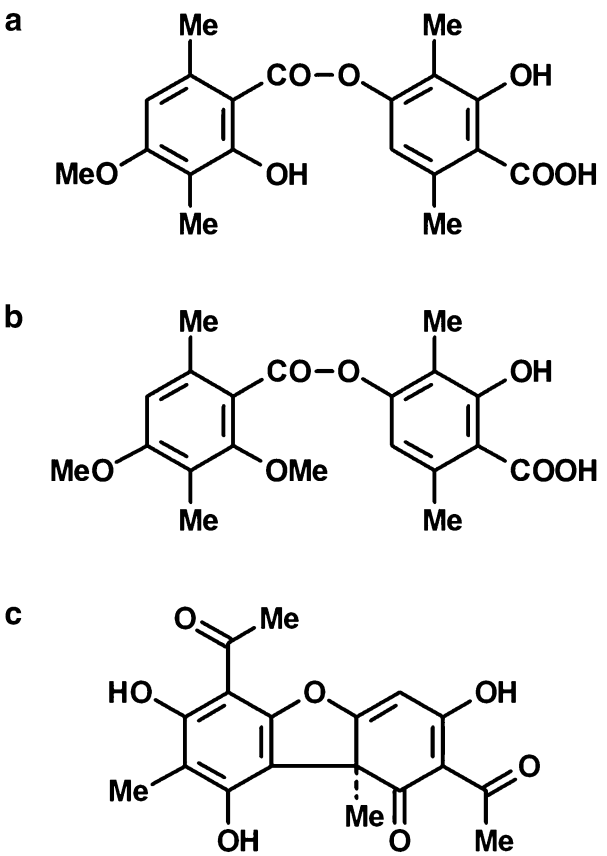

Fig. 2 Molecular structures of barbatic acid (**a**), diffractaic acid (**b**), and usnic acid (**c**)

of 1 mo in three different, independent experiments. In each, a layer of pure diffractaic or barbatic acid—extracted from metabolite-forming *Heterodea* mycobiont—was superimposed onto the nutrient media by using three different concentrations (0.5, 2, and 4 mg cm^{-2}; the quantity was chosen considering the overall thallus contents). The effect on the growth of the algae (starting with algal colonies, ca 3 mg fresh weight) was documented by a weekly analysis of images taken by a digital camera fixed to a light microscope. In another study, the effect of usnic acid (extracted from *H. beaugleholei*, Fig. 2c) on photobiont division rates was tested in a similar way. After 1 mo, the algal cells were harvested, weighed, and the obtained values were statistically evaluated by considering mean values and SDs.

Statistical Analyses Statistics were calculated by using ANOVA procedure of SigmaStat 3.11 for Windows; SigmaPlot 9.01 for Windows was used for figure preparation (Systat Software, Inc. 2004).

Results

HPLC analyses demonstrated that the voucher samples produce diffractaic acid as a major substance, whereas barbatic acid was detected as a minor satellite compound (Fig. 2a,b). Mycobiont cultures showed the highest growth rates on Murashige and Skoog (MS) medium (Stocker-Wörgötter 2001); development was accompanied by a more intense pigmentation than was found in cultures from other tested media (Fig. 1b).

Mycobiont cultures only began secondary metabolite production when they had been exposed to cold temperature stress (4°C for 6 wk) or UV-C light (254 nm, 1 hr per d over 2 wk). As a consequence of this treatment, cultures produced diffractaic acid, barbatic acid, and probably their precursors and intermediates. UV-C treatment doubled the proportion of produced substances and provoked a strong pigmentation.

In the UV-C experiment, diffractaic acid was the major substance, but cold temperature exposure resulted in large amounts of barbatic acid; overall values increased more than 60 times compared with voucher specimens (Fig. 3). In both experiments, the overall secondary metabolite contents were significantly higher than those of the voucher specimen. Based on the quantities of lichen acids in the thallus (4.80 mg cm^{-2} diffractaic acid and 0.36 mg cm^{-2} barbatic acid), the concentrations used for the algal test series were calculated as 0.5, 2, and 4 mg cm^{-2} on the surface of the agar plate.

After 3 d of incubation, algal growth was reduced considerably on the plates with barbatic acid. However, the algal biomass in the dishes that contained diffractaic and usnic acids and in the negative control, algal growth increased from 550 to 650% after 1 mo in culture (Table 1, Fig. 4). No significant differences were recognized between the diffractaic and usnic acid treatment and the control culture with no lichen metabolites. *Trebouxia* colonies were able to double their biomass at low barbatic acid concentrations (0.5 mg cm^{-2}), whereas the algal cells were seriously damaged at higher concentrations (2 mg cm^{-2}). Most of the cells died at the highest test concentration of 4 mg cm^{-2} (Figs. 5, and 6).

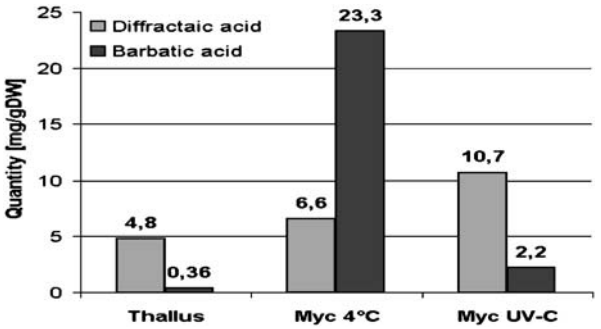

Fig. 3 Changes in lichen acid production of the cultures mycobionts (myc) due to temperature and UV-C stress in comparison with the thallus content

Discussion

As former studies have shown (Stocker-Wörgötter 2001; Hager and Stocker-Wörgötter 2005, Brunauer et al. 2006, 2007), the culturing of lichen mycobionts requires long-term experiments; numerous "case" studies have demonstrated that every "new" lichen fungus prefers a different composition of nutrient medium and specially adapted culture conditions. Many culture experiments are necessary to trigger the aposymbiotically grown mycobiont to produce specific secondary metabolites.

Table 1 Biomass increase of algal colonies on control and test-media after 1 mo (starting from 3 mg algal cells, N=3)

Medium (BBM)	Concentration (mg cm⁻2)	Mean (mg)	Increase (%)	SD
Control		19.13	537.66	18.23
+Barbatic acid	0.5	6.60	120.00	7.39
	2.0	2.58	−14.00	5.01
	4.0	1.95	−35.00	7.83
+Diffractaic acid	0.5	19.66	555.33	23.36
	2.0	20.65	588.33	28.47
	4.0	21.39	613.00	33.69
+Usnic acid	0.5	20.21	573.66	12.45
	2.0	20.44	581.33	37.51
	4.0	21.33	611.00	28.22

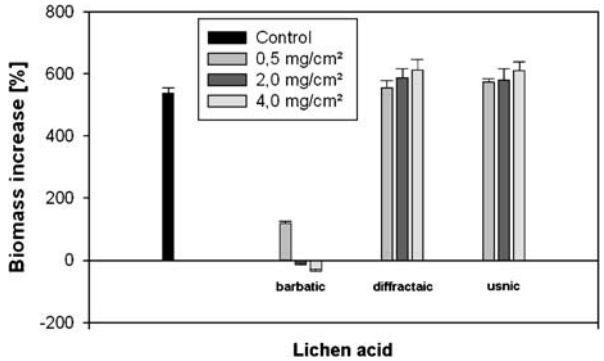

Fig. 4 Increase of algal colony biomass on the different lichen acid layers after 1 mo. *Error bars* represent SE

In some cases, lichen culturing is further complicated by the isolation of epi- and endolichenic fungi and algae. To identify the lichen mycobiont and to distinguish it from occasionally isolated, lichenicolous fungi, DNA canalyses of the ITS region have been performed. If the mycobiont produces lichen substances in culture, the fungus can be identified by its chemical profile, and the produced lichen substances can be used as chemical markers. Chemical analyses by HPLC, TLC, and microcrystallization tests cannot screen contaminations by other fungi.

Culture of *H. muelleri* mycobionts on MS medium enriched with sugars resulted in increased and optimized growth rates. The characteristic brown pigmentation found in cultures grown on MS is probably correlated with the brown pigment that is normally formed by the apothecia of the voucher specimens (Miyagawa et al. 1994).

Stress experiments with *H. muelleri* mycobiont cultures yielded surprising results, especially the more than 60-fold increase of barbatic acid that resulted from cold temperature treatment. Low temperatures can set off an increased production of secondary metabolites, as reported by Stocker-Wörgötter (2001) for *Umbilicaria mammulata*. The question as to why stress due to UV-C exposure resulted in an increased biosynthesis of medullary lichen acids (in the intact lichen formed by medullary hyphae) remains open. Further experiments, using different types of UV irradiations (A, B, and C) have to be performed, as different wavelengths may play a crucial role in secondary compound synthesis. According to Swanson and Fahselt (1997) and BeGora and Fahselt (2001), UV-A irradiation alone caused the production of higher contents of phenolic compounds in lichens than exposure to both UV-A and UV-B. A definite correlation between production of lichen acids and the effects of UV-light was recently reported by

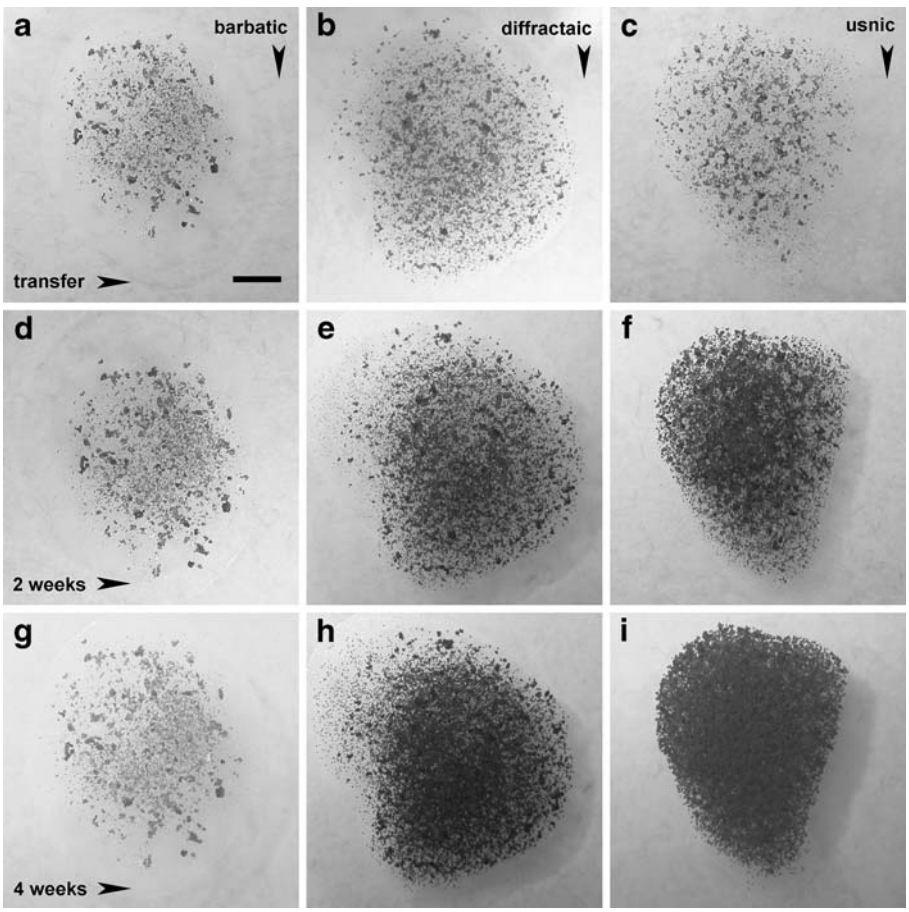

Fig. 5 Algal growth experiment example: growth over 1 mo of algal colonies on different lichen acid layers, concentration 2 mg cm^{-2}; *top*: algae on barbatic (**a**), diffractaic (**b**), and usnic acid (**c**) layer after transfer; middle: colonies on barbatic (**d**), diffractaic (**e**), and usnic acid (**f**) layer after 2 weeks; *bottom*: colonies on barbatic (**g**), diffractaic (**h**), and usnic acid (**i**) layer after 1 mo; *bar*=3 mm

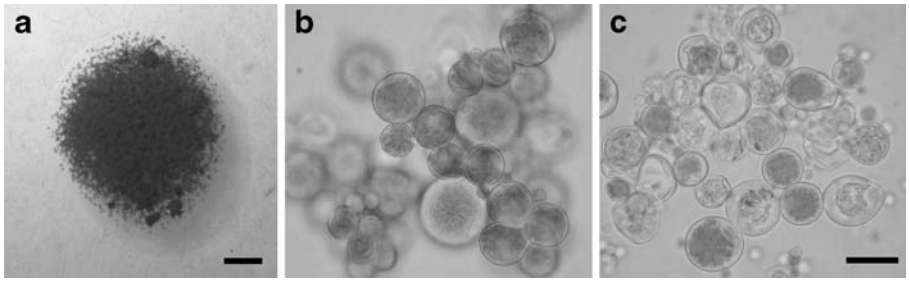

Fig. 6 Control colony after 1 mo (**a**); *bar*=3 mm. Algal cells of control colony (**b**), and of colony on 2 mg/cm^2 barbatic acid (**c**) after 1 mo; *bar*=10 μm

Solhaug et al. (2003) for parietin, an anthraquinone that is known to act as an UV-protecting agent in the cortex of *Xanthoria parietina*.

While barbatic and diffractaic acids are known to be biologically active metabolites that act as inhibitors of specific functions such as cell division and growth, usnic acid is a molecule with manifold activities (Table 2; Ingólfsdóttir 2002). Although the molecules are closely related, our experiments and those of others indicate that barbatic and diffractaic acids exhibit diverging biological activities. Barbatic acid, for example, impairs the function of photosystem II in higher plants, whereas diffractaic acid has fungicidal properties (Nishitoba et al. 1987; Dayan and Romagni 2001, 2002; Takahagi et al. 2006). In agreement with these experiments, our test series showed that the growth of photobionts exposed to barbatic acid concentrations higher than 0.5 mg.cm^{-2} was inhibited, and a significant loss of vitality and cell-bleaching was observed. At such high concentrations, algae were killed depending on the lengths of exposure times. Whether cell death of the algae was caused by barbatic acid inhibiting photosystem II or due to another mode of action remains to be determined. The concentration of 0.5 mg.cm^{-2}, which corresponds to the quantity present in the *H. muelleri* thallus, slowed down cell division rates of the *Trebouxia* photobionts. This result clearly shows that barbatic acid acts as a regulating agent for algal growth and mitosis in the lichen.

Photobionts incubated on BBM+d and BBM+u were not affected in comparison to the negative control. Although Schimmer and Lehner (1973) reported that usnic acid inhibited the growth of the free-living unicellular green alga *Chlamydomonas reinhardii*, it had no impact on the growth of *Trebouxia jamesii* in our experiments. The increase in algal biomass on BBM+u was similar to that of the negative control. As usnic acid is accumulated solely in the thallus cortex, this outcome was expected. Effects of usnic acid on

Table 2 Overview of known activity of the lichen acids tested in this study

Metabolite	Activity	Publication
Barbatic acid	Inhibition of EBV activation	Yamamoto et al. 1995
	Non-redox inhibitors of Leukotriene B4 biosynthesis	Kumar and Müller 1999b,c
	PS II inhibitor	Dayan and Romagni 2001
	Inhibition of PSII in atrazine-tolerant tobacco cells	Takahagi et al. 2006
Diffractaic acid	Inhibition of EBV activation	Yamamoto et al. 1995
	Analgesic and antipyretic agent	Okuyama et al. 1995
	Moderate inhibitor of arachidonate 5-lipoxygenase	Kumar and Müller 1999a,b,c
	Fungitoxic	Dayan and Romagni 2001
	Antiulcerogenic	Bayir et al. 2006
Usnic acid	Most potent antibiotic agent	Klosa 1953
	Growth inhibition of *Chlamydomonas reinhardii*	Schimmer and Lehner 1973
	Antitumor activities (Lewis lung-carcinoma test system)	Kupchan and Kopperman 1975
	Inhibits mitosis in *Allium*	Huovinen and Lampero 1989
	Antitranspiratory	Carbonnier et al. 1993
	Inhibition of EBV activation	Yamamoto et al. 1995
	Antipyretic and analgesic component	Okuyama et al. 1995
	In vitro activity against *Staphylococcus* and *Enterococcus*	Lauterwein et al. 1995
	Antimitotic effects	Cardarelli et al. 1997
	Antiproliferative activity, antisporiatic properties	Kumar and Müller 1999c
	Antimicrobial, fungicidal, herbicidal activity	Dayan and Romagni 2001
	Antifungal activity	Halama and Van Haluwin 2004
	anticancer properties	Mayer et al. 2005

epibiontic algae that grow on the lichen have to be tested. Usnic acid has various biological activities in several plants and even humans. According to Swanson and Fahselt (1997) it also has important functions in the lichen itself, e.g., as UV protection. While usnic acid located in the cortex could act against uncontrolled propagation of epibiontic algae without having any impact on the photobiont, compounds such as diffractaic acid, deposited and accumulated by medullary hyphae, could effectively restrict the growth of endolichenic fungi (Dayan and Romagni 2001).

It has to be emphasized that the two depsides tested, diffractaic and barbatic acids, exhibit two fundamentally different biological activities, although the molecules differ merely in one functional unit (diffractaic acid has a CH_3-group at the side chain of C2, barbatic acid has a hydrogen), a phenomenon often observed by closely related biologically active substances. Further investigations are planned to identify the effects of barbatic acid on functional aspects of the lichen symbioses The investigated substances and their derivatives will be tested also on the photobionts of other lichens and on free-living algae, and their influence on the photosynthesis will be evaluated.

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References

AHMADJIAN, V. 1970. The lichen symbiosis: its origin and evolution, Evolutionary Biology, vol. 4. pp. 163–184, in T. Dobzhansky, M. K. Hecht, and W. C. Steere (eds.). Appleton-Century Crofts, New York.

AHMADJIAN, V. 1973. Methods of isolating lichen symbionts and thalli, The Lichens. pp. 653–659, in V. Ahmadjian, and M. E. Hale (eds.). Academic Press, New York.

ARMALEO, D. 1995. Factors affecting depside and depsidone biosynthesis in a cultured lichen fungus. *Crypt. Bot* 5:14–21.

ARMALEO, D., and CLERC, P. 1995. A rapid and inexpensive method for the purification of DNA from lichens and their symbionts. *Lichenologist* 27:207–213.

ASAHINA, Y., and SHIBATA, S. 1954. Chemistry of lichen substances. JSPS, Tokyo.

BAYIR, Y., ODABASOGLU, F., CAKIR, A., ASLAN, A., SULEYMAN, H., HALICI, M., and KAZAZ, C. 2006. The inhibition of gastric mucosal lesion, oxidative stress and neutrophil-infiltration in rats by the lichen constituent diffractaic acid. *Phytomedicine* 13:584–590.

BEGORA, M. D., and FAHSELT, D. 2001. Usnic acid and atranorin concentrations in lichens in relation to bands of UV irradiance. *Bryologist* 104:134–140.

BRUNAUER, G., HAGER, A., KRAUTGARTNER, W. D., TÜRK, R., and STOCKER-WÖRGÖTTER, E. 2006. Experimental studies on *Lecanora rupicola* (L.) Zahlbr.: chemical and microscopical investigations of the mycobiont and re-synthesis stages. *Lichenologist* 38:577–585.

BRUNAUER, G., HAGER, A., GRUBE, M., TÜRK, R., and STOCKER-WÖRGÖTTER, E. 2007. Alterations in secondary metabolism of aposymbiotically grown mycobionts of *Xanthoria elegans* and cultured resynthesis stages. *Plant Physiol. Biochem* 45:146–151.

CARBONNIER, J., BATCHO, E., SHAKER-BAZARNOV, H., and MOLHO, D. 1993. Proprietes de l'Acide Usnique, Antitranspirant d'Origine Naturelle. *Rev. réseau amélior. product. agric. milieu aride* 5:19.

CARDARELLI, M., SERINO, G., CAMPANELLA, L., ERCOLE, P., DE CICCO NARDONE, F., ALESIANI, O., and OSIELLO, F. 1997. Antimitotic effects of usnic acid on different biological systems. *Cell. Mol. Life Sci* 53:667–672.

CULBERSON, C. F. 1969. Chemical and Botanical Guide to Lichen Products. University of North Carolina Press, Chapel Hill.

CULBERSON, C. F. 1970. Supplement to "Chemical and Botanical Guide to Lichen Products". *Bryologist* 73:177–377.

CULBERSON, C. F., and ARMALEO, D. 1992. Induction of a complete secondary-product pathway in a cultured lichen fungus. *Exp. Mycol* 16:52–63.

DAYAN, F. E., and ROMAGNI, J. G. 2001. Lichens as a potential source of pesticides. *Pestic Outlook* 12:229–232.

DAYAN, F. E., and ROMAGNI, J. G. 2002. Structural diversity of lichen metabolites and their potential for use, Advances in Microbial Toxin Research and its Biotechnological Exploration. pp. 151–161, in R. Upadhyaya (ed.). Kluwer Academic/Plenum Publisher, New York.

DEASON, T. R., and BOLD, H. C. 1960. Phycological studies. I. Exploratory studies of Texas soil algae. *Univ. Texas Publ* 6022:1–70.

GARDES, M., and BRUNS, T. D. 1993. ITS primers with enhanced specificity for Basidiomycetes. Application to the identification of mycorrhizae and rusts. *Mol. Ecol* 2:113–118.

GARGAS, A., DEPRIEST, P. T., GRUBE, M., and TEHLER, A. 1995. Multiple origins of lichen symbioses in fungi suggested by SSU rDNA phylogeny. *Science* 268:1492–1495.

GONZÁLEZ-TEJERO, M. R., MOLERO-MESA, J., CASARES-PORCEL, M., and MARTÍNEZ LIROLA, M. J. 1995. New contributions to the ethnopharmacology of Spain. *J. Ethnopharmacol* 45:157–165.

HAGER, A., and STOCKER-WÖRGÖTTER, E. 2005. Secondary chemistry and DNA-analyses of the Australian lichen *Heterodea muelleri* (Hampe) Nyl. and culture of the symbionts. *Symbiosis* 39:13–19.

HALAMA, P., and VAN HALUWIN, C. 2004. Antifungal activity of lichen extracts and lichenic acids. *BioControl* 49:95–107.

HAMADA, N., and MIYAGAWA, H. 1995. Secondary metabolites from isolated lichen mycobionts cultured under different osmotic conditions. *Lichenologist* 27:201–205.

HUNECK, S. 1999. The significance of lichens and their metabolites. *Naturwissenschaften* 86:559–570.

HUNECK, S. 2001. New Results on the Chemistry of Lichen Substances, Progress in the Chemistry of Organic Natural Products 81. in W. Herz, H. Falk, G. W. Kirby, and R. E. Moore (eds.). Springer, Wien, New York.

HUOVINEN, K., and LAMPERO, M. 1989. Usnic acid as a mitotic inhibitor in the *Allium* Test. *Planta Medica* 55:98.

INGÓLFSDÓTTIR, K. 2002. Usnic acid. *Phytochemistry* 61:729–736.

JAMES, T. Y., KAUFF, F., SCHOCH, C., MATHENY, B., HOFSTETTER, V., COX, C. J., CELIO, G., GUIEDAN, C., FRAKER, E., MIADLIKOWSKA, J., LUMBSCH, T., AUHUT, A., EEB, V., ARNOLD, A., AMTOFT, A., STAJICH, J. E., HOSAKA, K., SUNG, G., JOHNSON, D., O'ROURKE, B., CROCKETT, M., BINDER, M., CURTIS, J. M., SLOT, J. C., WANG, Z., WILSON, A. W., SCHUELLER, A., LONGCORE, J. E., O'DONNELL, K., MOZLEY-STANDRIDGE, S., PORTER, D., LETCHER, P. M., POWELL, M. J., TAYLOR, J. W., WHITE, M. M., GRIFFITH, G. W., DAVIES, D. R., HUMBER, R. A., MORTON, J. B., SUGIYAMA,

J., OSSMAN, A. Y., OGERS, J. D., PFISTER, D. H., HEWITT, D., HANSEN, K., HAMBLETON, S., SHOEMAKER, R. A., KOHLMEYER, J., VOLKMANN-KOHLMEYER, B., SPOTTS, R. A., SERDANI, M., CROUS, P. W., HUGHES, K. W., MATSUURA, K., LANGER, E., LANGER, G., UNTEREINER, W. A., LÜCKING, R., BUDEL, B., GEISER, D. M., APTROOT, D. M., DIEDERICH, P., SCHMITT, I., SCHULTZ, M., YAHR, R., HIBBETT, D. S., LUTZONI, F., MCLAUGHLIN, D. J., SPATAFORA, J. W., and VILGALYS, R. 2006. Reconstructing the early evolution of fungi using a six-gene phylogeny. *Nature* 443:818–822.

KLOSA, K. 1953. Chemische Konstitution und antibiotische Wirkung der Flechtenst. *Pharmazie* 8:435–442.

KROKEN, S., and TAYLOR, J. W. 2000. Phylogenetic species, reproductive mode, and specificity of the green alga *Trebouxia* forming lichens with fungal genus *Letharia*. *Bryologist* 103:645–660.

KUMAR, K. C. S., and MÜLLER, K. 1999a. Lichen metabolites. 1. Inhibitory action against leukotriene B_4 biosynthesis by a non-redox mechanism. *J. Nat. Prod* 62:817–820.

KUMAR, K. C. S., and MÜLLER, K. 1999b. Lichen metabolites. 2. Antiproliferative and cytotoxic activity of gyrophoric, usnic, and diffractaic acid on human keratinocyte growth. *J. Nat. Prod* 62:821–823.

KUMAR, K. C. S., and MÜLLER, K. 1999c. Depsides as non-redox inhibitors of leukotriene B4 biosynthesis and HaCaT cell growth. 1. Novel analogs of barbatic and diffractaic acid.. *Eur. J. Med. Chem* 34:1035–1042.

KUPCHAN, S. M., and KOPPERMAN, H. L. 1975. L-Usnic acid: tumor inhibitor isolated from lichens. *Experientia* 31:625.

LAUTERWEIN, M., OETHINGER, M., BELSNER, K., PETERS, T., and MARRE, R. 1995. In vitro activities of the lichen secondary metabolites vulpinic acid, (+)-usnic acid, and (−)-usnic acid against aerobic and anaerobic microorganisms. *Antimicrob. Agents Chemother* 39:2541–2543.

LUTZONI, F., KAUFF, F., COX, C. J., MCLAUGHLIN, D., CELIO, G., DENTINGER, B., PADAMSEE, M., HIBBETT, D., JAMES, T. Y., BALOCH, E., GRUBE, M., EEB, V., HOFSTETTER, V., SCHOCH, C., ARNOLD, A. E., MIADLIKOWSKA, J., SPATAFORA, J., JOHNSON, D., HAMBLETON, S., CROCKETT, M., SHOEMAKER, R., SUNG, G.-H., LÜCKING, R., LUMBSCH, T., O'DONNELL, K., BINDER, M., DIEDERICH, P., ERTZ, D., GUEIDAN, C., HANSEN, K., HARRIS, R. C., HOSAKA, K., LIM, Y.-W., METHENY, B., NISHIDA, H., PFISTER, D., OGERS, J., OSSMAN, A., SCHMITT, I., SIPMAN, H., STONE, J., SUGIYAMA, J., YAHR, R., and VILGALYS, R. 2004. Assembling the fungal tree of life: progress, classification, and evolution of subcellular traits. *Am. J. Bot* 91:1446–1480.

MAYER, M., O'NEILL, M. A., MURRAY, K. E., SANTOS-MAGALHAES, N. S., CARNEIRO-LEAO, A. M. A., THOMPSON, A. M., and APPLEYARD, V. C. L. 2005. Usnic acid: a non-genotoxic compound with anti-cancer properties. *Anti Canc. Drugs* 16:805–809.

MIYAGAWA, H., HAMADA, N., SATO, M., and UENO, T. 1994. Pigments from the cultured lichen mycobionts of *Graphis scripta* and *G. desquamescens*. *Phytochemistry* 36:1319–1332.

MÜLLER, K. 2001. Pharmaceutically relevant metabolites from lichens. *Appl. Microbiol. Biotechnol* 56:9–16.

NISHITOBA, Y., NISHIMURA, H., NISHIYAMA, T., and MIZUTAMI, J. 1987. Lichen acids, plant growth inhibitors from *Usnea longissima*. *Phytochemistry* 26:3181.

OKUYAMA, E., UMEYAMA, K., YAMAZAKI, M., KINOSHITA, Y., and YAMAMOTO, Y. 1995. Usnic acid and diffractaic acid as analgesic and antipyretic components of *Usnea diffracta*. *Planta Med* 61:113–115.

SCHIMMER, O., and LEHNER, H. 1973. Untersuchungen zur Wirkung von Usninsäure auf die Grünalge *Chlamydomonas reinhardtii*. *Arch. Microbiol* 93:145.

SOLHAUG, K. A., GAUSLAA, Y., NYBAKKEN, L., and BILGER, W. 2003. UV-induction of sun-screening pigments in lichens. *New Phytologist* 158:91–100.

STOCKER-WÖRGÖTTER, E. 2001. Experimental studies of the lichen symbiosis: DNA-analyses, differentiation and secondary chemistry of selected mycobionts, artificial resynthesis of two- and tripartite symbioses. *Symbiosis* 30:207–227.

STOCKER-WÖRGÖTTER, E., and ELIX, J. A. 2002. Secondary chemistry of cultured mycobionts: formation of a complete chemosyndrome by the lichen fungus of *Lobaria spathulata*. *Lichenologist* 34:351–359.

SWANSON, A., and FAHSELT, D. 1997. Effects of ultraviolet on polyphenolics of *Umbilicaria americana*. *Can. J. Bot* 75:284–289.

TAKAHAGI, T., IKEZAWA, N., ENDO, T., IFUKU, K., YAMAMOTO, Y., KINOSHITA, Y., TAKESHITA, S., and SATO, F. 2006. Inhibition of PSII in atrazine-tolerant tobacco cells by barbatic acid, a lichen-derived depside. *Biosci. Biotechnol. Biochem* 70:266–268.

UMEZAWA, H., SHIBAMOTO, N., NAGANAWA, H., AYUKAWA, S., MATSUZAKI, M., TAKEUCHI, T., KONO, K., and SAKAMOTO, T. 1974. Isolation of lecanoric acid, an inhibitor of histidine decarboxylase from a fungus. *J. Antibiot* 27:587–596.

WHITE, T. J., BRUNS, T., LEE, S., and TAYLOR, J. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics, PCR Protocols: A Guide to Methods and Applications. pp. 315–322, in M. A. Innis, D. H. Gelfand, J. J. Sninsky, and T. J. White (eds.). Academic Press, New York.

YAMAMOTO, Y., MIURA, Y., KINOSHITA, Y., HIGUCHI, M., MURAKAMI, A., OHIGASHI, H., and KOSHIMIZU, K. 1995. Screening of tissue cultures and thalli of lichens and some of their active constituents for inhibition of tumor promoter-induced Epstein–Barr virus activation. *Chem. Pharmaceut. Bull* 43:1388–1390.

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Barbarea vulgaris Glucosinolate Phenotypes Differentially Affect Performance and Preference of Two Different Species of Lepidopteran Herbivores

Hanneke van Leur · Louise E. M. Vet · Wim H. van der Putten · Nicole M. van Dam

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Abstract The composition of secondary metabolites and the nutritional value of a plant both determine herbivore preference and performance. The genetically determined glucosinolate pattern of *Barbarea vulgaris* can be dominated by either glucobarbarin (BAR-type) or by gluconasturtiin (NAS-type). Because of the structural differences, these glucosinolates may have different effects on herbivores. We compared the two *Barbarea* chemotypes with regards to the preference and performance of two lepidopteran herbivores, using *Mamestra brassicae* as a generalist and *Pieris rapae* as a specialist. The generalist and specialist herbivores did not prefer either chemotype for oviposition. However, larvae of the generalist *M. brassicae* preferred to feed and performed best on NAS-type plants. On NAS-type plants, 100% of the *M. brassicae* larvae survived while growing exponentially, whereas on BAR-type plants, *M. brassicae* larvae showed little growth and a mortality of 37.5%. In contrast to *M. brassicae*, the larval preference and performance of the specialist *P. rapae* was unaffected by plant chemotype. Total levels of glucosinolates, water soluble sugars, and amino acids of *B. vulgaris* could not explain the poor preference and performance of *M. brassicae* on BAR-type plants. Our results suggest that difference in glucosinolate chemical structure is responsible for the differential effects of the *B. vulgaris* chemotypes on the generalist herbivore.

Keywords Glucosinolates · Oviposition · Performance · Polymorphism · *Barbarea vulgaris* · *Pieris rapae* · *Mamestra brassicae* · Co-evolution

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H. van Leur (✉) · L. E. M. Vet · W. H. van der Putten · N. M. van Dam
Department of Multitrophic Interactions,
Netherlands Institute of Ecology (NIOO-KNAW),
Centre for Terrestrial Ecology,
P.O. Box 40, 6666 ZG Heteren, The Netherlands
e-mail: h.vanleur@nioo.knaw.nl

L. E. M. Vet
Laboratory of Entomology,
Wageningen University and Research Centre,
P.O. Box 8031, 6700 EH Wageningen, The Netherlands

W. H. van der Putten
Laboratory of Nematology,
Wageningen University and Research Centre,
P.O. Box 8123, 6700 ES Wageningen, The Netherlands

Introduction

Glucosinolates and their breakdown products are involved in plant defense against a wide variety of potential plant enemies. They can also serve as feeding and oviposition stimulants for specialist herbivores (Chew 1988; Louda and Mole 1991; Wittstock et al. 2003). The composition of glucosinolates is genetically variable within plant species, and influences the feeding choices of insect herbivores (Kroymann et al. 2001; Lambrix et al. 2001). A heritable glucosinolate polymorphism in *Barbarea vulgaris* results in two discrete chemotypes (Van Leur et al. 2006). In this study, we investigate the effect of these two chemotypes on the preference and performance of two lepidopterans.

The most common and genetically dominant chemotype of *B. vulgaris* forms mainly (*S*)-2-hydroxy-2-phenylethyl glucosinolate (glucobarbarin, BAR-type). Although depending on the presence of cofactors like ESP, pH, or metal ions (Burow et al. 2006), the most likely initial hydrolysis product of glucobarbarin is an isothiocyanate. Due to the 2-hydroxylation of the glucosinolate side chain,

it spontaneously cyclizes to 5-phenyloxazolidine-2-thione (Kjaer and Gmelin 1957). This glucosinolate breakdown product is known to reduce infection by the soil fungus *Plasmodiophora brassicae* (Ludwig-Müller et al. 1999). Hardly anything is known about other ecological activities of oxazolidine-2-thiones (Wittstock et al. 2003).

The less abundant and genetically recessive chemotype of *B. vulgaris* contains mainly 2-phenylethyl glucosinolate (gluconasturtiin, NAS-type). 2-Phenylethyl glucosinolate is also present in *Arabidopsis thaliana* and other *Brassicaceae* (Reichelt et al. 2002), predominantly in root tissues (Sang et al. 1984). Its most likely breakdown product is 2-phenylethyl isothiocyanate. Isothiocyanates are the predominant breakdown products of glucosinolates and are generally toxic to various herbivores (Wittstock et al. 2003). 2-Phenylethyl isothiocyanate negatively affects a broad range of phytophages, e.g., nematodes (Potter et al. 1999, 2000; Serra et al. 2002; Lazzeri et al. 2004), snails (Kerfoot et al. 1998), flies, aphids, mites (Lichtenstein et al. 1962), fungi (Sarwar and Kirkegaard 1998), and several generalist and specialist Lepidoptera (Wadleigh and Yu 1988; Borek et al. 1998). Despite counter-adaptations of specialists to reduce or circumvent negative effects of glucosinolates (Ratzka et al. 2002; Wittstock et al. 2003), isothiocyanates can still reduce survival and growth, and increase development time of specialists (Agrawal and Kurashige 2003). In contrast to the oxazolidine-2-thiones formed in BAR-type plants, which can increase the incidence of goiter in mammals, the 2-phenylethyl isothiocyanate formed in NAS-type plants has chemopreventive effects against tumorigenesis in mammalian organisms (Canistro et al. 2004).

Based on the specific biological effects of the expected breakdown products of glucobarbarin and gluconasturtiin, we hypothesized that NAS-type and BAR-type *B. vulgaris* plants have differential effects on insect herbivores. Generally, it is expected that chemical plant defenses are more effective against generalist herbivores than against specialists (Cornell and Hawkins 2003). Therefore, we compared the preference and performance on the two chemotypes of a generalist (*Mamestra brassicae*) and a specialist (*Pieris rapae*) herbivore, which are both well-studied, important crucifer pests (Theunissen et al. 1985; Finch and Kienegger 1997).

M. brassicae is a generalist feeding on plants in 70 species and 22 families, of which Brassicaceae are among the most preferred (Rojas et al. 2000). Even though *M. brassicae* can detect glucosinolates by receptor cells on the sensilla (Wieczorek 1976), and its oviposition is stimulated by damaged cabbage plants (Rojas 1999), no physiological adaptations of this species to glucosinolates have been yet described. The larvae of *P. rapae*, on the other hand, can detoxify glucosinolates by shifting hydrolysis products from isothiocyanates to less toxic nitriles by using the myrosinase directing nitrile-specifier protein (NSP). This enables *P. rapae* to consume foliage that is otherwise well defended (Wittstock et al. 2003, 2004). Female adults of *P. rapae* can detect intact glucosinolates in leaves of Brassicaceae with specialized receptor cells, and are stimulated to lay eggs on glucosinolate containing plants.

We assessed herbivore preference on the level of adult oviposition and larval feeding. Female oviposition preference initially determines the host of the larvae (Akhtar and Isman 2003). As the isothiocyanates from NAS-type plants are expected to be more toxic than the oxazolidine-2-thiones produced by BAR-type plants, we expected *M. brassicae* to be repelled by NAS-type plants and to prefer BAR-type plants for oviposition. Oviposition of *P. rapae* is affected by glucosinolates at the leaf surface (Renwick et al. 1992; Van Loon et al. 1992) and known to be stimulated by glucobarbarin as well as by gluconasturtiin in a dose-dependent way (Chew 1988; Huang and Renwick 1994; Huang et al. 1994a, b).

As larvae can eventually leave their initial host (van Dam et al. 2000), we also assessed larval preference. If the *B. vulgaris* chemotypes differ in toxicity or palatability, we expect to see the largest effect on larval preference and larval performance for the unadapted generalist *M. brassicae* and no or minor effects on the glucosinolate specialist *P. rapae*.

Although we observed no differences in morphology, growth, or germination between the chemotypes (van Leur and van Dam, unpublished results), pleiotropic effects or close linkages could possibly cause other genes and metabolites to be consistently different between the chemotypes. As the nutritional value of plants is important for herbivore performance and preference (Simpson and Simpson 1990; Berenbaum 1995), additionally, we analyzed the sugar content and amino acid level of the tissue on which the herbivores were feeding.

Materials and Methods

Plant Material B. vulgaris seeds were collected from 10 individual BAR and their nearest neighbor NAS-type maternal plants, which were freely cross pollinated in a natural population of *B. vulgaris*. The population was located in Elderveld, The Netherlands (51.95°N; 5.87°E) and consisted of 22% NAS-type plants (Van Leur et al. 2006). We selected offspring of maternal NAS-type plants "EL44" (68% BAR-type offspring) and "EL13" (62% BAR-type offspring). Plants were grown in a glasshouse, at 21°C (day) and 16°C (night), with 60% relative humidity and natural daylight supplemented with sodium lamps to maintain the minimum PAR at 225 μmol m^{-2} s^{-1} with a photoperiod of 16:8 (L:D). One week after germination on glass beads, seedlings were transplanted to a mixture of

peat soil (Potgrond 4, Lentse Potgrond BV., Lent, The Netherlands) and 20% sand. After 2 weeks, the seedlings were transplanted to 1.1-l pots, watered, and fertilized regularly with half strength Hoagland's nutrient solution with a doubled KH_2PO_4 content.

Chemical Analysis For quantification of glucosinolates, soluble sugars, and amino acids, one global extraction was used. In a 2-ml Eppendorf tube, 50.0 mg of lyophilized finely ground plant material were dissolved in 1.0 ml 70% MeOH in water (*v*/*v*), vortexed, and immediately boiled for 5 min to kill the remaining myrosinase activity. Tubes were placed in an ultrasonic bath for 15 min and centrifuged (10 min 10,000 rpm). The extraction was repeated for the pellet omitting the boiling step. For each sample, both supernatants were combined in a new 2-ml Eppendorf tube and supplemented individually with 70% MeOH to attain the average mass (N=3) of a 2-ml Eppendorf tube that contained 2.0 ml 70% MeOH. This "stock" extract was stored at −20°C until further analysis.

Half (1.0 ml) of the stock extract was used for glucosinolate analysis and applied to a DEAE-Sephadex A 25 column (EC 1990), desulfated with arylsulfatase (Sigma, St. Louis, MO, USA) and separated on a reversed phase C-18 column on HPLC with a CH_3CN–H_2O gradient as described in van Dam et al. (2004). Glucosinolate analysis was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with authentic standards isolated from *B. vulgaris* as previously described (Agerbirk et al. 2001), as well as standards kindly provided by M. Reichelt, MPI Chemical Ecology, and a certified rape seed standard (Community Bureau of Reference, Brussels, code BCR-367R). The same response factor was used for glucobarbarin as for gluconasturtiin (Buchner 1987). To calculate glucosinolate concentrations in the plant tissue, the obtained values were multiplied by two and divided by dry mass.

To analyze soluble sugar content, a 10-µl aliquot of the stock extract was diluted with 990 µl MilliQ water. Soluble sugars were analyzed by injecting 5 µl of the diluted extract on Dionex HPLC system, equipped with a Carbopac PA1 column (2×250 mm) and a Carbopac PA1 guard column (2×50 mm, Dionex, Sunnyvale, CA, USA). An isocratic gradient mixture of 10% 1 M NaOH and 90% MilliQ water was used to separate sugars at a flow rate of 0.25 ml/min. Column temperature was kept at 20°C. A "10-ppm" reference solution that contained 54.9 µM sorbitol and manitol, 29.21 µM trehalose, sucrose, and melbiose, and 55.51 µM glucose and fructose, was diluted to obtain 7.5, 5, and 2.5 ppm calibration standards to obtain a reference curve. After every 10 samples, an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of sugars in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

Amino acids were analyzed on a Dionex HPLC system by integrated pulsed amperometric detection. An aliquot of 20 µl of the stock extract was diluted with 980 µl MilliQ. Of this diluted extract, 25 µl were injected, and amino acids were separated with a ternary gradient (see DIONEX application update 152, Method 1, standard AAA gradient; condition 60/2 in Hanko and Rohrer 2004) on a 2×250 mm AminoPac© PA10 column with a 2×50 mm AminoPac© PA10 Guard column (Dionex, Sunnyvale, CA, USA). Eluents, flow rates, waveform, and working electrode conditions were all as specified under Method 1 in Dionex application update 152 and in Hanko and Rohrer (2004). The Sigma AA-S-18 amino acid standard (Sigma, St Louis, MO, USA) that contained 17 amino acids was supplemented with asparagine, glutamine, and tryptophane (2.5 µmol/ml each) to obtain a reference sample that contained the 20 most common amino acids. This reference solution was diluted to obtain calibration standard ranging from 1–8 µM for each amino acid, except for cysteine, which had a range of 0.5–4 µM. After every 10 samples, an additional standard was injected to check for deviations of retention times and the calibration curve. To calculate the molar concentration of the amino acids in the plant tissue, the concentration values were multiplied by 200 and divided by dry mass.

To determine the chemotype of each plant, glucosinolates were extracted from the first full grown leaf and analyzed on HPLC as described above. When the peak area of glucobarbarin divided by the peak area of gluconasturtiin was >10, the plant was considered a BAR-type. When this ratio was <0.1, it was considered a NAS-type.

Oviposition Insects were obtained from the Laboratory of Entomology of Wageningen University the Netherlands. Stock colonies of *P. rapae* and *M. brassicae* were maintained on *Brassica oleracea* var. *gemnifera* L., cultivar Cyrus, in a climatized room at 20–22°C, 50–70% relative humidity, and a photoperiod of 16:8 (L:D). We used ca. 4-month-old half-sib plants of family EL44 that were clipped 1 month before use to ensure abundant fresh leaf material. At the start of the experiment, plants were moved from the glasshouse to a climatized room at 21°C (day) 16°C (night), 60% relative humidity, illuminated to 200 PAR at plant height (Philips Master TLD 50W/840 HF and 60 W lights) and a photoperiod of 16:8 (L:D). In the same room, 1- to 3-day-old adult insects were held in a mesh cage (40×45×65 cm^3) provided with sugar solution. Males and females were held together to mate for at least 24 h. Oviposition preference was assessed by introducing individual pairs into one of the 11 oviposition mesh cages (40×45×65 cm^3) each containing one BAR-type and one NAS-type plant and

a source of sugar. Oviposition was checked every day at 10.30 and 15.30 h. *M. brassicae* oviposition was recorded 24 h after the first egg up to 7 days after introduction (N=68). *P. rapae* oviposition was recorded for at least 5 h after the first egg and up to a maximum of 24 h in total (N=35). Eggs deposited on the cage, pot, or on the label were not included in the analyses. To obtain sufficient replicates, four randomly chosen plants were used twice for *M. brassicae*, but oviposition preference was never tested on the same combination of plants.

Larval Preference For *P. rapae* as well as for *M. brassicae*, we used plants from EL44 and EL13 half sibs (one plant per chemotype per half-sib family and per herbivore). Two 0.9 cm diameter discs of each chemotype were cut from fully expanded leaves and placed in a circle (in an alternated design) in a 12 cm diameter Petri dish. We tested neonate larvae and 5-day-old larvae, which, until use, were feeding on *Brassica oleracea* var. *gemnifera* L., cultivar Cyrus leaves. The larvae were released individually at the center of the Petri dish with equal distance to all leaf discs (N=20 per species per age). After 4 h, the amount of leaf material consumed was recorded visually and categorized as follows: 0 = no damage, 1 = only consumption of the lower leaf layers, 2 = less then 10% was consumed, 3 = between 10% and 50% of the leaf disc was consumed, and 4 = more than 50% was consumed. We were not able to assign consumption categories in the experiment with neonates because they caused so little damage, but 5-day-old larvae showed distinct feeding patterns.

Larval Performance Larval performance was tested by forcing neonate larvae to stay on selected plants and measuring larval biomass and survival every other day. We selected forty 6-week-old plants (20 EL44 half sibs and 20 EL13 half sibs; 10 of each chemotype per half-sib family). To obtain a total of 80 plants, every plant was multiplied by cutting it into two halves and growing each plant in fresh pots. After 3 weeks, the plants were used to test performance of *M. brassicae.* Larvae were kept on the plant using 25 mm diameter meshed clip cages. After 4 days, these clip cages were replaced by 55 mm diameter meshed clip cages. After 8 days, *M. brassicae* larvae had consumed such large amounts of leaf tissue that the experiment had to be stopped. The remaining larvae were removed and weighed. All plants were clipped and after 3 weeks of regrowth used to asses performance of *Pieris* in the same way as we did for *M. brassicae*. However, *P. rapae* larvae were kept on the plants till day 18 when most larvae had pupated. Until day 4, each larva was feeding on a single leaf that we harvested to measure the consumed leaf area and sugar, glucosinolate, and amino acid content. These samples were considered to provide an estimate for the chemotype differences over the first 8 days of feeding.

The long-term performance of *M. brassicae* larvae was studied in a second experiment, in which three neonate larvae were placed on one plant (15 BAR and 15 NAS-type plants of EL 13). The larvae could freely move on the plant, but could not move to other plants due to a plastic cylinder and a water barrier around each plant. After 8 and 13 days, we determined larval mass and the number of dead or lost larvae. After 13 days on BAR-type plants, all but five larvae had died or were lost, so that we stopped the experiment. As dead larvae had often dried out, we excluded their biomass from all calculations.

Statistical Analysis In the oviposition preference experiments, total egg load may differ among individuals. Therefore, the number of eggs on each chemotype per individual female was treated as a paired sample. Per paired sample, we considered the chemotype with the highest egg load as the preferred type. Overall preference was tested with a Sign test. The number of eggs per plant and number of clusters per plant were not normally distributed, and were analyzed with the Wilcoxon matched-pair signed-ranks test. As there were no paired observations for cluster size, these data were analyzed with the Mann–Whitney U test.

To analyze larval preference, we compared the average food consumption category between the two chemotypes by using the Wilcoxon matched-pair signed-ranks test.

In the larval performance experiments, the larval masses were not normally distributed and therefore analyzed for differences between the chemotypes with separate Mann–Whitney U tests for each day followed by Bonferroni correction for multiple comparisons (for *M. brassicae* α=0.05/4=0.0125; for *P. rapae* α=0.05/8=0.0062). Data on the leaf consumption of each herbivore on the two chemotypes were also tested with the Mann–Whitney U test. The relationships between larval biomass and leaf consumption and between larval biomass and total glucosinolate concentration were tested by using Pearson product–moment correlations. The data were log-transformed to get a normal distribution. Before analysis of variance (ANOVA) total glucosinolate, sugar, and amino acid content data were log-transformed to meet the assumptions of ANOVA. Statistical analyses were performed with STATISTICA (data analysis software system), version 7.1. (StatSoft (2005); http://www.statsoft.com).

Results

Oviposition Preference Generalist and specialist adult herbivores did not prefer one chemotype over the other. Of the 68 *M. brassicae* females tested, 33.8% oviposited on the plants, 47.1% preferred to oviposit on the cage, whereas

19.1% did not oviposit at all within 1 week. The chemotypes did not receive significantly different numbers of eggs (Table 1). *M. brassicae*, which is a gregarious species, deposited a total of 19 clusters on BAR-type and 19 on NAS-type plants. The number of clusters per plant and the average cluster size, ranging from 6 to 465 eggs per cluster, were not significantly different between chemotypes (Table 1).

Of the 35 *P. rapae* pairs tested, 32 females oviposited on plants, two females did not oviposit at all, and one female died. With an average of 53.5±5.1 eggs per female, 61% deposited most eggs on NAS-type plants, and one deposited an equal amount of eggs on both chemotypes. The average numbers of eggs deposited on each chemotype did not significantly differ between chemotypes (Table 1). In total, BAR-type plants received 861 eggs and NAS-type plants 850 (Table 1).

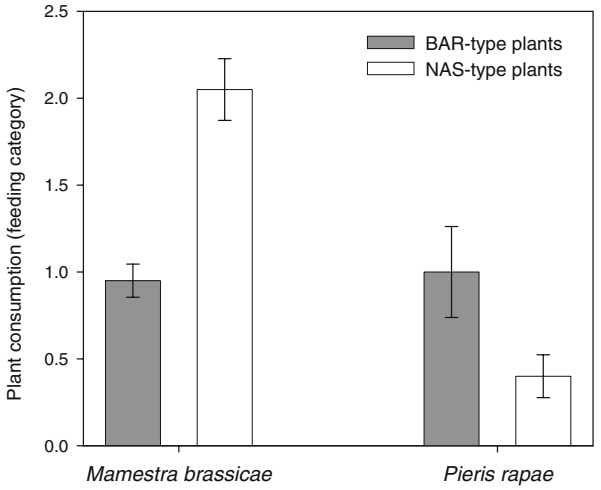

Fig. 1 Consumption of BAR-type (*grey bars*) and NAS-type (*white bars*) leaf discs by 5-day-old larvae of *Mamestra brasscicae* and *Pieris rapae* (feeding category averaged per Petri dish ± SE)

Larval Preference Of the 20 5-day-old *M. Brassicae* larvae tested, only one preferred to feed on BAR-type leaf discs, whereas 19 preferred the NAS-type (Sign test: $Z=3.67$, $P<0.001$). This preference for NAS-type leaf discs was confirmed when corrected for the consumed quantity (feeding categories). *M. brassicae* larvae consumed more from NAS-type leaf discs than from BAR-type (Fig. 1; Wilcoxon matched-pairs test on feeding categories: *M. brassicae* $N=20$, $Z=3.743$, $P<0.001$). In contrast, the 20 5-day-old *P. rapae* larvae did not show a significant preference. Five larvae preferred NAS-type, 11 BAR-type, and four consumed from BAR-type as well as NAS-type leaf discs (Sign test: $Z=1.25$, $P=0.211$). Although *P. rapae* larvae consumed more from BAR-type leaves, the difference in feeding categories was not significant (Fig. 1; Wilcoxon matched-pairs test on feeding categories: *P. rapae N=20,* $Z=1.629$, $P=0.103$). Consequently, we found no significant preference of *P. rapae* larvae for either chemotype.

Larval Performance and Chemotypes Over the first 8 days, the larval biomass accumulation of the generalist *M. brassicae* was affected by chemotype (Mann–Whitney *U* test: $P<0.001$ for each day of recording). When forced to stay on NAS-type plants, *M. brassicae* larvae grew exponentially (Fig. 2a). On BAR-type plants, however, *M. brassicae* larvae hardly increased in biomass and were moribund. This difference in performance was positively correlated to the difference in leaf consumption after 4 days of feeding (correlation analysis: $r=0.942$, $r^2=0.887$, $P<0.01$). Consequently, *M. brassicae* larvae caused more damage to NAS-type leaves than to BAR-type (Fig. 3a; Mann–Whitney *U* test: $U=12$, $Z=-7.583$, $P<0.001$). Larval survival after 8 days on the plants was in line with these results. On NAS-type plants, all larvae were still alive, whereas on BAR-type plants 37.5% of the *M. brassicae* larvae had died. The results of the second experiment, in which the larvae could move freely on the plants, confirmed the results of the clip cage experiment. After 13 days, 89% ±10 of the larvae had stayed and survived on

Table 1 Oviposition of *Mamestra brassicae* and *Pieris rapae* on BAR-type and NAS-type *Barbarea vulgaris*

	Mamestra brassicae				*Pieris rapae*			
	BAR	NAS	*P*	Z	BAR	NAS	*P*	*Z*
Preference[a]	14	11	0.838	0.204	12	19	0.472	0.720
Eggs[b]	220±40.9	193±51.7	0.399	0.843	26.9±18.2	26.6±14.5	0.922	0.100
Clusters/plant[c]	1.36±0.2	1.73±0.3	0.951	0.061				
Cluster size[d]	162±23.4	112±32.9	0.800	0.254				

[a] Times when that chemotype received most eggs in a pairwise comparison
[b] Average number of eggs per plant ± SE
[c] Average number of clusters per plant ± SE
[d] Average number of eggs per cluster ± SE

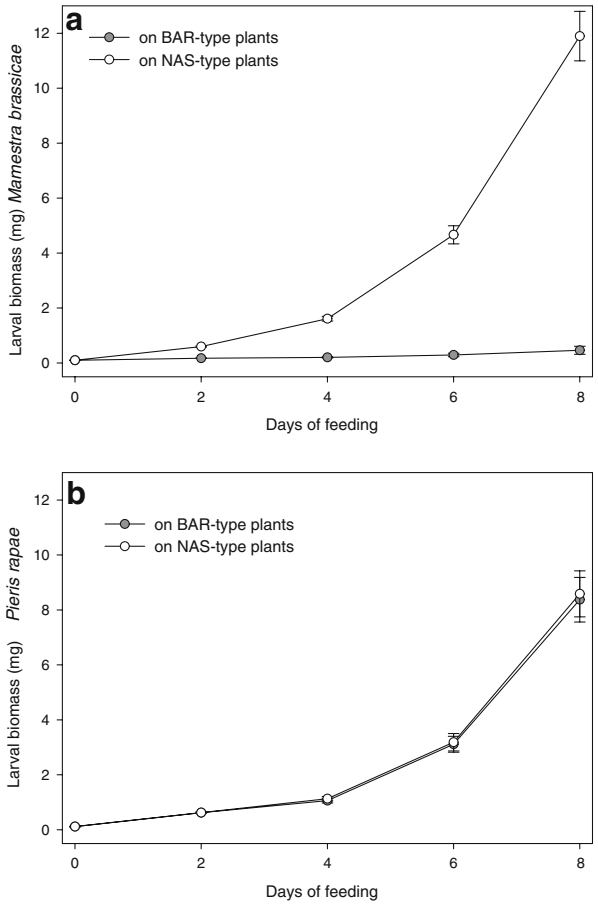

Fig. 2 Biomass accumulation of **a** *Mamestra brassicae* and **b** *Pieris rapae* larvae during 8 days of feeding BAR-type (*grey bars*) and NAS-type (*white bars*) *Barbarea vulgaris* plants ± SE

NAS-type plants and weighed 42.13±2.69 mg, whereas only 20% ±10 of the larvae had stayed and survived on BAR-type plants and weighed only 4.11±0.02 mg.

In contrast to *M. brassicae* larvae, *P. rapae* larvae grew and survived equally well on both chemotypes. *P. rapae* larval biomass accumulation over time showed no significant difference between chemotypes over the first 18 days (Fig. 2b; Mann–Whitney *U* test: $P>0.05$ for each day of recording) and was positively correlated to leaf consumption after 4 days (correlation: $r=0.768$, $r^2=0.589$, $P<0.01$). Leaf damage due to *P. rapae* feeding was not different between the chemotypes (Fig. 3b; Mann–Whitney *U* test: $U=673$, $Z=-1.217$, $P=0.223$). After 8 days, larval mortality was 7.5% on NAS-type and 12.5% on BAR-type plants. On day 14, the first *P. rapae* larvae started pupating. After 16 days on BAR-type plants, 45% had pupated, and on NAS-type plants this was 47.5%. At the end of the experiment, after 18 days, larval weights were still not significantly different on the chemotypes (on BAR-type: 161.9±7.37 mg, on NAS-type: 175.24±5.63 mg; Mann–Whitney *U* test: $U=238$, $Z=-1.131$, $P=0.265$).

Larval Performance and Total Glucosinolate Content The leaves on which larvae were feeding differed in glucosinolate composition (glucobarbarin vs. gluconasturtiin) and in total glucosinolate content (Fig. 3c,d). In both chemotypes, we detected glucobarbarin, gluconasturtiin, glucosibarin, glucobrassicin, 4-methoxyglucobrassicin, and neoglucobrassicin. In both families, BAR-type leaves had on average 1.5 times higher total glucosinolate levels than NAS-type leaves (Fig. 3c; ANOVA type effect: $F_{1,143}=22.831$, $P<0.001$). Because *M. brassicae* fed more on NAS plants, overall a negative—but weak—correlation of total glucosinolate content with the consumed leaf area was observed (correlation: $r(X,Y)=-0.26$, $r^2=0.065$, $P<0.05$). To analyze in more detail whether total glucosinolate level determines larval consumption and performance, we also examined the correlation *within* each chemotype. Within chemotypes, the range of glucosinolate concentrations was substantial (factor 6–7), but there was no negative correlation between glucosinolate level and larval biomass (Fig. 4; Correlation: within BAR, $r(X,Y)=0.15$, $r^2=0.024$, $P=0.932$; within NAS, $r(X,Y)=0.28$, $r^2=0.078$, $P=0.090$). Moreover, larvae on BAR-type leaves with similar low levels of glucosinolates as NAS-types consumed and performed considerably worse (Fig. 4; glucosinolate levels <35 μmol g^{-1} dry mass). There was also no difference in total glucosinolate level from BAR-type leaves on which the larvae survived or died (with living larvae: 31.32±2.95 μmol g^{-1}, with dead larvae: 32.81±6.49 μmol g^{-1}; ANOVA: $F_{1,29}=0.059$, $P>0.05$; Fig. 4). As the performance of *P. rapae* larvae was similar among chemotypes and families, there was no correlation of larval biomass with total glucosinolate level.

Larval Performance and Nutritional Value Total sugar content of both chemotypes was composed of five consistently detected sugars, which were glucose, sucrose, sorbitol, fructose, and trehalose (from high to low average concentration). Total amino acid content of both chemotypes was composed of 11 consistently detected amino acids, which were threonine, isoleucine, arginine, serine, glutamate, aspartate, glutamine, asparagine, phenylalanine, tyrosine, and histidine (from high to low average concentration). Because the individual sugars and amino acids showed effects similar to the total levels (results not shown), we only present data on total levels.

In contrast to the total glucosinolate level, sugar and amino acid content did not differ between the chemotypes (Fig. 3e–h). Although in the *M. brassicae* experiment the total sugar content was overall higher in BAR-type plants, there was a significant interaction between chemotype and family (Fig. 3e; ANOVA type effect: $F_{1,\ 75}=12.014$, $P<0.001$; type × family $F_{1,\ 75}=10.252$, $P<0.05$). Similar interactions between chemotype and family were shown for amino acid content of the plants in the *M. brassicae*

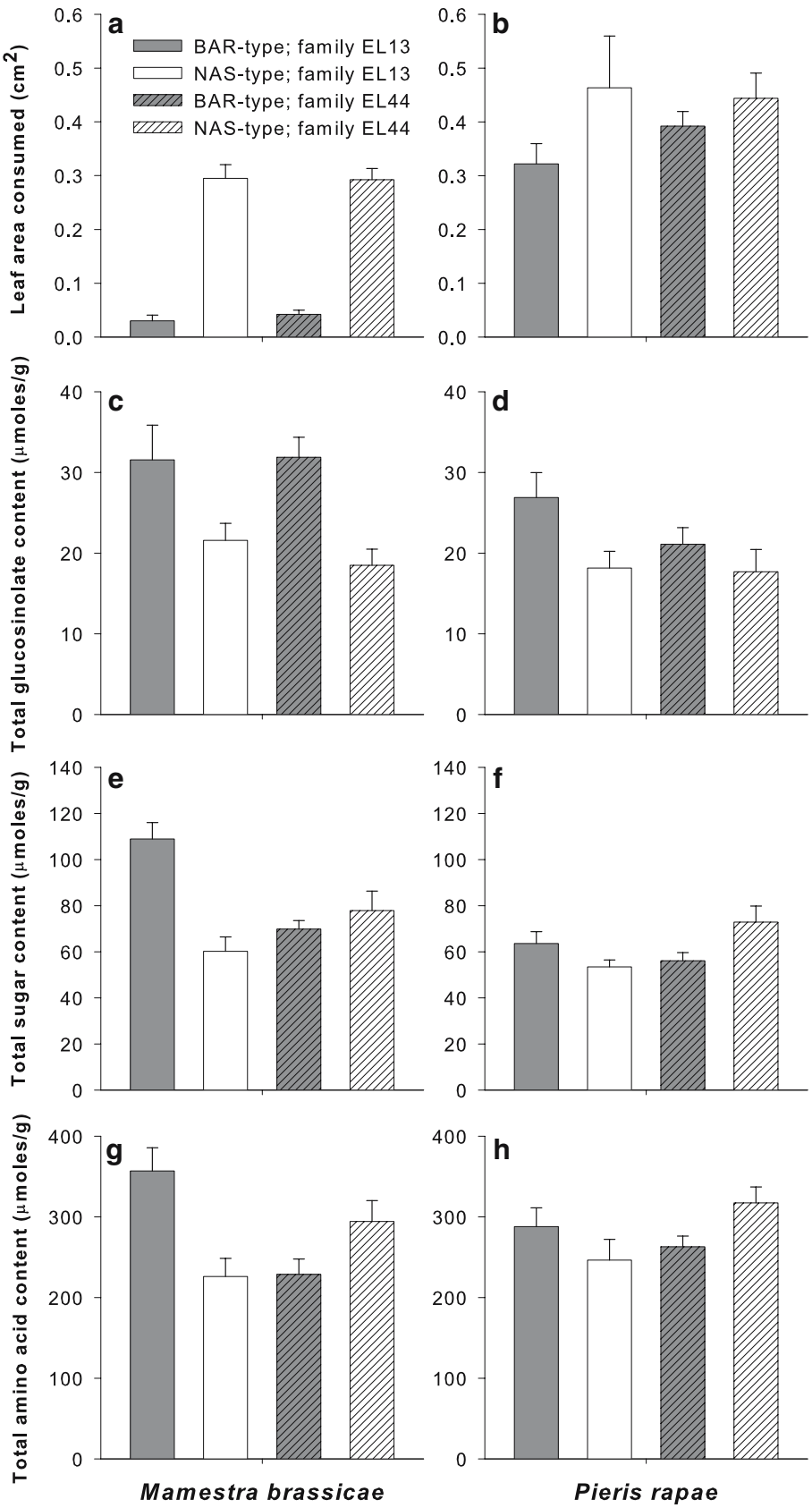

Fig. 3 Leaf characteristics after 4 days of larval feeding of *Mamestra brassicae* (*left graphs*) or *Pieris rapae* (*right graphs*) on *Barbarea vulgaris* ± SEM: **a** and **b** consumed leaf area (cm^2), **c** and **d** total glucosinolate content, **e** and **f** total sugar content, **g** and **h** total amino acid content. Results are depicted per chemotype (*grey bars* BAR-type plants; *white bars* NAS-type plants) and per half-sib family (*no hatching* EL13, *hatching* EL44)

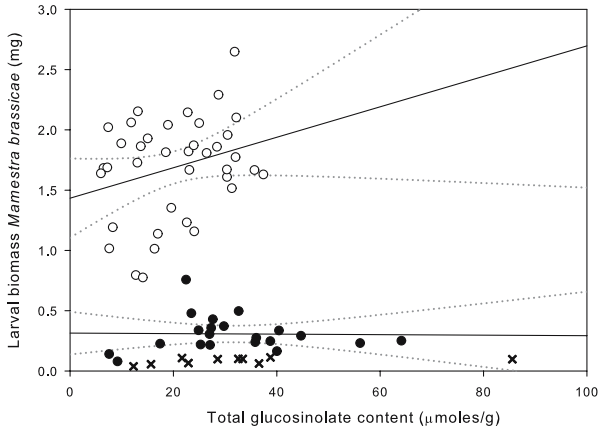

Fig. 4 Correlation of total glucosinolate content and the larval biomass after 4 days feeding of *Mamestra brassicae* experiment on NAS-type (*white dots*), BAR-type (*black dots*), and dead larvae of on BAR-type (*black crosses*) *Barbarea vulgaris* plants with their correlations per chemotype (*black line*) and their 95% confidence intervals (*dotted lines*)

experiment and for sugars and amino acid content of plants in the *P. rapae* experiment. In all chemotype × family interactions, the BAR-type of the EL13 half-sib family had higher levels of primary metabolites than the NAS-type of that family (Fig. 3e–h). In the EL44 half-sib family, the sugar and amino acid level differences of the chemotypes were negligible in plants during the *M. brassicae* experiment or even opposite to those of the EL13 during the *P. rapae* experiment. As sugar and amino acid content did not vary consistently with chemotype, these cannot explain the differences in larval performance and preference between the chemotypes.

Discussion

NAS and BAR chemotypes of *B. vulgaris* differentially affected preference and performance of the generalist herbivore *M. brassicae* but not the specialist *P. rapae*. Larvae of *M. brassicae* grew exponentially and had 100% survival on NAS-type leaves, but hardly grew and had a high mortality when feeding on BAR-type plants. As the dose-dependent effect of total glucosinolate content on herbivores is well-known (Li et al. 2000; Agrawal and Kurashige 2003; Mewis et al. 2006), the poor performance of *M. brassicae* could have been caused by the on average 1.5 times higher total glucosinolate content of BAR-type plants compared to NAS-type plants (Van Leur et al. 2006). However, we did not observe any dose-dependent effect *within* the chemotypes. Moreover, *M. brassicae* larvae on BAR-type plants with total glucosinolate levels similar to NAS-type plants still performed significantly worse. Hence, we exclude total glucosinolate level as explanatory factor for the differences found between chemotypes on larval biomass and leaf consumption.

In addition to total glucosinolate content, sugar and amino acid contents differed between chemotypes, but the pattern was inconsistent among half-sib families. The statistical interaction between chemotype and family found for sugar and amino acid levels was not found for herbivore performance and preference (Fig. 3). Therefore, total sugar content and total amino acid content can not explain the observed effects on *M. brassicae*.

In a different *B. vulgaris* polymorphism, described by Agerbirk et al. (2003b), there were P- and G-type plants that differed in resistance to *Plutella xylostella*. Other than our BAR/NAS polymorphism, the P-type and G-types also differed in trichome density, did not co-occur in natural populations, were hard to cross, and neither of the Danish types had a high content of gluconasturtiin in the leaves (Agerbirk, personal communication).The resistance of the G-type to *P. xylostella* was due to a difference in saponin content (Agerbirk et al. 2003a). Based on these findings, we compared saponin levels between BAR and NAS-type plants by using LC-TOF-MS (see supplemental data). This analysis revealed that the levels of the saponin described by Agerbirk et al. (2003a) and a saponin described by Shinoda et al. (2002) did not differ between the NAS and BAR chemotypes (supplemental data). Therefore, we exclude these saponins as an explanatory factor for the poor performance of *M. brassicae* on BAR-type plants.

The observed effects are likely caused by difference in glucosinolate structures and their break-down products. BAR-type plants were more toxic and deterrent to *M. brassicae* than the NAS-type plants. This suggests that *M. brassicae* can deal effectively with gluconasturtiin and isothiocyanates, but not with glucobarbarin and resulting oxazolidine-2-thiones. The latter compounds have received hardly any attention in chemical–ecological literature. Generalist herbivores possess broad-spectrum detoxification enzymes such as P450 enzymes and mixed-function oxidases (MFO) that enable them to deal with a wide range of allelochemicals, including glucosinolates (Schoonhoven et al. 1998; Li et al. 2000). Which detoxification mechanism is present in *M. brassicae* and why it can handle the presumably more toxic isothiocyanates, but not the oxazolidine thiones is unknown. The non-different performance of *P. rapae* on the chemotypes may indicate that NSP enzymes are equally effective in redirecting the hydrolysis pathways of both chemotypes towards the generally less toxic nitriles (Wittstock et al. 2004). Identification of the bioactive compounds could be acquired by using bioassay-guided fractionation.

Neither herbivore species significantly preferred one of the chemotypes as a host plant for oviposition. Even though isothiocyanates are known to elicit anemotaxis in herbi-

vores at extremely low concentrations (Finch and Skinner 1982), the concentrations of volatile cues emitted by our undamaged plants may have been below the detection limit (Finch et al. 1978). Upon damage or induction by herbivores, volatile levels may rise and affect oviposition preference (Rothschild and Schoonhoven 1977; Bruinsma et al. 2007). Next to chemical suitability, the surface on which to oviposit can be an important factor (Renwick and Chew 1994). In our experiment, however, leaf surface structure was unlikely to affect oviposition preference between chemotypes because the chemotypes did not have apparent differences in leaf surface (e.g., trichomes) in contrast to the completely different *B. vulgaris* polymorphism reported by Agerbirk et al. (2003b).

As *M. brassicae* larvae performed poorly on BAR-type plants, the lack of oviposition preference of adults seems to be non-adaptive. The discrepancy between larval performance and oviposition preference on *B. vulgaris* has also been shown for the Diamondback moth (*Plutella xylostella*) (Serizawa et al. 2001; Badenes-Perez et al. 2006). It may occur when insects or plants are new to an area and there has not been enough time for evolutionary adaptation (Agosta 2006). Whether this is the case for *M. brassicae* and the *B. vulgaris* chemotypes is unknown.

Co-evolutionary theory suggests that the variation of plant defense compounds is maintained by sequential adaptations of specialist herbivores and plants (Agrawal et al. 1999; Cornell and Hawkins 2003). The good performance of *P. rapae* on both chemotypes suggests that neither chemotype is effectively defended against this specialist. On the contrary, BAR-type plants are defended against the generalist *M. brassicae.* Although we have no experimental evidence indicating which chemotype is the newest form, the effective defense of BAR-type plants against *M. brassicae* suggests an evolutionary adaptive step in which BAR-type evolved from NAS-type. This matches with the biosynthetic origin of these glucosinolates. Gluconasturtiin is presumably the precursor that is hydroxylated to produce glucobarbarin. Moreover, gluconasturtiin occurs in five times more genera than glucobarbarin (Fahey et al. 2001). This does not preclude, however, that the NAS-types found locally in the Netherlands are due to a loss-of-function mutation from the BAR-type. In natural populations of *B. vulgaris*, there are only 0–22% NAS-type plants (Van Leur et al. 2006). This results in a potentially limited genetic basis for NAS-type plants compared to BAR-type plants, which could contribute to linkage disequilibrium between the BAR/NAS locus and other loci (for instance closely linked loci). However, the natural population from which we selected seed batches for these experiments had a phenotype frequency close to a Hardy–Weinberg equilibrium (22% potential NAS-type fathers observed instead of 25% expected). We estimate the chance of linkage disequilibrium effects in our experiments to be relatively small. Additionally, there are no *a priori* indications of closely linked loci that affect herbivore performance.

For the plant, the benefit of increasing its defense against *M. brassicae* will be larger than increasing its defense against *P. rapae*. Being gregarious and larger, *M. brassicae* larvae are more harmful to the chosen individual (many larvae feeding from one plant) and will more strongly reduce plant fitness than the solitary *P. rapae* (only one or few larvae feeding on a plant). Therefore, irrespective of the degree of specialization of the herbivores, it will be more important for *B. vulgaris* to be defended against *M. brassicae* than against *P. rapae.*

Based on our results, we would expect BAR-type plants in the field to suffer less from herbivory than NAS-type plants. Besides being the chemotype that is most severely damaged by *M. brassicae* larvae, the NAS chemotype is also the recessive genotype. Therefore, if herbivorous insects are the only selective force, we expect that natural selection will drive natural populations towards 100% BAR-types. European populations of *B. vulgaris* indeed are mainly dominated by BAR-type plants, but a minority number of populations has still up to 22% NAS-type plants. This indicates that in these populations there may be other factors that play a role in maintaining this chemical polymorphism. Other factors that may differ between the two types and that determine plant fitness, e.g., belowground herbivory, higher trophic level interactions, and inter- and intraspecific competition, need to be included in future studies.

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References

AGERBIRK, N., OLSEN, C. E., BIBBY, B. M., FRANDSEN, H. O., BROWN, L. D., NIELSEN, J. K., and ENWICK, J. A. A. 2003a. A saponin correlated with variable resistance of *Barbarea vulgaris* to the diamondback moth *Plutella xylostella*. *J. Chem. Ecol.* 29:1417–1433.

AGERBIRK, N., ORGAARD, M., and NIELSEN, J. K. 2003b. Glucosinolates, flea beetle resistance, and leaf pubescence as taxonomic

characters in the genus *Barbarea* (Brassicaceae). *Phytochemistry* 63:69–80.

AGERBIRK, N., PETERSEN, B. L., OLSEN, C. E., HALKIER, B. A., and NIELSEN, J. K. 2001. 1,4-Dimethoxyglucobrassicin in *Barbarea* and 4-hydroxyglucobrassicin in *Arabidopsis* and *Brassica*. *J. Agric. Food Chem.* 49:1502–1507.

AGOSTA, S. J. 2006. On ecological fitting, plant-insect associations, herbivore host shifts, and host plant selection. *Oikos* 114:556–565.

AGRAWAL, A. A., GORSKI, P. M., and TALLAMY, D. W. 1999. Polymorphism in plant defense against herbivory: Constitutive and induced resistance in *Cucumis sativus*. *J. Chem. Ecol.* 25:2285–2304.

AGRAWAL, A. A., and KURASHIGE, N. S. 2003. A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *J. Chem. Ecol.* 29:1403–1415.

AKHTAR, Y., and ISMAN, M. B. 2003. Larval exposure to oviposition deterrents alters subsequent oviposition behavior in generalist, *Trichoplusia ni* and specialist, *Plutella xylostella* moths. *J. Chem. Ecol.* 29:1853–1870.

BADENES-PEREZ, F. R., NAULT, B. A., and SHELTON, A. M. 2006. Dynamics of Diamondback moth oviposition in the presence of a highly preferred non-suitable host. *Entomol. Exp. Appl.* 120:23–31.

BERENBAUM, M. R. 1995. Turnabout is fair play—Secondary roles for primary compounds. *J. Chem. Ecol.* 21:925–940.

BOREK, V., ELBERSON, L. R., MCCAFFREY, J. P., and MORRA, M. J. 1998. Toxicity of isothiocyanates produced by glucosinolates in Brassicaceae species to black vine weevil eggs. *J. Agric. Food Chem.* 90:109–112.

BRUINSMA, M., VAN, DAM, N. M., VAN, LOON, J. J. A., and DICKE, M. 2007. Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores. *J. Chem. Ecol.* 33:655–668.

BUCHNER, R. 1987. Approach to determination of HPLC response factors for glucosinolates, pp. 50–58, in J. P. Wathelet (ed.). Glucosinolates in RapeseedMartinus Nijhoff Publishers, Dordrecht, The Netherlands.

BUROW, M., MARKERT, J., GERSHENZON, J., and WITTSTOCK, U. 2006. Comparative biochemical characterization of nitrile-forming proteins from plants and insects that alter myrosinase-catalysed hydrolysis of glucosinolates. *FEBS J.* 273:2432–2446.

CANISTRO, D., DELLA, CROCE, C., IORI, R., BARILLARI, J., BRONZETTI, G., POI, G., CINI, M., CALTAVUTURO, L., PEROCCO, P., and PAOLINI, M. 2004. Genetic and metabolic effects of gluconasturtiin, a glucosinolate derived from cruciferae. *Mutat. Res.* 545:23–35.

CHEW, F. S. 1988. Biological effects of glucosinolates, pp. 155–181, in H. G. Cutler (ed.). Biologically Active Natural ProductsACS, Washington DC.

CORNELL, H. V., and HAWKINS, B. A. 2003. Herbivore responses to plant secondary compounds: A test of phytochemical coevolution theory. *Am. Nat.* 161:507–522.

EC. 1990. Oil seeds—Determination of glucosinolates High Performance Liquid Chromatography. Official Journal of the European Communities L 170/28. Annex VIII:03.07.27–34.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

FINCH, S., and KIENEGGER, M. 1997. A behavioural study to help clarify how undersowing with clover affects host-plant selection by pest insects of *brassica* crops. *Entomol. Exp. Appl.* V84:165–172.

FINCH, S., and SKINNER, G. 1982. Trapping cabbage root flies in traps baited with plant extracts and with natural and synthetic isohiocyanates. *Entomol. Exp. Appl.* 31:133–139.

FINCH, S., SKINNER, G., and FREEMAN, G. M. 1978. Distribution and analysis of cabbage root fly pupal populations. *Ann. Appl. Biol.* 88:351–356.

HANKO, V. P., and OHRER, J. S. 2004. Determination of amino acids in cell culture and fermentation broth media using anion-exchange chromatography with integrated pulsed amperometric detection. *Anal. Biochem.* 324:29–38.

HUANG, X., and ENWICK, J. A. A. 1994. Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi* oleracea. *J. Chem. Ecol.* 20:1025–1037.

HUANG, X. P., ENWICK, J. A. A., and SACHDEVGUPTA, K. 1994a. Oviposition stimulants in Barbarea–Vulgaris for Pieris–Rapae and P–Napi–Oleracea—Isolation, identification and differential activity. *J. Chem. Ecol.* 20:423–438.

HUANG, X. P., ENWICK, J. A. A., and SACHDEVGUPTA, K. 1994b. Oviposition Stimulants in *Barbarea vulgaris* for *Pieris rapae* and *P. napi* Oleracea—Isolation, identification and differential activity. *J. Chem. Ecol.* 20:423–438.

KERFOOT, W. C., NEWMAN, R. M., and HANSCOM, Z. 1998. Snail reaction to watercress leaf tissues: reinterpretation of a mutualistic 'alarm' hypothesis. *Freshw. Biol.* 40:201–213.

KJAER, A., and GMELIN, R. 1957. Isothiocyanates 28. A new isothiocyanate glucoside (glucobarbarin) furnishing (-)-5-phenyl-2-oxazolidinethione upon enzymic hydrolysis. *Acta Chem. Scand.* 11:906–907.

KROYMANN, J., TEXTOR, S., TOKUHISA, J. G., FALK, K. L., BARTRAM, S., GERSHENZON, J., and MITCHELL-OLDS, T. 2001. A gene controlling variation in *Arabidopsis* glucosinolate composition is part of the methionine chain elongation pathway. *Plant Physiol. (Rockv)* 127:1077–1088.

LAMBRIX, V., EICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D. J., and GERSHENZON, J. 2001. The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13: 2793–2807.

LAZZERI, L., CURTO, G., LEONI, O., and DALLAVALLE, E. 2004. Effects of glucosinolates and their enzymatic hydrolysis products via myrosinase on the root-knot nematode *Meloidogyne incognita* (Kofoid et White) Chitw. *J. Agric. Food Chem.* 52:6703–6707.

LI, Q., EIGENBRODE, S. D., STRINGAM, G. R., and THIAGARAJAH, M. R. 2000. Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J. Chem. Ecol.* 26:2401–2419.

LICHTENSTEIN, E. P., STRONG, F. M., and MORGAN, D. G. 1962. Identification of 2-phenylethylisothiocyanate as an insecticide occurring naturally in the edible parts of turnips. *J. Agric. Food Chem.* 10:30–33.

LOUDA, S., and MOLE, S. 1991. Glucosinolates: chemistry and ecology, pp. 123–164, in G. A. Rosenthal, and M. R. Berenbaum (eds.). Herbivores. Their interactions with secondary plant metabolites. Vol. 1Academic, San Diego, CA.

LUDWIG-MÜLLER, J., BENNETT, R. N., KIDDLE, G., IHMIG, S., UPPEL, M., and HILGENBERG, W. 1999. The host range of *Plasmodiophora brassicae* and its relationship to endogenous glucosinolate content. *New. Phytol.* 141:443–458.

MEWIS, I., TOKUHISA, J. G., SCHULTZ, J. C., APPEL, H. M., ULRICHS, C., and GERSHENZON, J. 2006. Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67:2450–2462.

POTTER, M. J., VANSTONE, V. A., DAVIES, K. A., KIRKEGAARD, J. A., and ATHJEN, A. J. 1999. Reduced susceptibility of *Brassica napus* to *Pratylenchus neglectus* in plants with elevated root levels of 2-phenylethyl glucosinolate. *J. Nematol.* 31:291–298.

POTTER, M. J., VANSTONE, V. A., DAVIES, K. A., and ATHJEN, A. J. 2000. Breeding to increase the concentration of 2-phenylethyl glucosinolate in the roots of *Brassica napus*. *J. Chem. Ecol.* 26:1811–1820.

RATZKA, A., VOGEL, H., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and KROYMANN, J. 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U S A* 99:11223–11228.

REICHELT, M., BROWN, P. D., SCHNEIDER, B., OLDHAM, N. J., STAUBER, E., TOKUHISA, J., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and GERSHENZON, J. 2002. Benzoic acid glucosinolate esters and other glucosinolates from *Arabidopsis thaliana*. *Phytochemistry* 59:663–671.

RENWICK, J. A. A., and CHEW, F. S. 1994. Oviposition behavior in lepidoptera. *Annu. Rev. Entomol.* 39:377–400.

RENWICK, J. A. A., ADKE, C. D., SACHDEV-GUPTA, K., and STÄDLER, E. 1992. Leaf surface chemicals stimulating oviposition by *Pieris rapae* (Lepidoptera: Pieridae) on cabbage. *Chemoecology* 3:33–38.

ROJAS, J. C. 1999. Influence of host plant damage on the host-finding behavior of *Mamestra brassicae* (Lepidoptera: Noctuidae). *Environ. Entomol.* 28:588–593.

ROJAS, J. C., WYATT, T. D., and BIRCH, M. C. 2000. Flight and oviposition behavior toward different host plant species by the cabbage moth, *Mamestra brassicae* (L.) (Lepidoptera: Noctuidae). *J. Insect Behav.* 13:247–254.

ROTHSCHILD, M., and SCHOONHOVEN, L. M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266:352–355.

SANG, J. P., MINCHINTON, I. R., JOHNSTONE, P. K., and TRUSCOTT, R. J. W. 1984. Glucosinolate profiles in the seed, root and leaf tissue of cabbage, mustard, rape seed, radish and swede. *Can. J. Plant. Sci.* 64:77–93.

SARWAR, M., and KIRKEGAARD, J. A. 1998. Biofumigation potential of brassicas—II. Effect of environment and ontogeny on glucosinolate production and implications for screening. *Plant Soil* 201:91–101.

SCHOONHOVEN, L. M., JERMY, T., and VAN, LOON, J. J. A. 1998. Insect-Plant Biology. From physiology to evolution. Chapman & Hall, London.

SERIZAWA, H., SHINODA, T., and KAWAI, A. 2001. Occurrence of a feeding deterrent in *Barbarea vulgaris* (Brassicales: Brassicaceae), a crucifer unacceptable to the diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *Appl. Entomol. Zool.* 36:465–470.

SERRA, B., OSA, E., IORI, R., BARILLARI, J., CARDOSO, A., ABREU, C., and OLLIN, P. 2002. In vitro activity of 2-phenylethyl glucosinolate, and its hydrolysis derivatives on the root-knot nematode *Globodera rostochiensis* (Woll.). *Sci. Hortic. (Amst)* 92:75–81.

SHINODA, T., NAGAO, T., NAKAYAMA, M., SERIZAWA, H., KOSHIOKA, M., OKABE, H., and KAWAI, A. 2002. Identification of a triterpenoid saponin from a crucifer, *Barbarea vulgaris*, as a feeding deterrent to the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* 28:587–599.

SIMPSON, S. J., and SIMPSON, C. L. 1990. The Mechanisms of Nutritional Compensation by Phytophagous Insects, pp. 111–160, in E. A. Bernays (ed.). Insect–Plant Interactions, vol. 2.CRC Press, Boca Raton, FL.

THEUNISSEN, J., OUDEN, H., and WIT, A. K. H. 1985. Feeding capacity of caterpillars on cabbage, a factor in crop loss assessment. *Entomol. Exp. Appl.* V39:255–260.

VAN, DAM, N. M., HADWICH, K., and BALDWIN, I. T. 2000. Induced responses in *Nicotiana attenuata* affect behavior and growth of the specialist herbivore *Manduca sexta*. *Oecologia* 122:371–379.

VAN, DAM, N. M., WITJES, L., and SVATOS, A. 2004. Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytol.* 161:801–810.

VAN, LEUR, H., AAIJMAKERS, C. E., and VAN, DAM, N. M. 2006. A heritable glucosinolate polymorphism within natural populations of *Barbarea vulgaris*. *Phytochemistry* 67:1214–1223.

VAN, LOON, J. J. A., BLAAKMEER, A., GRIEPINK, F. C., VAN, BEEK, T. A., SCHOONHOVEN, L. M., and DE, GROOT, A. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3:39–44.

WADLEIGH, R. W., and YU, S. J. 1988. Detoxification of isothiocyanate allelochemicals by glutathione transferase in three lepidopterous species. *J. Chem. Ecol.* 14:1279–1288.

WIECZOREK, H. 1976. The glycoside receptor of the larvae of *Mamestra brassicae* L. (Lepidoptera, Noctuidae). *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 106:153–176.

WITTSTOCK, U., AGERBIRK, N., STAUBER, E. J., OLSEN, C. E., HIPPLER, M., MITCHELL-OLDS, T., GERSHENSON, J., and Vogel, H. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U S A* 101: 4859–4864.

WITTSTOCK, U., KLIEBENSTEIN, D. J., LAMBRIX, V., EICHELT, M., and GERSHENZON, J. 2003. Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores, pp. 101–125, in J. T. Romeo (ed.). Recent Advances in Phytochemistry: Integrative Phytochemistry: From Ethnobotany to Molecular Ecology, vol. 37Pergamon Press, Oxford.

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Performance of Generalist and Specialist Herbivores and their Endoparasitoids Differs on Cultivated and Wild *Brassica* Populations

Rieta Gols · Tibor Bukovinszky · Nicole M. van Dam · Marcel Dicke · James M. Bullock · Jeffrey A. Harvey

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Abstract Through artificial selection, domesticated plants often contain modified levels of primary and secondary metabolites compared to their wild progenitors. It is hypothesized that the changed chemistry of cultivated plants will affect the performance of insects associated with these plants. In this paper, the development of several specialist and generalist herbivores and their endoparasitoids were compared when reared on a wild and cultivated population of cabbage, *Brassica oleracea*, and a recently established feral *Brassica* species. Irrespective of insect species or the degree of dietary specialization, herbivores and parasitoids developed most poorly on the wild population. For the specialists, plant population influenced only development time and adult body mass, whereas for the generalists, plant populations also affected egg-to-adult survival. Two parasitoid species, a generalist (*Diadegma fenestrale*) and a specialist (*D. semiclausum*), were reared from the same host (*Plutella xylostella*). Performance of *D. semiclausum* was closely linked to that of its host, whereas the correlation between survival of *D. fenestrale* and host performance was less clear. Plants in the Brassicaceae characteristically produce defense-related glucosinolates (GS). Levels of GS in leaves of undamaged plants were significantly higher in plants from the wild population than from the domesticated populations. Moreover, total GS concentrations increased significantly in wild plants after herbivory, but not in domesticated or feral plants. The results of this study reveal that a cabbage cultivar and plants from a wild cabbage population exhibit significant differences in quality in terms of their effects on the growth and development of insect herbivores and their natural enemies. Although cultivated plants have proved to be model systems in agroecology, we argue that some caution should be applied to evolutionary explanations derived from studies on domesticated plants, unless some knowledge exists on the history of the system under investigation.

Keywords Crucifer · *Diadegma fenestrale* · *Diadegma semiclausum* · Direct defense · Glucosinolates · Induction · *Mamestra brassicae* · Pieris rapae · *Plutella xylostella*

R. Gols (✉) · T. Bukovinszky · M. Dicke
Laboratory of Entomology, Plant Science Group,
Wageningen University,
Wageningen, The Netherlands
e-mail: rieta.gols@wur.nl

N. M. van Dam · J. A. Harvey
Department of Multitrophic Interactions,
Centre for Terrestrial Ecology, Netherlands Institute of Ecology,
Heteren, The Netherlands

J. M. Bullock
Centre for Ecology and Hydrology,
Wallingford, Oxfordshire, UK

Introduction

The occurrence of pests in agroecosystems has long promoted the study of insect–plant interactions in crop plants, such as cabbage, lima bean, maize, cotton, and tomato (Takabayashi et al. 1994; Turlings et al. 1995; De Moraes et al. 1998; Geervliet et al. 2000; Thaler et al. 2001). Studies with crop plants have generated a wealth of data, highlighting a number of important mechanisms that influence the structure and function of multitrophic interactions and communities (Root 1973; Sheehan 1986; Khan et al. 1997; Gols et al. 2005). However, critics have argued that the biology and ecology of crop plants is often dramatically different from wild populations, thus bringing

into some question the evolutionary relevance of the conclusions generated from data that rely on crop plants (Benrey et al. 1998; van der Meijden and Klinkhamer 2000). For instance, plant breeding programs have been reported to disrupt the original plant defense strategies that were present in the wild progenitors of cultivated species (Evans 1993). Artificial selection of some crop plants, aimed at accentuating a specific plant trait or group of traits (e.g., the production of edible structures), has been shown to reduce the level of undesired constituents, such as defense compounds, while enhancing others (such as primary metabolites including proteins and sugars). Many of the undesired secondary plant compounds (SPC) are known to have evolved and function as putative defenses against herbivores, whereas the desired primary plant compounds act as nutrients and thus may actually enhance the performance of herbivores (Schoonhoven et al. 2005).

Levels of SPC are dynamic and vary with such factors as season, soil conditions, and leaf age (reviewed by Schoonhoven et al. 2005). Moreover, plants may increase their levels of defenses in response to feeding damage (Karban and Baldwin 1997; Agrawal 1999a), which may reduce the costs of defenses by avoiding the allocation of resources to defense when the attacker is absent. Secondary plant compounds have also been shown to affect negatively the development of higher trophic levels that attack these herbivores, such as predators, parasitoids, and even hyperparasitoids (Barbosa et al. 1986, 1991; Francis et al. 2001; Harvey et al. 2003, 2005, 2007; Ode et al. 2004). Consequently, changes in plant chemistry, mediated by artificial selection, may influence the behavior and development of consumers over several trophic levels, and this may ultimately lead to broader effects on the communities associated with these plants (Harvey et al. 2003; Ode 2006). Most importantly, in wild plants, defense mechanisms have not been constrained by the "directional selection" that characterizes many crop plants. Therefore, to understand the evolution of plant defenses against insect herbivores, multitrophic interactions should also be studied in wild conspecifics of the cultivated plant species where plant defenses are the likely result of a range of biotic and abiotic selection pressures.

One appropriate plant family for studying the effects of artificial *versus* natural selection on multitrophic interactions is the Brassicaceae, which contains such important crops as cabbages and various types of mustard (Gómez-Campo and Prakash 1999). Of all plants that have been domesticated, few have been manipulated to produce so many different cultivars as *Brassica oleracea* L. (e.g., cabbage, broccoli, cauliflower, and Brussels sprout; Gómez-Campo and Prakash 1999). Wild types of *B. oleracea* grow naturally along rocky coastlines of Britain and France (Mitchell and Richards 1979). It has been speculated that the wild populations in the UK are derived from plants that were cultivated by the Greeks and Romans in the Mediterranean region between 1,000 and 2,000 BC (Mitchell 1976). These early cultivated forms were introduced to Britain, but have been naturalized for centuries (Mitchell 1976). However, more recent evidence points also at an Atlantic origin of domestication (Song et al. 1990).

Plants in the Brassicaceae characteristically produce secondary metabolites called glucosinolates (hereafter GS) (Fahey et al. 2002). After tissue damage, myrosinases catalyze the hydrolysis of GS into (iso)thiocyanates and nitriles (Mithen 2001; Fahey et al. 2002), which play a role in defense against insect herbivores (Rask et al. 2000). Generalist herbivores produce enzymes that can detoxify a wide range of substrates (Krieger et al. 1971), whereas specialists have evolved enzyme systems that can detoxify specific plant compounds that are associated with herbivore diet (e.g., Johnson 1999; Ratzka et al. 2002). Thus, generalist herbivores are usually more sensitive to high levels of specific allelochemicals compared to specialists. (see e.g., Blau et al. 1978; Giamoustaris and Mithen 1995).

Specialist feeding on brassicaceous plants are adapted to plants containing GS, and they detoxify, excrete, or even sequester these harmful metabolites (Müller et al. 2001; Ratzka et al. 2002; Wittstock et al. 2004). Moreover, some insects use these compounds as indicators of food plant suitability (Nayar and Thorsteinson 1963; Renwick and Lopez 1999). Not all GS are equally effective as stimulants, and high levels of GS can reduce the performance of herbivores that are specialized on brassicaceous species (Stowe 1998; Li et al. 2000; Traw and Dawson 2002; Agrawal and Kurashige 2003). In addition, GS concentrations can increase in response to herbivore feeding damage (Bodnaryk 1992; Agrawal 1999b) and negatively affect subsequent herbivory by both generalists and specialists (Agrawal 1999b; Bartlet et al. 1999; Traw and Dawson 2002).

This study compares the development of several species of herbivores and endoparasitoids when reared on three *Brassica* populations that differ in their degree of domestication. Insects were reared on a cultivated and a wild population of *B. oleracea*, and a recently escaped (feral) *Brassica* species. Levels of GS were measured as indicators of direct plant defense. Initially, the development of two specialists on Brassicaceae, *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Pieris rapae* L. (Lepidoptera: Pieridae), and a generalist herbivore, *Mamestra brassicae* L. (Lepidoptera: Noctuidae), were examined when reared on the three populations. Finally, the development of a specialist and generalist parasitoid reared on the same host, *P. xylostella, were compared. Separate cohorts of P. xylostella were parasitized by two species of endoparasitoids, Diadegma semiclausum Hellén (Hymenoptera: Ich-*

neumonidae) and a related species, D. fenestrale Holmgren (Hymenoptera: Ichneumonidae). These two parasitoids differ in host specialization, with D. semiclausum restricted to P. xylostella and D. fenestrale attacking several other hosts that feed on non-brassicaceous species (Legaspi 1986; Azidah et al. 2000).

The following hypothesis was tested: specialist herbivores and parasitoids are less affected than generalists by differences in host plant chemistry between various *Brassica* populations that differ in their degree of domestication. It is proposed that changes in plant biology via domestication have significant effects on community level interactions and processes.

Methods and Materials

Plants The *B. oleracea* variety gemmifera (Brussels sprout) cv. Cyrus was used. Compared to other vegetable crops of *B. oleracea*, Brussels sprout cultivars contain relatively high levels of GS (Kushad et al. 1999; Rosa 1999), but considerably lower levels than the wild *B. oleracea* populations in Dorset, Great Britain (Moyes et al. 2000; Gols et al. 2008). Seeds from several plants (>10) were collected from a wild population of *B. oleracea* growing on chalk cliffs along the south coast of Great Britain, near Swanage, Dorset ("Old Harry," 50°38′N, 1°55′E). This population contains intermediate levels of GS compared to other Dorset wild populations (Moyes et al. 2000). A feral *Brassica* population, which was found in a roadside hollow about 15 km east of Wageningen (51°95′N, 5°78′E, The Netherlands), was also included.

In addition to comparing the development of different herbivores, we also compared the development of a specialist and generalist parasitoid reared on the same host (see section on *Plutella xylostella* and *Diadegma* species). To discriminate between food-plant quality mediated through the host and host quality itself, a second closely related wild brassicaceous plant species, black mustard, *Brassica nigra* L., was included in one of the experiments. Seeds of *B. nigra* were collected from a natural population growing in a small patch along the River Rhine, near Wageningen, The Netherlands (51°94′N, 5°62′E).

Seeds from the different populations were germinated in the first week of March 2005. Seedlings were transferred to 1.1 l pots filled with potting soil ("Lentse potgrond" no. 4, Lent, The Netherlands). Plants were grown in a greenhouse at 20–30°C, 40–80% r.h, with a photoperiod of at least 16 hr. If the light dropped below 500 μmol photons/m^2/sec during the 16-hr photoperiod, supplementary illumination was supplied by high-pressure mercury lamps. Plants were watered daily. After the plants were 4 wk old, they were fertilized once a week with Kristalon Blauw (N–P–K) 19–6–20–3 micro (2.5 mg/l), which was applied to the soil. *B. oleracea* plants were 7 wk old when they were used in experiments and attained similar amounts of biomass (25–30 g per plant). Plants from all three populations were in the vegetative state and developed new leaves during the experiments. Fertilization and watering continued during the experiments. *Brassica nigra* plants were 5 wk old and were not fertilized because the soil still contained enough nutrients for optimal growth. *B. nigra* matures much faster than *B. oleracea*.

Insects All insects used originated from cabbage fields in the vicinity of Wageningen. Cultures of all the herbivores have been maintained in the laboratory on Brussels sprouts cv. Cyrus for many years in climate rooms at 22±2°C, 40–80% r.h, with a light regime of 16:8 L/D. The two parasitoid species were collected in the summer of 2004 and were thereafter reared on plants heavily infested with *P. xylostella* larvae for several generations. After pupation on the walls of the rearing cage, parasitoid cocoons were carefully removed and transferred to a clean cage. Emerged adult wasps were provided with water and honey *ad libitum*. For parasitism, we used females that were 5 to 10 d old after adult emergence.

Glucosinolate Analyses As an indicator of direct defense, GS concentrations in leaf tissues of *B. oleracea* were measured. Leaf samples were taken during the performance experiments (see below) from three treatment groups: plants that were undamaged, plants damaged by unparasitized *P. xylostella*, and plants damaged by larvae that had been parasitized by *D. semiclausum*. When leaf samples were taken, the damaged plant groups had been exposed to herbivore feeding for 7 d. Undamaged control plants were maintained in the same greenhouse, but were physically separated from the plants with caterpillars. Larvae, feces, and pupae were removed from leaves. All fully developed leaves were harvested with the exception of the oldest leaves, which had turned yellow and did not contain feeding damage. Leaves were removed with a razor blade, pooled per plant, and stored at −80°C. Samples were later freeze-dried and pulverized with a mortar and pestle. Fifty milligram aliquots of freeze-dried material were weighed in 2-ml centrifuge tubes. GS were extracted and purified as described in van Dam et al. (2004) and were separated on a reverse phase C-18 column (Alltima C-18, 3 μm,150× 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (Dionex, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a DIONEX PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. Peaks were integrated at

229 nm, for which standard response factors have been defined (EC 1990). The different GS were identified based on their retention times, and UV spectra were compared to those of pure compounds (sinigrin, Sigma, St. Louis, MO, USA; glucotropaeolin and glucobrassicin were kindly provided by M. Reichelt, Max Planck Institute for Chemical Ecology, Jena, Germany), or compared to a certified oil seed reference (EC Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland).

Insect Performance To investigate the effect of domestication on plant direct defenses, the different herbivores and parasitoids (see below) were reared on the three plant populations. For all insects, egg-to-adult development time, adult dry mass, and survival (to adult) were determined when reared on the different populations. Adult dry mass was obtained by weighing adults on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, CA, USA) that had been dried to constant weight at 80°C (3 d). Plants with insects were maintained in a greenhouse under the same conditions as described in the *Plant* section. Plants of the same population that received the same insect treatment were placed together, and caterpillars were allowed to develop and move around freely on plants until they reached the final instar. Different herbivore treatments were randomly positioned in a greenhouse, but were all placed in a similar position relative to the light sources to minimize microclimatic differences among plant populations and treatments.

Plutella xylostella and Diadegma Species To obtain eggs of *P. xylostella*, more than 150 adult moths were released with a 50:50 sex ratio in a plastic cage (37×40×30 cm). Folded strips of Parafilm served as substrate for females to lay eggs on. Females were allowed to oviposit on the Parafilm overnight. Subsequently, the strips with eggs were incubated for 4 d at 22°C until the eggs hatched. Pieces of Parafilm with neonate larvae were placed on top of individual plants of each of the three plant populations. Larvae were allowed to feed on these plants until they reached the third instar (L3).

For each plant population, one cohort of 60 larvae was transferred to new plants and served as an unparasitized control. A second cohort of 130 larvae was parasitized by *D. semiclausum*, and a third cohort of 180 larvae was parasitized by *D. fenestrale*. For parasitism, individual female wasps of both species were presented with a L3 *P. xylostella* host. A host was considered as parasitized when the female wasp was observed to insert into and remove her ovipositor from the larva. Individual female wasps were allowed to oviposit in up to 10 separate hosts. After this, they were removed. Parasitized larvae were transferred to new plants of the same population on which the larvae had fed previously. Five plants were used for the unparasitized controls and nine for each of the parasitoid treatments. The number of plants provided ample food for all larvae to complete their development.

When caterpillars molted into L4, strips of corrugated cardboard were placed on top of the plants, as *P. xylostella* prefers to pupate in secluded areas. After pupation, cocoons were collected and stored in labeled vials until adult emergence. When the moths or wasps emerged, the time of eclosion and sex were recorded. Individuals were killed by freezing at −20°C and stored for dry mass determination. Vials with cocoons ready to emerge were checked every 2 hr. Development time for *P. xylostella* was measured in full days, as the exact time of oviposition had not been recorded. In the case of the two *Diadegma* species, the median time point of the period needed to parasitize the hosts (3–4 hr) was used as the time of oviposition.

To further investigate whether food plant quality or host quality was a more important factor in the development of *D. fenestrale*, the experiments described above with *P. xylostella* were repeated on a second (and closely related) wild brassicaceous species, *B. nigra*. A separate study (Gols et al. unpublished) has shown that *B. nigra* is a qualitatively superior plant for the development of *P. xylostella*, compared with *B. oleracea*. We reared 33 unparasitized larvae on three *B. nigra* plants, and 180 larvae parasitized by *D. fenestrale* on 10 plants. We recorded egg-to-adult development time, adult biomass, and survival as before. The plants provided ample food for all the larvae to complete their development.

Pieris rapae Neonate larvae were obtained from the general culture and transferred to seven plants of each population with a distribution of six larvae per plant. When larvae had developed into L5, they were transferred to plastic containers that contained some leaf material from the plants they had fed on previously. After pupation, pupae were collected and placed in new plastic containers lined with filter paper. At adult emergence, the time of eclosion and sex were recorded, and the individuals were killed by freezing, followed by dry mass determination (as above). Containers with pupae ready to emerge were checked every 2 hr. Development time was measured in full days, as the exact time of oviposition had not been recorded.

Mamestra brassicae Like *P. xylostella*, adult *M. brassicae* are primarily nocturnal. Females do not need plants as an oviposition substrate and readily lay batches of eggs onto the surface of paper. From the general culture, we obtained paper sheets with *M. brassicae* eggs that were laid the previous night. These sheets were incubated at 22±2°C (5 d) until the eggs hatched. Neonate larvae were transferred to 10 plants of each population, with a density

of five larvae per plant. Once they had reached late L5, *M. brassicae* larvae were collected from the three populations, counted, and transferred to plastic containers (15×12×6 cm) that contained 2 cm of potting soil mixed with vermiculite (1:1) and some leaf material from the plant on which they had been feeding previously. After the larvae had pupated, they were collected and placed in new plastic containers filled with a layer of vermiculite. At moth emergence, the date of eclosion was recorded, and the individuals were killed by freezing, followed by dry mass determination. Containers with pupae ready to emerge were checked every 2 h. Development was measured in full days as the exact time of oviposition had not been recorded.

Statistical Analysis Data on adult dry mass and development time were analyzed by using ANOVA with plant population and sex and their interaction as factors. All larvae within one plant population were considered as independent samples. The Tukey–Kramer method was used for multiple comparisons of the means. For each insect species, a *G* test for heterogeneity was performed on survival rates of the three plant populations with H_0: survival on each of the three plant populations is equal.

Concentrations of individual GS compounds were log (x+1) transformed to meet assumptions of normality. To examine differences in GS content, a Mixed Model was used with plant population and plant treatment (intact, damaged by unparasitized *Plutella*, damaged by parasitized *Plutella*) as the fixed factors in the model. There was no random factor in the analysis, and the estimation of effects in the model was based on restricted likelihood maximization. When the main factors or their interactions were significant, specific linear contrasts were applied to separate further factor levels. When necessary, correction for unbalanced sample sizes was carried out by using the Satterthwaite correction. Analysis was carried out with SAS 8.02 (1999–2001 ©SAS Institute, Inc).

Results

Glucosinolate Analyses GS analyses of leaf tissues revealed considerable quantitative and some qualitative variation among the different *Brassica* populations (Fig. 1). Three pentyl-derived (C5) GS, glucoalyssin, gluconapoleiferin, and glucobrassicanapin were only detected in the feral population, whereas the other 10 compounds were present in all three populations (Fig. 1). Total GS concentrations in undamaged plants were 3.2 and 1.4 times higher in plants of the wild population than in the cultivated and feral populations, respectively (Fig. 1). Furthermore, concentrations of all individual compounds were significantly different among the three populations (statistics not shown, but all significance levels were lower than 0.05).

Differences between the cultivated and feral populations on the one side, and the wild population on the other side, became even more pronounced after the plants were induced by larval *P. xylostella* feeding (Fig. 1). In the cultivated and feral plants, total levels of GS remained at similar levels before and after induction by *P. xylostella* feeding (cultivar: t_{50}=0.82, P=0.42; feral population: t_{50}=0.39, P=0.70), whereas in the wild population, concentrations were 1.5–2 times higher after herbivore feeding (t_{50}=3.11, P<0.001). Levels of individual GS changed differentially in response to herbivore feeding. The indole GS, glucobrassicin, was induced by *P. xylostella* feeding in all the plant populations (t_{50}=6.90, P<0.001). Moreover, glucobrassicin accounted for almost 70% of the GS composition in the wild population after induction, and for only 53% and 35% in the cultivated and feral population, respectively. In contrast, the relative amount of glucobrassicin in undamaged plants was only 21%, 5%, and 25% in the cultivated, feral, and wild populations, respectively. A second indole GS, neoglucobrassicin, was also induced in response to herbivory in the feral (t_{50}=2.55, P=0.01) and the wild population (t_{50}=4.76, P<0.001), but not in the cultivated population (t_{50}=0.37, P=0.71) in which levels of this compound were very low.

Not all GS concentrations increased after herbivory. Sinigrin was reduced after *P. xylostella* larval feeding in the cultivated (t_{50}=2.4, P=0.02) and the wild population (t_{50}=3.67, P<0.001), but not in the feral population (t_{50}=0.07, P=0.95). Similarly, levels of glucoiberin decreased in response to *P. xylostella* feeding in the cultivar (t_{50}=2.79, P=0.007) and the wild population (t_{50}=4.22, P<0.001), but not in the feral population (t_{50}=0.02, P=0.99). In the feral population, both sinigrin and glucoiberin were present in much lower concentrations than in the other two populations (Fig. 1). In plants damaged by parasitized and unparasitized larvae of *P. xylostella*, concentrations of individual GS were not significantly different (statistics not shown, but all significance levels were lower than 0.05).

Insect Performance: Herbivores In *P. xylostella,* plant population and sex had an effect on egg-to-adult development time (plant population: $F_{2,\ 131}$=5.95, P=0.003; sex: $F_{1,\ 131}$=5.52, P=0.02; Fig. 2a). Female *P. xylostella* developed faster than males; the fastest development time was observed for females reared on the cultivated and the feral population. For adult biomass, the interaction between plant population and sex was significant ($F_{2,\ 132}$=11.1, P<0.001). Plant population had a strong effect on female but not on male biomass (Fig. 2b). The heaviest females were

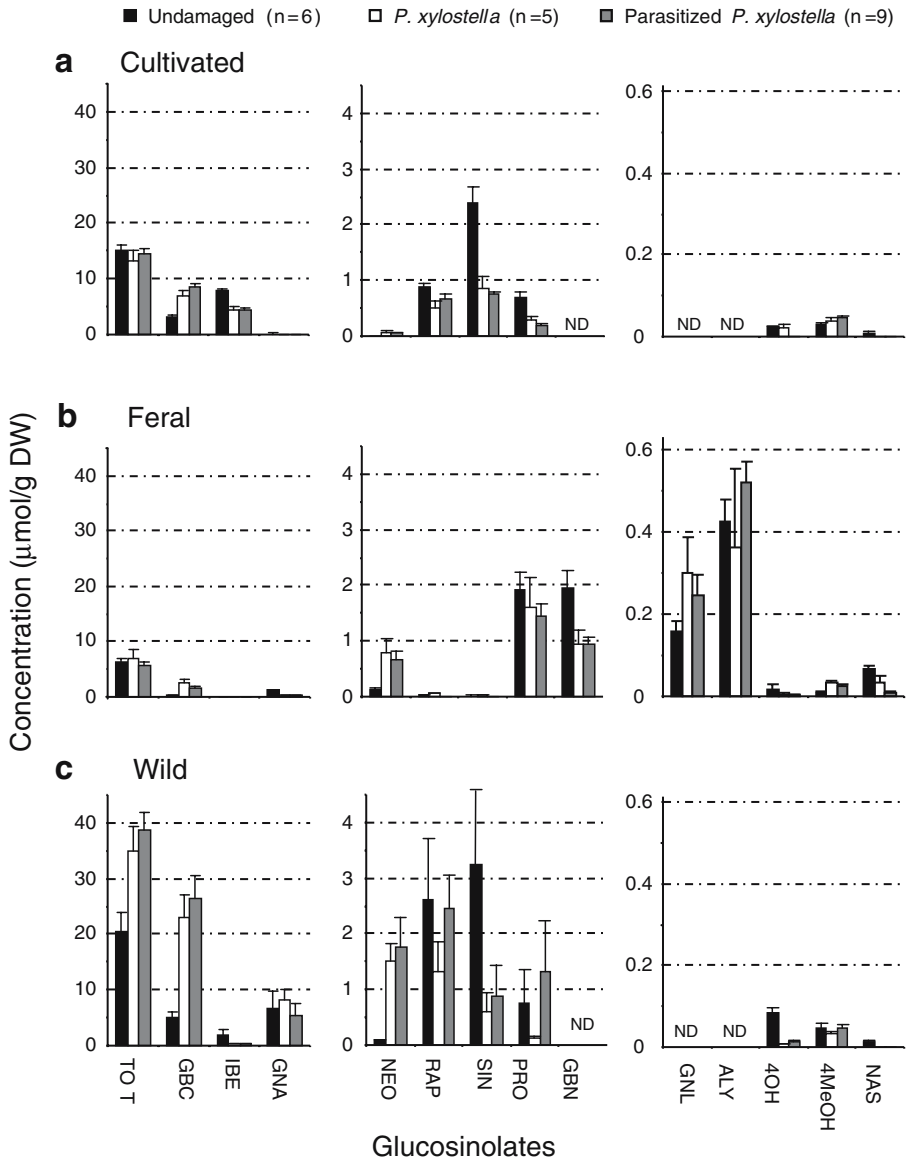

Fig. 1 Glucosinolate (GS) concentrations (mean ± SE) in leaf tissues of a cultivated (**a**), feral (**b**), and wild (**c**) *Brassica* population. Concentrations were measured in leaf tissue from plants that were undamaged (black bars), damaged by unparasitized *P. xylostella* larvae (white bars) and damaged by parasitized (*D. semiclausum*) larvae (gray bars). GS abbreviations and scientific names: *TOT* total GS concentration, *GBC* glucobrassicin (= indol-3-ylmethyl GS), *IBE* glucoiberin (= 3-methylsulfinyl propyl GS), *GNA* gluconapin (= 3-butenyl GS), *NEO* neoglucobrassicin (= 1-methoxyindol-3-ylmethyl GS), *RAP* glucoraphanin (= 4-methylsulfinyl butyl GS), *SIN* sinigrin (= 2-propenyl GS), *PRO* progoitrin (= 2(*R*)-2-hydroxy-3-butenyl GS), *GBN* glucobrassicanapin (= 4-pentenyl GS), *GNL* gluconapoleiferin (= 2-hydroxy-4-pentenyl GS), *ALY* glucoallyssin (= 5-methylsulfinyl pentyl GS), *4OH* 4-hydroxyglucobrassicin (= 4-hydoxyindol-3-ylmethyl GS), *4MeOH*, 4-methoxyglucobrassicin (= 4-methoxyindol-3-ylmethyl GS), and *NAS* gluconasturcin (= 2-phenylethyl GS). *ND* not detectable

recovered from the cultivar, and the lightest from the wild population. In contrast, males were significantly lighter than females ($F_{1,\ 132}$=317, P<0.001) and obtained similar biomasses on all three plant populations (Fig. 2b). For *P. xylostella*, plant population did not affect larval survival to the adult stage (χ^2=0.96, df=2, P=0.62, Fig. 3).

Development time also varied with the population on which the *P. rapae* larvae had been reared ($F_{2,\ 105}$=7.8, P< 0.001, Fig. 4a). Egg-to-adult development time of males was shortest on the cultivar, longer on the feral, and longest on the wild population (Fig. 4a). Males took longer to complete their development than females ($F_{1,\ 105}$=7.5, P= 0.007). Plant population also had a significant effect on adult biomass ($F_{2,\ 101}$=17.5, P<0.001, Fig. 4b). Whereas adult biomass in *P. rapae* did not differ between the cultivated and the feral line, biomass of butterflies reared on the wild population was lower (Fig. 4b). On average, females were marginally heavier than males ($F_{1,\ 101}$=3.2, P=0.08). Irrespective of plant population, more than 92%

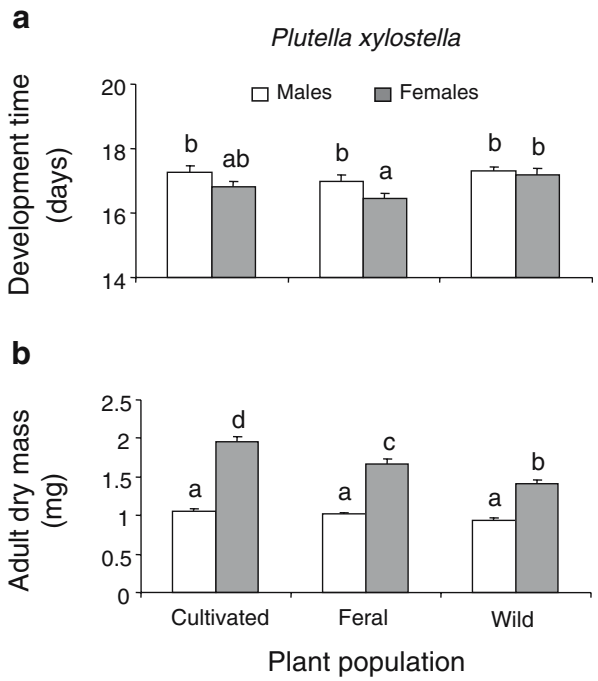

Fig. 2 Egg-to-adult development time (**a**) and adult dry mass (**b**) of *P. xylostella* males (white bars) and females (gray bars) reared on either a cultivated, a feral, or wild *Brassica* population. Bars (mean ± SE) with different letters are significantly different from each other (Tukey multiple comparisons, α=0.05). Numbers of individuals (*N*) were on the cultivar, escape, and wild population, respectively: males, 21, 22, and 31; females 23, 22, 18

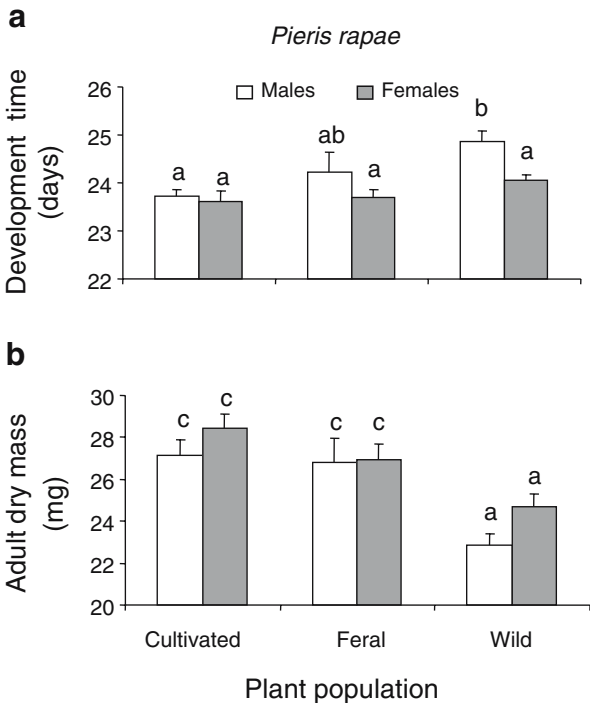

Fig. 4 Egg-to-adult development time (**a**) and adult dry mass (**b**) of *P. rapae* males (white bars) and females (gray bars) reared on either a cultivated, feral, or wild *Brassica* population. Bars (mean ± SE) with different letters are significantly different from each other (Tukey multiple comparisons, α=0.05). Numbers of individuals (*N*) were on the cultivated, feral, and wild population, respectively: males, 22, 10, and 19; females 16, 20, 20

of all *P. rapae* larvae successfully developed into adults, and survival rates were not significantly different (χ^2=0.22, *df*=2, *P*=0.90, Fig. 3).

In the case of the generalist herbivore, *M. brassicae*, the effect of host-plant population on adult biomass was more pronounced compared to the two specialists. It is difficult to determine the sex of adult moths, therefore, the data were pooled. Adult mass on the cultivated line was twice as high, compared to the wild population, and was also significantly higher on the feral population ($F_{2,\ 49}$=8.28, *P*<0.001, Fig. 5b). Plant population did not affect development time ($F_{2,\ 49}$=0.33, *P*=0.72, Fig. 5a). Unlike the two specialists, survival of *M. brassicae* was affected by plant population (χ^2=20.4, *df*=2, *P*<0.001, Fig. 3). The percentage of *M. brassicae* larvae that developed successfully into adults was highest on the cultivated population (58%), slightly lower on the feral population (42%), and the smallest (4%) on the wild population.

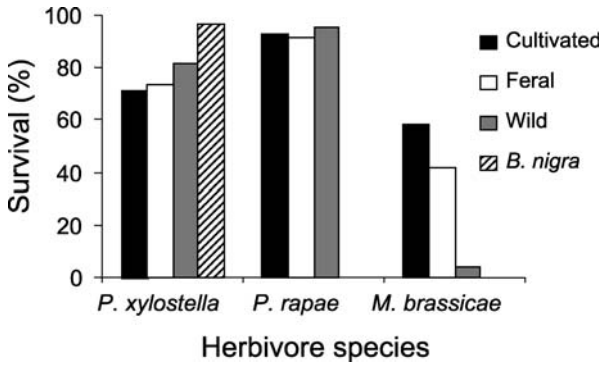

Fig. 3 Larval to adult survival of two specialist herbivores, *P. xylostella* and *P. rapae*, and one generalist, *Mamestra brassica*, when reared on either a cultivated, feral or wild *Brassica* population. *P. xylostella* was also reared on a wild population of *B. nigra*

Insect Performance: Parasitoids In the specialist parasitoid, *D. semiclausum*, plant population had an effect on egg-to-adult development time ($F_{2,\ 275}$=6.49, *P*=0.002, Fig. 6a). The parasitoid developed fastest on the feral population and developed more slowly on the cultivated and wild populations of *B. oleracea* (Fig. 6a). Egg-to-adult development time was longer in females than in males ($F_{1,\ 275}$=13.0, *P*<0.001). Furthermore, plant population had an effect on adult biomass in *D. semiclausum*, ($F_{2,\ 277}$= 11.3, *P*<0.001, Fig. 6b). The heaviest *D. semiclausum* wasps emerged from hosts that were reared on the cultivated population, whereas the lightest emerged from host reared on the wild and feral populations. *D. semiclausum* females were heavier than males ($F_{1,\ 277}$=57.4,

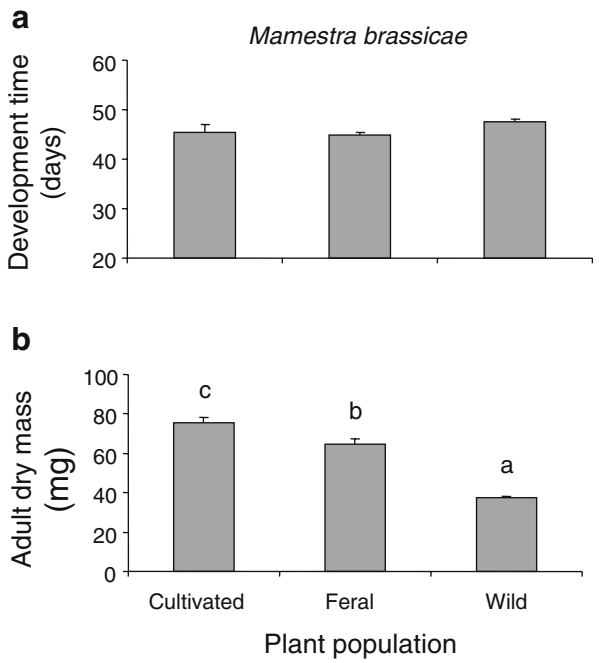

Fig. 5 Egg-to-adult development time (**a**) and adult dry mass (**b**) of *M. brassicae* reared on either a cultivated, feral, or wild *Brassica* population. Bars (mean ± SE) with different letters are significantly different from each other (Tukey multiple comparisons, α=0.05). Numbers of individuals (*N*) were on the cultivated, feral, and wild population, respectively: 21, 29, and 2

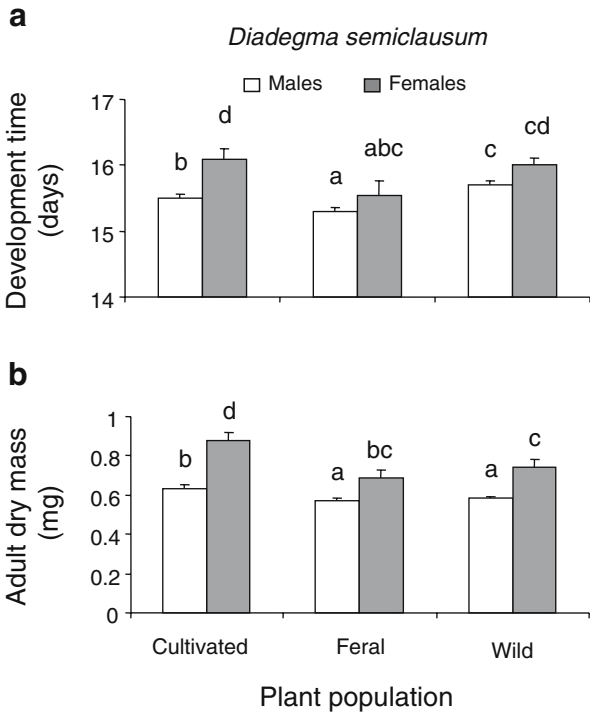

Fig. 6 Egg-to-adult development time (**a**) and adult dry mass (**b**) of *D. semiclausum* males (white bars) and females (gray bars) reared on *P. xylostella* feeding on either a cultivated, feral, or wild *Brassica* population. Bars (mean ± SE) with different letters are significantly different from each other (Tukey multiple comparisons, α=0.05). Numbers of individuals (*N*) were on the cultivated, feral, and wild population, respectively: 87, 69, and 81 for males and 11, 12, 10 for females

P<0.001). Between 67 and 80% *D. semiclausum* successfully completed development to eclosion on the three *Brassica* populations (Fig. 7). Survival rates were not significantly different on the three plant populations (χ^2= 3.77, *df*=2, *P*=0.15).

As with *D. semiclausum*, egg-to-adult development time in *D. fenestrale* varied with plant population ($F_{2,\ 69}$=5.27, *P*=0.007, Fig. 8a). However, dry mass in male *D. fenestrale* wasps did not vary significantly with the population on which the hosts had been reared ($F_{2,\ 69}$=0.25, *P*=0.78, Fig. 8b). As only thirteen *D. fenestrale* females in total successfully developed, data on females were excluded from the analysis and are not presented in the figures. The most dramatic effect of plant population was on the survival of *D. fenestrale* (Fig. 7), which was significantly different for the three plant populations (χ^2=12.2, *df*=2, *P*=0.002). Only 9% of the parasitized hosts reared on the wild population successfully produced *D. fenestrale* wasps. By contrast, 33% survived on the cultivar and 16% on the feral population. Moreover, the lowest number of surviving *D. fenestrale* parasitoids, eight out of 172 (or 4.6%), was obtained on *B. nigra*. Male wasps (*N*=5) developing in hosts reared on *B. nigra* plants were lighter (0.432± 0.033 mg, mean ± SE) and developed slower (17.8±0.37 d) than males reared from hosts on *B. oleracea* (see Fig. 6).

In contrast, healthy *P. xylostella* moths performed better on *B. nigra* than on *B. oleracea,* in terms of survival (97%, Fig. 3) and adult mass, 1.985±0.068 mg (*N*=16) and 1.104± 0.068 mg (*N*=16), for females and males, respectively (see also Fig. 2b). However, development time, 17.8±0.26 d for both female and male moths, was slightly longer (see also Fig. 2a).

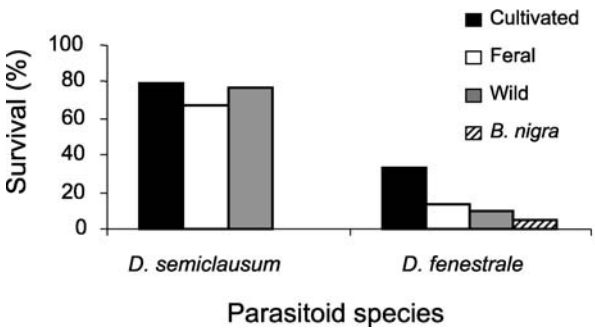

Fig. 7 Egg-to-adult survival of a specialist parasitoid, *D. semiclausum*, and a generalist parasitoid, *D. fenestrale*, when reared from the host, *P. xylostella,* on either a cultivated, feral, or wild *Brassica* population. *D. fenestrale* was also reared from *P. xylostella* on a wild population of *B. nigra*

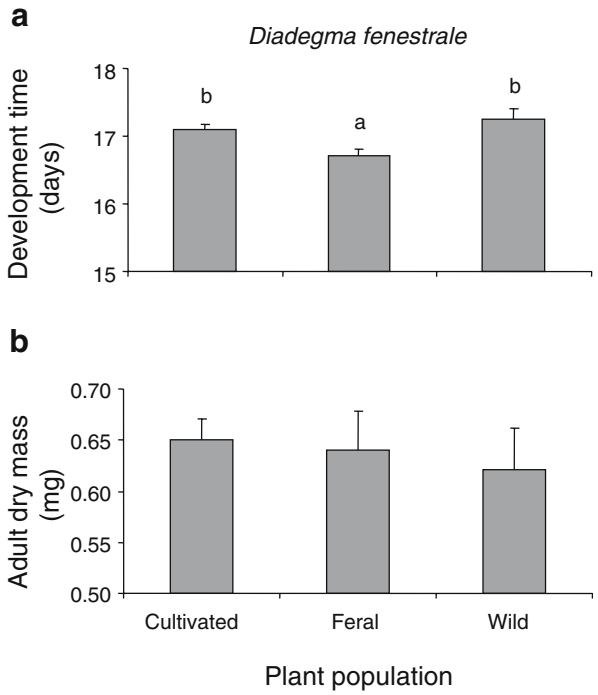

Fig. 8 Egg-to-adult development time (**a**) and adult dry mass (**b**) of *D. fenestrale* males reared on *P. xylostella* feeding on either a cultivated, feral, or wild *Brassica* population. Bars (mean ± SE) with different letters are significantly different from each other (Tukey multiple comparisons, α=0.05). Numbers of individuals (*N*) were on the cultivar, escape, and wild population, respectively: 44, 18, and 14

Discussion

Through artificial selection via domestication, levels of primary and secondary compounds in domesticated plants are often altered compared with their progenitors. The results of this study revealed that GS concentrations in leaf tissue varied significantly among the three different plant populations. Much higher concentrations of GS were recorded in wild *B. oleracea* than in cultivated and feral population. GS levels in the wild population studied in this paper were similar to concentrations reported earlier in other wild populations of *B. oleracea* (Mithen et al. 1995; Moyes et al. 2000). The most striking differences in GS concentrations were observed after induction by herbivore feeding, especially in the wild population. In wild plants, total GS concentrations were 1.7 times higher in induced plants than in uninduced conspecific plants, and were 2.7 and 4.9 times higher after herbivory than in induced plants of the cultivated and feral population, respectively. By contrast, the total GS concentrations in induced plants remained at the same level as in undamaged plants in the cultivated and feral populations, although differences were found for levels of individual compounds.

The feral population differed from the other two populations with respect to pentyl-derived GS, which were absent in the cultivated and wild population. These C5 GS usually are not found in *B. oleracea* crops (Rosa 1999), indicating that this population may have crossed with a closely related species such as *B. napus* or *B. rapa*, both of which contain pentyl GS (Giamoustaris and Mithen 1995). Moreover, both *B. napus* and *B. rapa* are cultivated and grow naturally in the Netherlands. In all plant populations, concentrations of the indole GS, glucobrassicin, increased the most after herbivore feeding, but this compound was dominant only in the wild population, accounting for 70% of the GS content. Several studies have reported on herbivore-induced changes in GS concentrations in both cultivated and wild brassicaceous species. In line with our results, previous studies have shown that levels of indole GS increase in response to insect wounding (Bodnaryk 1992; Agrawal et al. 1999; Bartlet et al. 1999; Gols et al. 2008). We found that concentrations of some of the aliphatic GS were lower in plants that had been damaged by *P. xylostella* than in undamaged plants. Previous studies that used different herbivores reported that levels of aliphatic GS were either unaffected (Bodnaryk 1992; Bartlet et al. 1999; Traw and Dawson 2002; Gols et al. 2008) or even increased (Traw and Dawson 2002) in response to insect wounding. These results suggest differential induction of aliphatic GS by different herbivores (see also Traw and Dawson 2002).

Glucosinolates also play a major role in determining a plant's nutritional quality, not only for humans and livestock but also for pathogens and insect herbivores (Chew 1988; Mithen 1992). This study shows that plants from a wild population of *B. oleracea* are less suitable for the development of several herbivores than plants from a cultivated and a feral population. However, the severity of these effects differed between the specialists *P. rapae* and *P. xylostella* on one hand, and the generalist *M. brassicae* on the other. In *P. rapae*, emerging adult butterflies were smaller and took longer to complete development when reared on wild plants than on the other two populations. These effects were less pronounced in *P. xylostella*; however, where adult body mass was more negatively affected than development time. Importantly, in both of the specialist herbivores, survival was high, irrespective of the plant population on which the larvae had been reared. By contrast, adult body mass and survival in the generalist herbivore, *M. brassicae*, were significantly lower when reared on the wild *B. oleracea* strain. This reveals that costs in terms of reduced fitness are higher for generalist herbivores than for specialists when they feed on the more toxic wild plants. Similarly, Giamoustaris and Mithen (1995) found a negative relationship between GS content in oilseed rape (*B. napus*) and the amount of leaf damage by generalist herbivores, but a positive relationship for specialists. Levels of GS, especially indole GS, were higher in the wild population of *B. oleracea* than levels of GS found in *B. napus* by Giamoustaris and Mithen (1995). These results suggest that indole GS,

especially neoglucobrassicin, which is present in very low concentrations in the cultivar, may play a role in reducing the performance of insect herbivores.

Specialist insect herbivores, in contrast with generalists, may use GS as indicators of host plant suitability. For example, GS serve as feeding stimulants for insect herbivores specialized on plants belonging to the Brassicaceae (Nayar and Thorsteinson 1963; David and Gardiner 1966; Renwick and Lopez 1999). However, not all GS are equally active as feeding stimulants (e.g., Nayar and Thorsteinson 1963), and high levels of GS can even be toxic for specialists (Agrawal and Kurashige 2003). As such, high levels of specific GS may be responsible for the reduced performance of the specialists *P. xylostella* and *P. rapae* when reared on the wild population. Furthermore, levels of the enzyme myrosinase, which catalyzes hydrolysis of GS into the more toxic (iso)thiocyanates and nitriles (Rask et al. 2000; Mithen 2001), may also have differed among the populations. In addition, host plant quality is not determined only by the presence of allelochemicals. Nutrients, such as proteins and carbohydrates, as well as digestibility reducers, also play a role (Slansky 1993). It is possible that levels of limiting nutrients such as nitrogen and other defense-related compounds also vary across the three populations and thus amplify differences in performance caused by GS (Slansky and Feeny 1977).

The performance of the two parasitoid species reared on *P. xylostella* also varied with the plant population on which the host had been reared. However, there were also significant differences in performance between the specialist parasitoid, *D. semiclausum*, and the congeneric generalist parasitoid, *D. fenestrale*. The development of *D. semiclausum* was directly affected by the development of the host. Adult body mass was reduced when developing on wild *B. oleracea* plants, whereas development time and survival were unaffected, revealing that *D. semiclausum* ontogeny is affected by quantitative changes in host quality as mediated through the diet of their host. Alternatively, the development of *D. fenestrale* was characterized less by direct differences in host quality than by indirect population-related variations in plant quality. Although *P. xylostella* survival was high (>80%) on all *B. oleracea* populations, as well as on *B. nigra* plants, mortality in *D. fenestrale* was much higher on the wild *Brassica* populations.

As in most endoparasitoids, larvae of *D. fenestrale* primarily consume host hemolymph and fat body during early development. They only begin to indiscriminately attack other tissues later during development (the so-called "destructive feeding phase"). In this way, they do not kill the host until the last possible moment. Larvae of *P. xylostella* are known to utilize enzymes that convert GS into desulfo-GS in their gut, which are then excreted with their feces (Ratzka et al. 2002). Because of their polarity, GS presumably do not permeate the host-gut membrane but effectively remain in the gut before they are excreted. Consequently, the larvae of *D. fenestrale* probably ingest little, if any, GS when feeding on hemolymph. However, during the destructive feeding phase, the parasitoid larvae undoubtedly consume the host gut and its contents, and this is when the toxic effects of plant allelochemicals on non-adapted parasitoids may be realized. In hosts parasitized by *D. fenestrale,* mortality mainly occurred just before the parasitoids would have been expected to pupate (personal observation) supporting this argument. Furthermore, the gut of endoparasitoid larvae is not externally connected until after emergence from the host. The excretion of wastes into internal host tissues would facilitate bacterial infection and precocious death of both the host and the developing parasitoid (Harvey et al. 2003). Thus, low concentrations of allelochemicals that are ingested by parasitoid larvae are stored and presumably accumulate in their tissues. This may account for the high mortality recorded here with *D. fenestrale* that developed in *P. xylostella* caterpillars reared on mustard and wild cabbage plants, which contain high levels of GS.

All insects used in this study have been reared on the Cyrus cultivar for many generations and may have adapted to this plant population. However, *P. xylostella* developed more successfully on *B. nigra* plants than on the cultivar, with moths enjoying higher survival and larger body mass. Furthermore, the performance of *Mamestra brassicae* and *Pieris rapae* was almost similar on the feral and the cultivated population. We cannot exclude that the observed differences are the result of rearing history rather than true plant effects, but the fact that the insects have no history with the recently cultivated feral population of *B. oleracea* and developed with equal success as on the cultivar suggests that plant quality is affected by domestication. Moreover, the development of the insects was more strongly negatively affected on the wild "Old Harry" population than on the feral population.

In summary, this study has shown that plant quality in terms of development of herbivores and their natural enemies differs significantly between wild and cultivated populations of *B. oleracea*. The identity of the food plant and the degree of specialization exhibited by the herbivores and their parasitoids influenced the degree to which plant population affected performance. Most importantly, these results demonstrate that artificial selection in which certain plant traits are accentuated at the expense of others can alter a significant part of a plant's evolved physiology. This may in turn have large impacts on insect communities that are associated with these plants. In wild plants, defense mechanisms have evolved under natural selection pressures from herbivores and pathogens and by the effects of natural enemies on herbivore populations. The reduction of the levels of direct defenses in cultivated plants could partly

explain why these plants have often become more susceptible to attack from a wide range of herbivores and pathogens. To better understand the relative contribution of insect herbivory as a selective agent on the evolution of plant defenses, these traits should be studied in wild populations in which defense mechanisms have not been constrained by the "directional selection" that characterizes many species of crop plants. Future studies should examine insect communities associated with plant populations, including cultivars that differ in resistance against insect herbivores in plots in which the structural heterogeneity is also manipulated. This will facilitate a better understanding of the role that artificial selection has played in shaping the structure of communities associated with cropping systems.

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References

AGRAWAL, A. A. 1999a. Induced plant defense: Evolution of induction and adaptive phenotypic plasticity, pp. 251–268, in A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses Against Pathogens and Herbivores. Biochemistry, Ecology and AgricultureAPS Press, St. Paul, Minnesota.

AGRAWAL, A. A. 1999b. Induced responses to herbivory in wild radish: Effects on several herbivores and plant fitness. *Ecology* 80:1713–1723.

AGRAWAL, A. A., and KURASHIGE, N. S. 2003. A role for isothiocyanates in plant resistance against the specialist herbivore Pieris rapae. *J. Chem. Ecol.* 29:1403–1415.

AGRAWAL, A. A., STRAUSS, S. Y., and STOUT, M. J. 1999. Costs of induced responses and tolerance to herbivory in male and female fitness components of wild radish. *Evolution* 53:1093–1104.

AZIDAH, A. A., FITTON, M. G., and QUICKE, D. L. J. 2000. Identification of the *Diadegma* species (Hymenoptera: Ichneumonidae, Campopleginae) attacking the diamondback moth, Plutella xylostella (Lepidoptera: Plutellidae) . *Bull. Entomol. Res.* 90:375–389.

BARBOSA, P., SAUNDERS, J. A., KEMPER, J., TRUMBULE, R., OLECHNO, J., and MARTINAT, P. 1986. Plant allelochemicals and insect parasitoids: Effects of nicotine on Cotesia congregata (Say) (Hymenoptera, Braconidae) and Hyposoter annulipes (Cresson) (Hymenoptera, Ichneumonidae). *J. Chem. Ecol.* 12:1319–1328.

BARBOSA, P., GROSS, P., and KEMPER, J. 1991. Influence of plant allelochemicals on the tobacco hornworm and its parasitoid, Cotesia congregate. *Ecology* 72:1567–1575.

BARTLET, E., KIDDLE, G., WILLIAMS, I., and WALLSGROVE, R. 1999. Wound-induced increases in the glucosinolate content of oilseed rape and their effect on subsequent herbivory by a crucifer specialist. *Entomol. Exp. Appl.* 91:163–167.

BENREY, B., CALLEJAS, A., RIOS, L., OYAMA, K., and DENNO, R. F. 1998. The effects of domestication of Brassica and Phaseolus on the interaction between phytophagous insects and parasitoids. *Biol. Control* 11:130–140.

BLAU, P. A., FEENY, P., CONTARDO, L., and ROBSON, D. S. 1978. Allylglucosinolate and herbivorous caterpillars: A contrast in toxicity and tolerance. *Science* 200:1296–1298.

BODNARYK, R. P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31:2671–2677.

CHEW, F. S. 1988. Biological effects of glucosinolates, pp. 155–181, in H. G. Cutler (ed.). Biologically Active Natural Products for Potential Use in AgricultureAmerican Chemical Society, Washington.

DAVID, W. A. L., and GARDINER, B. O. 1966. Mustard oil glucosides as feeding stimulants for Pieris brassicae larvae in a semi-synthetic diet. *Entomol. Exp. Appl.* 9:247–255.

DE MORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

EC. 1990. Oil seeds — determination of glucosinolates High Performance Liquid Chromatography. Official Journal of the European Communities L 170/28. Annex VIII:03.07.27–34.

EVANS, L. T. 1993. Crop Evolution, Adaptation and Yield. Cambridge University Press.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2002. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 59:237–237.

FRANCIS, F., LOGNAY, G., WATHELET, J. P., and HAUBRUGE, E. 2001. Effects of allelochemicals from first (Brassicaceae) and second (Myzus persicae and Brevicoryne brassicae) trophic levels on Adalia bipunctata. *J. Chem. Ecol.* 27:243–256.

GEERVLIET, J. B. F., VERDEL, M. S. W., SNELLEN, H., SCHAUB, J., DICKE, M., and VET, L. E. M. 2000. Coexistence and niche segregation by field populations of the parasitoids Cotesia glomerata and C. rubecula in the Netherlands: Predicting field performance from laboratory data. *Oecologia* 124:55–63.

GIAMOUSTARIS, A., and MITHEN, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (Brassica napus ssp oleifera) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126:347–363.

GOLS, R., BUKOVINSZKY, T., HEMERIK, L., HARVEY, J. A., VAN LENTEREN, J. C., and VET, L. E. M. 2005. Reduced foraging efficiency of a parasitoid under habitat complexity: Implications for population stability and species coexistence. *J. Anim. Ecol.* 74:1059–1068.

GOLS, R., WAGENAAR, R., BUKOVINSZKY, T., VAN DAM, N. M., DICKE, M., BULLOCK, J. M., and HARVEY, J. A. 2008. Genetic variation in the defense chemistry of wild cabbage populations and its effects on native herbivores and their endoparasitoids. Ecology, in press.

GÓMEZ-CAMPO, C., and PRAKASH, S. 1999. Origin and domestication, pp. 33–58, in C. Gomez-Campo (ed.). Developments in Plant Genetics and Breeding, 4. Biology of Brassica Coenospecies. Elsevier, Amsterdam.

HARVEY, J. A., VAN DAM, N. M., and GOLS, R. 2003. Interactions over four trophic levels: Foodplant quality affects development of a hyperparasitoid as mediated through a herbivore and its primary parasitoid. *J. Anim. Ecol.* 72:520–531.

HARVEY, J. A., VAN NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in Plantago lanceolata on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31:287–302.

HARVEY, J., GOLS, R., WAGENAAR, R., and BEZEMER, T. 2007. Development of an insect herbivore and its pupal parasitoid reflect differences in direct plant defense. *J. Chem. Ecol.* 33:1556–1569.

JOHNSON, K. S. 1999. Comparative detoxification of plant (*Magnolia virginiana*) allelochemicals by generalist and specialist Saturniid silkmoths. *J. Chem. Ecol.* 25:253–269.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago.

KHAN, Z. R., AMPONGNYARKO, K., CHILISWA, P., HASSANALI, A., KIMANI, S., LWANDE, W., OVERHOLT, W. A., PICKETT, J. A., SMART, L. E., WADHAMS, L. J., and WOODCOCK, C. M. 1997. Intercropping increases parasitism of pests. *Nature* 388:631–632.

KRIEGER, R. I., FEENY, P. P., and WILKINSON, C. F. 1971. Detoxication enzymes in guts of caterpillars: An evolutionary answer to plant defenses. *Science* 172:579–581.

KUSHAD, M. M., BROWN, A. F., KURILICH, A. C., JUVIK, J. A., KLEIN, B. P., WALLIG, M. A., and JEFFERY, E. H. 1999. Variation of glucosinolates in vegetable crops of *Brassica oleracea*. *J. Agric. Food Chem.* 47:1541–1548.

LEGASPI, B. A. C. 1986. Host discrimination in two species of ichneumonid wasps, *Diadegma* spp., attacking larvae of Plutella xylostella. *Entomol. Exp. Appl.* 41:79–82.

LI, Q., EIGENBRODE, S. D., STRINGHAM, G. R., and THIAGARAJAH, M. R. 2000. Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J. Chem. Ecol.* 26:2401–2419.

MITCHELL, N. D. 1976. The status of *Brassica oleracea* subsp. *oleracea* (wild cabbage) in the British Isles. *Watsonia* 11:97–103.

MITCHELL, N. D., and RICHARDS, A. J. 1979. Biological flora of the British isles—*Brassica oleracea* L. ssp. *oleracea* (B. sylvestris (L.) Miller). *J. Ecol.* 67:1087–1096.

MITHEN, R. 1992. Leaf glucosinolate profiles and their relationship to pest and disease resistance in oilseed rape. *Euphytica* 63:71–83.

MITHEN, R. F. 2001. Glucosinolates and their degradation products. *Adv. Bot. Res.* 35:213–262.

MITHEN, R., RAYBOULD, A. F., and GIAMOUSTARIS, A. 1995. Divergent selection for secondary metabolites between wild populations of *Brassica oleracea* and its implications for plant–herbivore interactions. *Heredity* 75:472–484.

MOYES, C. L., COLLIN, H. A., BRITTON, G., and RAYBOULD, A. E. 2000. Glucosinolates and differential herbivory in wild populations of *Brassica oleracea*. *J. Chem. Ecol.* 26:2625–2641.

MÜLLER, C., AGERBIRK, N., OLSEN, C. E., BOEVÉ, J. L., SCHAFFNER, U., and BRAKEFIELD, P. M. 2001. Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly Athalia rosae. *J. Chem. Ecol.* 27:2505–2516.

NAYAR, J. K., and THORSTEINSON, A. J. 1963. Further investigations into the chemical basis of insect–host plant relationships in an oligophagous insect, *Plutella maculipennis* (Curtis) (Lepidoptera-Plutellidae). *Can. J. Zool.* 41:923–929.

ODE, P. J. 2006. Plant chemistry and natural enemy fitness: Effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol.* 51:163–185.

ODE, P. J., BERENBAUM, M. R., ZANGERL, A. R., and HARDY, C. W. 2004. Host plant, host plant chemistry and the polyembryonic parasitoid *Copidosoma sosares*: Indirect effects in a tritrophic interaction. *Oikos* 104:388–400.

RASK, L., ANDREASSON, E., EKBOM, B., ERIKSSON, S., PONTOPPIDAN, B., and MEIJER, J. 2000. Myrosinase: Gene family evolution and herbivore defense in Brassicaceae. *Plant Mol. Biol.* 42:93–113.

RATZKA, A., VOGEL, H., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and KROYMANN, J. 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U. S. A.* 99:11223–11228.

RENWICK, J. A. A., and LOPEZ, K. 1999. Experience-based food consumption by larvae of *Pieris rapae*: Addiction to glucosinolates? *Entomol. Exp. Appl.* 91:51–58.

ROOT, R. B. 1973. Organization of a plant-arthropod association in simple and diverse habitats: The fauna of collards (*Brassica oleracea*). *Ecol. Monogr.* 43:95–120.

ROSA, E. A. S. 1999. Chemical composition, pp. 315–357, in C. Gomez-Campo (ed.). Developments in Plant Genetics and Breeding, 4. Biology of Brassica Coenospecies. Elsevier Science B.V., Amsterdam.

SCHOONHOVEN, L. M., VAN LOON, J. J. A., and DICKE, M. 2005. Insect-Plant Biology (2nd ed.). Oxford University Press, USA.

SHEEHAN, W. 1986. Response by specialist and generalist natural enemies to agroecosystem diversification—a selective review. *Environ. Entomol.* 15:456–461.

SLANSKY, F. 1993. Nutritional ecology: The fundamental quest for nutrients, pp. 29–91, in N. E. Stamp, and T. M. Casey (eds.). Caterpillars: Ecological and Evolutionary Constraints on ForagingChapman and Hall, New York.

SLANSKY, F., and FEENY, P. 1977. Stabilization of the rate of nitrogen accumulation by larvae of the cabbage butterfly on wild and cultivated food plants. *Ecol. Monogr.* 47:209–228.

SONG, K., OSBORN, T. C., and WILLIAMS, P. H. 1990. Brassica taxonomy based on nuclear restriction fragment length polymorphisms (Rflps). 3. Genome relationships in *Brassica* and related genera and the origin of *Brassica oleracea* and *B. rapa* (syn campestris). *Theor. Appl. Genet.* 79:497–506.

STOWE, K. A. 1998. Realized defense of artificially selected lines of *Brassica rapa*: Effects of quantitative genetic variation in foliar glucosinolate concentration. Environ. *Entomol.* 27:1166–1174.

TAKABAYASHI, J., DICKE, M., and POSTHUMUS, M. A. 1994. Volatile herbivore-induced terpenoids in plant mite interactions: Variation caused by biotic and abiotic factors. *J. Chem. Ecol.* 20:1329–1354.

THALER, J. S., STOUT, M. J., KARBAN, R., and Duffey, S. S. 2001. Jasmonate-mediated induced plant resistance affects a community of herbivores. *Ecol. Entomol.* 26:312–324.

TRAW, M. B., and DAWSON, T. E. 2002. Reduced performance of two specialist herbivores (Lepidoptera: Pieridae, Coleoptera: Chrysomelidae) on new leaves of damaged black mustard plants. *Environ. Entomol.* 31:714–722.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., ROSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U. S. A.* 92:4169–4174.

VAN DAM, N. M., WITJES, L., and SVATOS, A. 2004. Interactions between aboveground and belowground induction of glucosinolates in two wild *Brassica* species. *New Phytol.* 161:801–810.

VAN DER MEIJDEN, E., and KLINKHAMER, P. G. L. 2000. Conflicting interests of plants and the natural enemies of herbivores. *Oikos* 89:202–208.

WITTSTOCK, U., AGERBIRK, N., STAUBER, E. J., OLSEN, C. E., HIPPLER, M., MITCHELL-OLDS, T., GERSHENSON, J., and VOGEL, H. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U. S. A.* 101:4859–4864.

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Phenolic Metabolites in Leaves of the Invasive Shrub, *Lonicera maackii*, and Their Potential Phytotoxic and Anti-Herbivore Effects

Don Cipollini · Randall Stevenson · Stephanie Enright · Alieta Eyles · Pierluigi Bonello

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Abstract *Lonicera maackii* is an invasive shrub in North America for which allelopathic effects toward other plants or herbivores have been suspected. We characterized the major phenolic metabolites present in methanol extracts of *L. maackii* leaves. In addition, we examined the effects of methanol–water extracts of *L. maackii* leaves on seed germination of a target plant species and on feeding preference and growth rate of a generalist insect herbivore. A total of 13 individual major and minor compounds were detected in crude leaf extracts by high-performance liquid chromatography coupled to electronspray ionization-tandem mass spectrometry (ESI-MS/MS). Extracts were dominated by two major flavones, apigenin and luteolin, and their glucoside derivatives, apigenin-7-glucoside and luteolin-7-glucoside. Quantities of these compounds, along with chlorogenic acid, varied between two sampling points. Leaf extracts that contained these compounds were inhibitory to seed germination of *Arabidopsis thaliana*. In addition, treatment of artificial diet with leaf extracts deterred feeding of the generalist herbivore, *Spodoptera exigua*, in choice experiments but had no effect on growth rate in short-term no-choice bioassays. Purified apigenin tended to deter feeding by *S. exigua* and inhibited seed germination of *A. thaliana*. We conclude that leaves of *L. maackii* contain phenolic compounds, including apigenin and chlorogenic acid, capable of having biological effects on other plants and insects.

Keywords *Arabidopsis thaliana* · Feeding deterrents · Flavones · Invasive plants · Phenolics · Phytotoxicity · *Spodoptera exigua*

D. Cipollini (✉) · R. Stevenson · S. Enright
Department of Biological Sciences, Wright State University,
208 Biological Sciences Building, 3640 Colonel Glenn Hwy,
Dayton, OH 45435, USA
e-mail: don.cipollini@wright.edu

A. Eyles · P. Bonello
Department of Plant Pathology, The Ohio State University,
201 Kottman Hall, 2021 Coffey Road,
Columbus, OH 43210, USA

Present address:
A. Eyles
Cooperative Research Centre for Forestry,
TIAR/University of Tasmania,
Private Bag 12,
Hobart 7001, Australia

Introduction

After receiving a great deal of scrutiny in the 1970s and 1980s, allelopathy has received renewed attention in part because of its potential to explain the dramatic success of some invasive plants (Hierro and Callaway 2003). Allelochemicals produced by invasive plants can contribute to their success and spread in several ways. For example, allelochemicals can inhibit growth of competing vegetation through direct or indirect means, thus providing the invader with a competitive advantage (Callaway and Aschehoug 2000; Ridenour and Callaway 2001). In addition to effects on other plants, allelochemicals produced by invasive plants can also contribute to insect and disease resistance and thus confer a competitive advantage to the invader in the introduced range (e.g., Haribal and Renwick 1998). In some cases, the same compound (or closely related structures) can have effects on multiple receiver species (Bais et al. 2002).

Lonicera maackii (Amur honeysuckle, Caprifoliaceae) is a Eurasian shrub that is invasive in eastern North America. The plant has devastating effects on forest understory

vegetation, which is due in large part to the intense shading this shrub imposes, along with competition for soil resources (e.g., Collier et al. 2002). Some of its effects may be mediated by allelopathic chemicals, but this phenomenon has been little studied. Trisel (1997) showed that both whole leaves and aqueous leaf extracts of *L. maackii* could inhibit germination of *Fraxinus* seeds and survival of *Acer* seedlings. Dorning and Cipollini (2006) showed negative effects of aqueous leaf and root extracts on germination of several wild herbaceous species, while Cipollini et al. (2008) showed negative effects of aqueous leaf extracts on growth and reproduction of *Arabidopsis thaliana*. While none of these studies addressed allelopathy in an ecological context, they demonstrated the potential phytotoxic effects of *L. maackii*. In addition to its effects on plants, *L. maackii* is relatively unaffected by herbivores and disease in North America (Trisel 1997; D. Cipollini, personal observation). These observations suggest that *L. maackii* contains compounds in its leaves (and other tissues) that are capable of negatively affecting other organisms. To date, no studies on the chemistry of *L. maackii* leaves or other tissues have been published, but tissues of other *Lonicera* species are known to contain a variety of flavonoids and their glucoside derivatives, phenolic acids, and iridoids (e.g., Flamini et al. 1997; Skulman et al. 2004).

In this study, we characterized and quantified the major phenolic metabolites present in methanol extracts of *L. maackii* leaves by high-performance liquid chromatography (HPLC)-electronspray ionization-tandem mass spectrometry (ESI-MS/MS). We also examined the effects of methanol–water fractions of *L. maackii* leaves on seed germination of a target plant species and on feeding preference and growth rate of a generalist insect herbivore. Finally, we examined the effect of selected flavonoids identified in methanol extracts on seed germination and insect feeding preference.

Materials and Methods

Chemicals and Reagents Methanol (HPLC grade) and acetic acid (HPLC grade) were purchased from Fisher (Pittsburgh, PA, USA). Ultrapure water was prepared with a Milli-Q water system (Millipore, Bedford, MA, USA). Standards of chlorogenic acid, apigenin-7-glucoside, luteolin, and luteolin-7-glucoside were purchased from Extrasynthese (Genay, France). Apigenin was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Leaf Extraction Leaves were randomly collected from three shrubs growing in the Wright State University Woods, Dayton, Ohio on September 2005 and again on July 2006. For HPLC-ultraviolet (UV) and HPLC-MS analyses, three 0.100 g leaf samples were extracted twice with 0.500 ml of 100% methanol over 48 h in the dark at 4°C. In the extraction of plant secondary metabolites, methanol is commonly used to successfully extract a wide range of polar metabolites, many of which should also be present in aqueous extracts (e.g., Eyles et al. 2007). Extracts were transferred to a 1.5-ml microcentrifuge tube and centrifuged (13,000 rpm for 5 min) to remove solids. Samples were stored at −20°C.

Analysis of Phenolics with HPLC-UV and HPLC-ESI-MS/MS HPLC with UV detection and HPLC-ESI-MS were used to analyze the phenolic compounds present in the crude extracts (e.g., Eyles et al. 2007). LC-MS analyses were conducted on a Hewlett Packard HPLC system (Palo Alto, CA, USA) model HP 1100 equipped with a HP DAD G1315A detector coupled to a Q-tof I (Micromass). The same column and gradient program as detailed by Bonello and Blodgett (2003) was used; however, the mobile phase consisted of water/acetic acid (A; 98:2, *v*/*v*) and methanol/acetic acid (B; 98:2, *v*/*v*), and the flow rate was set at 1 ml/min. Detailed description of the operating conditions for HPLC-ESI-MS can be found in Eyles et al. (2007). Concentrations were determined for the five dominant phenolics in three independent samples from September 2005 and in three independent samples from July 2006. Concentrations were determined in relation to standard curves generated with relevant standards. Mean concentrations of each compound during each sampling period were compared statistically by using *t* tests for unequal variances.

Insect Bioassay Leaves from *L. maackii* were randomly collected from a single shrub from the same population used for chemical analysis then lyophilized and ground in a Wiley mill. An extract was prepared in 33% methanol using a tissue/solvent ratio of 1 g:15 ml. This mixture was stirred at room temperature for 24 h then filtered through several layers of cheesecloth and filter paper with a vacuum system. The liquid was evaporated under vacuum, and the residue was reconstituted in distilled water at a tissue/water ratio of 3 g:5 ml. This extract [0.6 g dry weight leaf equivalent per milliliter (referred to hereafter as gle/ml)] was stored at −20°C until further use.

Spodoptera exigua (beet armyworm, Noctuidae), was used as a model for the response of generalist insect herbivores to *L. maackii* allelochemicals. This species has a broad host range and overlaps in distribution across much of the native and introduced ranges of *L. maackii* (distribution maps of plant pests, 1972, CAB International, Map 302). Eggs of *S. exigua* were obtained from Benzon Research (Carlisle, PA, USA), and larvae were raised on an

artificial *S. exigua* diet (Southland Products, Lake Village, AR, USA) in an incubator at 22°C with a 16:8 light/dark period until they were 7 days old. Insects were starved for 3 h before starting the bioassay.

The original honeysuckle extract (0.6 gle/ml) was serially diluted by half with water to make a total of six extract concentrations. Artificial diet was prepared using a 1:10 ratio of diet to boiling water. Immediately after mixing, the diet was poured into 96-well microplates (with 0.300 ml wells) then leveled with a spatula. Once the diet plugs were cool, several were placed in each of the six extract concentrations in 15 ml Falcon tubes (enough to submerge all plugs) and were allowed to absorb the extract at 4°C for 24 h. An additional set of plugs was placed in distilled water to serve as the control. After 24 h, diet plugs were lightly blotted then weighed. Each 0.25 g diet plug absorbed 0.025 ml of extract with this procedure. Thus, the concentration in the diet that received the initial 0.6 gle/ml extract was 0.042 gle/g of diet.

No-Choice Bioassay For each extract concentration, one treated diet plug was placed in a 3.5 cm Petri dish along with a preweighed *S. exigua* larva. There were eight replicate plates for each extract concentration. Petri dishes were sealed with Parafilm, and insects were allowed to feed for 24 h in the incubator. After that time, diet plugs and insects were reweighed to calculate a relative growth rate (RGR; milligram mass gained per milligram insect mass per day) of larvae and a relative consumption rate (RCR; milligram diet eaten per milligram insect mass per day). A separate set of diet plugs from the 0.6 gle/ml extract and from the distilled water treatment was set up as above, except without an insect, to measure diet mass lost due to evaporation. The average mass loss was 0.010 g, which was accounted for in the final mass of all diet plugs. RGR and RCR were compared among extract concentrations with one-way analysis of variance (ANOVA) of log-transformed data on SAS version 9.1 (SAS Institute, Cary, NC, USA).

Choice Bioassay One diet plug containing 0.042 gle/g of diet was placed in a 3.5 cm Petri dish, along with a control diet plug and a pre-weighed *S. exigua* larva. Diet plugs were placed so they were not touching, and the insect was placed at an equal distance from each plug. There were eight replicate plates in this bioassay. Petri dishes were sealed with Parafilm, and insects were allowed to choose diet plugs and feed for 24 h in the incubator. Each diet plug in each dish was weighed after 24 h to calculate an antifeedant index $(AI)=[(C-E)/(C+E)]\times 100$, where C equals the amount of control diet consumed and E equals the amount of extract-treated diet consumed (Rodriguez et al. 1999). This index reveals the amount of the control diet consumed relative to the treated diet standardized by the total amount of feeding done by the insect. Negative values indicate stimulation of feeding, while positive values indicate deterrence of feeding. A value of zero would indicate no effect of the extract on feeding behavior. Mass lost to drying was accounted for in the calculations. A one-sided t test was used to examine whether the AI for this experiment differed significantly from zero.

Seed Germination Bioassay We used *A. thaliana* (Ecotype Col-0) as a representative target species, since its germination, growth, and reproduction were greatly inhibited by aqueous extracts of *L. maackii* leaves (Dorning and Cipollini 2006; Cipollini et al. 2008). Its seed-germination response to aqueous extracts of *L. maackii* also paralleled that of the native annual *Impatiens capensis* (Dorning and Cipollini 2006). Although it possesses general allelochemical detoxification mechanisms like most plants (Baerson et al. 2005), *A. thaliana* has been used as a model in several studies of phytotoxicity because of its general sensitivity to allelochemicals (e.g., Bais et al. 2003; Pennacchio et al. 2005). Wild *A. thaliana* also interacts with *L. maackii* in nature. Its range overlaps with that of *L. maackii* throughout North America, Europe, and Asia (USDA, NRCS 2007), and both species can be found growing in close proximity in the same field sites in North America (D. Cipollini, personal observation). Methanol–water extracts were prepared from *L. maackii* leaves as for the insect bioassay. The extract (0.6 gle/ml) was serially diluted by half with water to make a total of six extract concentrations. For each concentration, ten seeds (Col-0) were placed in a Petri dish (3.5 cm diameter) lined with Whatman® filter paper, along with 0.5 ml of extract. Three replicate Petri dishes were set up for each extract concentration, plus three replicates with distilled water. Seed germination was recorded at 4 and 7 days, and distilled water was added daily to each dish as needed. Numbers of seeds germinated on day 7 were compared among extract concentrations with one-way ANOVA.

Bioassays with Selected Flavones After confirming that apigenin and luteolin were major components of the phenolic metabolites present in *L. maackii* leaves, we conducted a bioassay of feeding deterrence and seed germination inhibition by using commercial standards. In addition, given that the concentration of luteolin in fresh leaves was tenfold higher than apigenin in our July samples, the bioassays were conducted to reflect the in situ relative proportion of apigenin and luteolin. Flavones were dissolved in methanol and diluted in water before their introduction in the diet. In the bioassay of feeding deterrence, a set of diet plugs containing apigenin (0.02 mg/g diet), a set containing luteolin (0.2 mg/g diet),

and a set containing both apigenin (0.01 mg/g diet) and luteolin (0.1 mg/g diet) were prepared. These concentrations are near but lower than those recorded for fresh leaves on a mass basis (see Table 2), thus, effects should be conservative. Concentration of methanol in the diet plugs was 1.3%. Each flavone-treated diet plug was presented to a *S. exigua* caterpillar along with a solvent-only diet plug, and feeding choice bioassays were conducted and analyzed as above. There were eight replicates of each flavone treatment in this experiment. One-sided *t* tests were used to examine whether the AI for each flavone treatment differed significantly from zero.

For the seed germination experiment, we placed 0.015 ml of 0.05% methanol or solutions of apigenin (0.063 mg/ml), luteolin (0.63 mg/ml) and apigenin (0.031 mg/ml) + luteolin (0.31 mg/ml) in 0.05% methanol in separate 2 ml centrifuge tubes. Relevant levels of flavones in soils under *L. maackii* where seeds may contact them are unknown. The amount of flavone present in each ml of these solutions is slightly less than that expected to be found in 1 g of fresh leaf, on average (see Table 2). Five *A. thaliana* seeds were placed in each tube, and the number of seeds germinated (showing radicle emergence from the seed coat) in each tube was examined after 4 and 7 days. There were three replicate tubes for each solution, and the experiment was repeated once. We analyzed numbers of seeds germinated on day 7 with a three-way ANOVA, with trial, apigenin (+ or −), luteolin (+ or −), and their interaction as effects.

Results

A total of 13 individual compounds were detected in honeysuckle crude extracts by HPLC-ESI-MS (Fig. 1, Table 1). Among them, five compounds were characterized (4, 9, 10, 12, and 13), initially on the basis of their MS and MS/MS (MS^2) data and by comparison of their UV spectra with literature data. Their identities were unambiguously confirmed by spiking samples with their respective standards, which were also used to construct standard curves for quantification purposes. Another four compounds (1, 2, 7, and 8) were tentatively characterized on the basis of their UV, MS, and mass spectrometry fragmentation behavior with literature data. The sensitive MS system also detected many smaller peaks that have yet to be analyzed. In general, however, honeysuckle crude extract was dominated strongly by two major flavones, apigenin (13) and luteolin (12), and their glucoside derivatives, apigenin-7-glucoside (10) and luteolin-7-glucoside (9), respectively (Fig. 1).

These compounds displayed UV spectra that were characteristic of flavones (Markham 1982). Furthermore, MS experiments of compound 10 showed a dominant fragment at *m/z* 269, indicating an apigenin derivative, while compounds 8 and 9 yielded an intense ion at *m/z* 285, indicating luteolin derivatives. The neutral loss of 162 Da indicates a hexoside for compounds 9 and 10. Compound 8 exhibited a loss of 294 Da, which most likely results from a disaccharide moiety composed of a hexose and a xylose unit. In a similar manner, compound 7 was tentatively identified as eriodictyol hexoside (Table 1). Samples collected at different times of the year generally contained the same major metabolites, although there was quantitative variation among samples (Table 2). Namely, higher concentrations of chlorogenic acid, luteolin, and luteolin-7-glucoside were detected in the July 2006 sample than in the September 2005 sample, while the inverse was true for apigenin.

In the no-choice bioassay of insect feeding and growth, the *S. exigua* larvae consumed at least some of the diet plug that they were offered in all cases. However, there were no

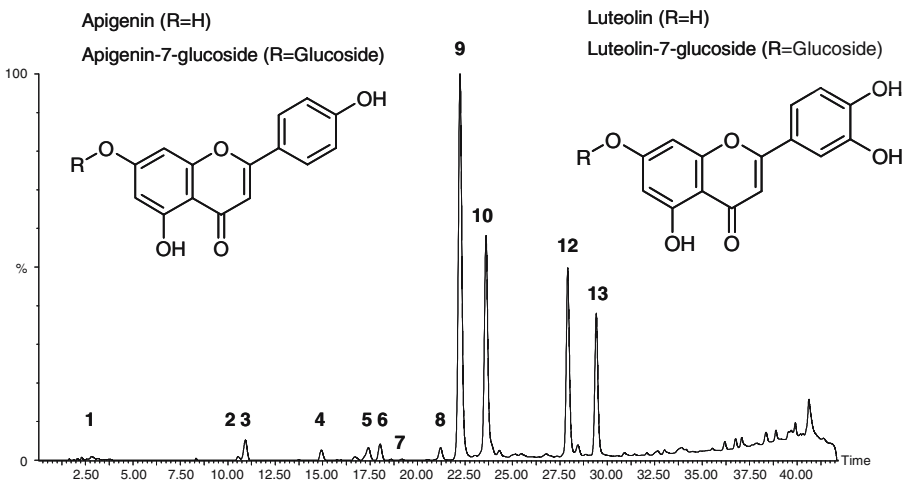

Fig. 1 A representative HPLC-UV chromatographic profile at 280 nm of *L. Maackii* leaf extract (100% methanol) collected in September 2005, showing the chemical structures of the most dominant phenolics. For peak assignments, see Table 1

Table 1 Characterization of phenolic compounds from methanol extracts of *L. maackii* leaves by HPLC-UV and HPLC-ESI-MS

No. 1	Rt (min)	$[M-H]^-$	Main Product Ions by ESI-MS[a]	UV λ_{max} (nm)	Assigned Identity[b]	References
1	2.81	191	127, 173, 171	274	Quinic acid	Clifford et al. 2006
2	10.54	325	163	ND#	Coumaric acid hexoside	Maatta et al. 2003
3	10.92	373	193, 167, 149, 179	245	Unknown	
4	14.93	353	191	sh 300, 327	Chlorogenic acid[c]	Zhu et al. 2004
5	16.69	561	329, 155	275	Unknown	
6	17.40	451	225	sh 290, 312	Unknown	
7	18.02	449	287, 151	283	Eriodictyol hexoside	Hvattum 2002
8	21.21	579	285	265, 352	Luteolin diglycoside	
9	22.24	447	285	255, 350	Luteolin-7-glucoside[c]	Zhu et al. 2004
10	23.61	431	269	267, 339	Apigenin-7-glucoside[c]	Zhu et al. 2004
11	24.31	551	195, 357, 327, 345, 133, 227	338	Unknown	
12	27.92	285	241, 217, 199, 151	254, 351	Luteolin[c]	Wu et al. 2004
13	29.42	269	225, 149, 151, 201	267, 339	Apigenin[c]	Wu et al. 2004

ND Not detected, *sh* shoulder
[a] Ordered by decreasing intensity
[b] Tentative identification based on MS data and UV spectrum consistent with literature
[c] Identification based on retention time, mass spectral, and UV spectrum consistent with those of standard

significant differences in RCR or RGR among concentrations of *L. maackii* extract ($F_{6,49}$=0.44, P=0.849; $F_{6,49}$=1.16, P=0.342, data not shown). In the choice bioassay of insect feeding and growth, most insects had sampled the *L. maackii* extract-treated diet plugs but had a clear preference for the control diet plugs. The AI for this experiment was 55.56 (±8.56 SE), which differed significantly from zero (t=6.54, P<0.001), indicating strong feeding preference for the control diet relative to the treated diet.

In the seed germination bioassay, *L. maackii* extracts strongly inhibited *A. thaliana* germination. After 4 days of incubation, all seeds in the water control had germinated, while the only seeds to germinate in the *L. maackii* extracts were those at the lowest concentration (0.019 gle/ml; $F_{6,14}$=125.1, P<0.001). By 7 days, this number rose slightly, and only one other seed germinated in the next highest extract concentration (0.038 gle/ml; $F_{6,14}$=111.0, P<0.001; Fig. 2).

The diets containing apigenin alone [AI=7.25 (±6.67 SEM)] or apigenin + luteolin [AI=5.29 (±6.41 SEM)] tended to deter feeding by *S. exigua*, but neither AI differed significantly from zero (t=1.09, P=0.156; t=0.823, P=0.218, respectively). The diet that contained luteolin alone [AI=−7.32 (±4.11 SEM)] marginally stimulated feeding (t=1.70, P=0.059). Across two trials, solutions that contained apigenin had only about half as much seed germination by day 7 as solutions without apigenin (apigenin, $F_{1,17}$=15.30, P=0.001, Fig. 3), a pattern that emerged by day 4 (data not shown). Luteolin had no significant independent or interactive effect.

Discussion

L. maackii is an invasive shrub in North America for which allelopathic effects have been suspected but little studied. Here, we identified several phenolic metabolites in methanol extracts of leaves of this plant and showed that methanol–water extracts substantially inhibited germination of a target plant, in accordance with studies that use aqueous extracts (Dorning and Cipollini 2006). In addition, feeding by a generalist insect herbivore was deterred by the presence of methanol–water extracts in an artificial diet. One flavone identified in crude extracts tended to deter herbivore feeding and inhibited seed germination on its own.

Methanol extracts were dominated by two major flavones, apigenin and luteolin, and their glucoside deriv-

Table 2 Concentration (mg/g FW) of major phenolic compounds from methanol extracts of *L. maackii* leaves collected during two sampling periods

Sample Date	Chlorogenic Acid	Apigenin	Apigenin-7-Glucoside	Luteolin	Luteolin-7-Glucoside
July 2006	0.737 (0.348)a	0.109 (0.013)a	0.652 (0.112)	1.044 (0.127)a	7.036 (1.863)a
September 2005	0.0595 (0.034)b	0.263 (0.030)b	0.695 (0.152)	0.239 (0.028)b	1.762 (0.435)b

Numbers represent means (±SE) of three samples. Means with different letters are significantly different at α=0.05.

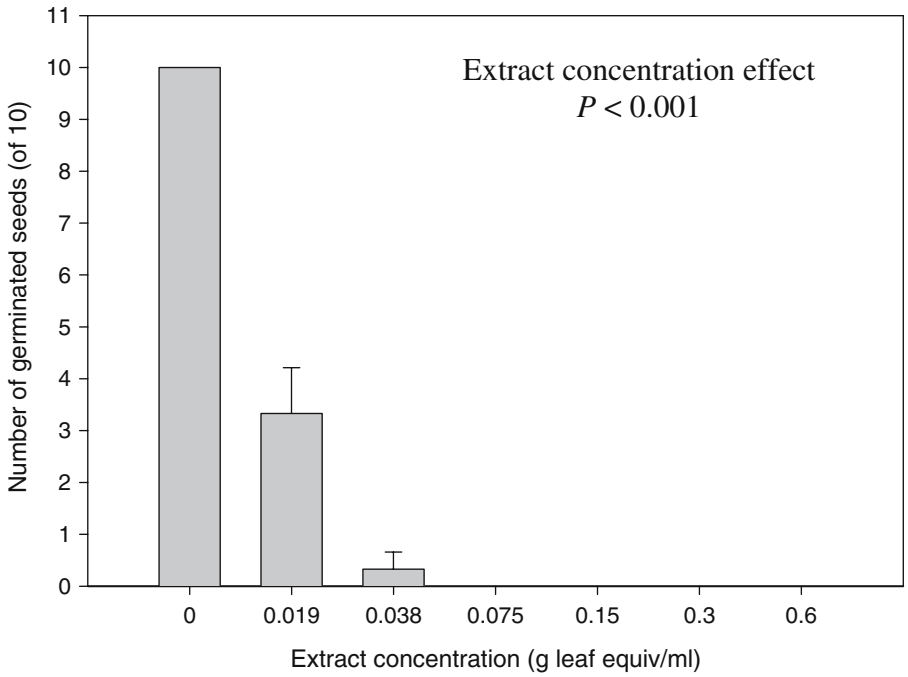

Fig. 2 Effects of methanol–water extracts of *L. Maackii* leaves on germination of *A. thaliana* seeds after 7 days of incubation. *Bars* are means (±SE) of three replicates, each containing ten seeds at each extract concentration. $p<0.001$

atives. These compounds are widespread in the plant kingdom and are well known to be bioactive (Iwashina 2003). These flavones have been studied extensively in the human health literature and possess anti-inflammatory, anti-tumor, and a variety of other health benefits (Verbeek et al. 2004; Graf et al. 2005). They have been less studied in the context of allelopathy, but there is some evidence that apigenin derivatives from *Cistus ladanifer* can reduce growth rate of target plant species and are detected in soils around *C. ladanifer* plants (Chaves et al. 2001). In this paper, we showed that apigenin alone inhibited the germination of *A. thaliana* seeds, while luteolin had no effect. Because these flavones are poorly soluble in water in their crystalline form, they are likely underrepresented in aqueous extracts of *L. maackii* previously noted for their phytotoxicity (Trisel 1997; Dorning and Cipollini 2006; Cipollini et al. 2008). As the results of Chaves et al. (2001) indicate, however, similar flavones do accumulate in soils

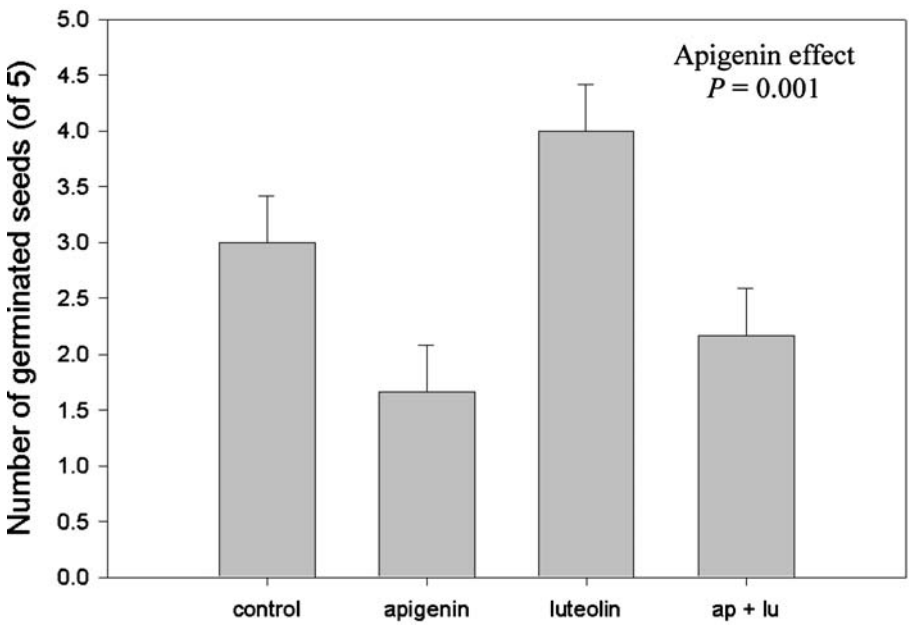

Fig. 3 Effects of purified apigenin, luteolin, and their combination on germination of *A. thaliana* seeds. *Bars* are means (±SE) of two trials, with three replicates of each treatment in each trial and with each replicate containing five seeds. $p<0.001$

around producing plants, so they may be relevant in the field. As for effects on insect performance, both apigenin and luteolin have been noted as phytoestrogens with the ability to disrupt molting cycles (Oberdorster et al. 2001). Although preferring to feed on them, *Spodoptera littoralis* larvae and pupae were smaller when continuously fed alfalfa plants in which apigenin concentrations were increased by herbivore feeding (Agrell et al. 2003). We saw no effect of extracts on relative growth rates of *S. exigua* in our 24-h no-choice bioassay, but longer-term effects on insect growth and development could be important. In contrast, extracts markedly deterred feeding on treated diets relative to a control diet in our choice bioassay. When offered in diet plugs, apigenin alone tended to deter feeding by *S. exigua*, while luteolin tended to stimulate it. While plant resistance to feeding will depend upon the overall balance of stimulants and deterrents, feeding deterrent effects of apigenin and other compounds could be partly responsible for the strong deterrence of crude extracts and for the relative lack of herbivory on *L. maackii* observed in the field in North America (Trisel 1997). A related flavone glycoside derived from apigenin, isovitexin 6″-*O*-β-D-glucopyranoside, significantly deters feeding and performance of *Pieris napi oleraceae* larvae on *Alliaria petiolata*, another invasive plant in North America (Haribal and Renwick 1998).

The July sample of *L. maackii* leaves contained substantial amounts of chlorogenic acid, and coumaric acid hexoside may have been present in our samples. Chlorogenic acid is highly extractable from plant tissues in water or methanol–water mixtures but poorly extractable by 100% methanol (Sripad et al. 1982), so levels found in our crude methanol extracts probably underestimated the actual amount in fresh tissue. There is a wide literature on both the phytotoxic and anti-insect effects of chlorogenic acid and coumaric acid (e.g., Dowd and Vega 1996; Blum and Gerig 2006), so we assume that these compounds contributed to the phytotoxic and feeding-deterrent effects of crude extracts demonstrated in this study.

Levels of chlorogenic acid displayed temporal variation in concentration with July samples reaching much higher concentration than September samples. This suggests that effects of this compound on either plants or herbivores may be more evident in summer months when maximum levels are present in leaves. Likewise, concentrations of luteolin and lueolin-7-glucoside were higher in July samples than in September samples, while concentrations of apigenin-7-glucoside were similar, and apigenin samples were higher in September than in July. Although yearly environmental variation could contribute to this pattern, such temporal variation is consistent with the role of selected flavones in UV light protection in plants, which would presumably be of greater importance during July. Tegelberg and Julkunen-Titto (2001) showed that concentrations of luteolin-7-glucoside in *Salix myrsifolia* leaves were significantly increased by UV-B light exposure, while concentrations of apigenin-7-glucoside were unaffected, a pattern that matches the temporal pattern seen in our study.

While the major effects of *L. maackii* on understory plant communities are likely driven by resource competition, we conclude that leaves of *L. maackii* contain phenolic compounds capable of having biological effects on other plants and herbivorous insects. Our implication of phenolics does not preclude the involvement of other metabolites of *L. maackii* in species interactions, including iridoids known to be produced by *Lonicera* species (Flamini et al. 1997) or other compounds (some of which may be novel) that would not be detected by our extraction and analytical techniques. In turn, while the major phenolic compounds identified here are not novel in the North American flora, nor is the genus *Lonicera* (USDA, NRCS 2007), the quantity introduced to the environment by *L. maackii* may depart from that of the North American flora for several reasons. *L. maackii* has a much longer leafing season than almost any other North American deciduous woody shrub (Harrington et al. 1989); thus, the length of time that it is physiologically active and capable of producing putative allelochemicals in growing leaves (or other tissues) is extended for this plant. In addition, *L. maackii* can reach a very high density and leaf-area index in moderate to high light environments (Hutchinson and Vankat 1997), which is not the case for the more sparsely distributed North American *Lonicera* species. Thus, the input of putative allelochemicals into the environment is likely high in dense patches of *L. maackii*.

Allelochemical levels vary temporally and likely among individual leaves and shrubs of *L. maackii*. Thus, the relative phytotoxicity and anti-insect properties of leaves or extracts of this plant likely vary throughout the season and in different habitats. In turn, recipient plant and herbivore communities vary in composition throughout the season and may be differentially exposed and/or sensitive to *L. maackii* allelochemicals. Along with seeds of *A. thaliana*, aqueous extracts of *L. maackii* leaves and roots inhibited germination of the herbaceous annual, *I. capensis*, and the herbaceous biennial, *A. petiolata*, while having no effect on seeds of *L. maackii* itself (Dorning and Cipollini 2006). However, many of the herbaceous plants that interact with *L. maackii* in North America are perennial, so it will be important to examine a wide range of native plants for their response to *L. maackii* allelochemicals. Variation must certainly exist in the response of herbivores to *L. maackii*, which have not yet been widely surveyed. While allelochemical levels in leaves may be related quantitatively to the preference and performance of leaf-associated herbivores and pathogens, allelopathic effects on other plants in

the field will depend on concentrations and stability of putative allelochemicals reaching the understory. Phenolics are readily metabolized by soil microbes which can greatly alter their bioactivity (Blum 1998). Nothing is yet known about levels or degradation rates of putative allelochemicals in soils around *L. maackii* plants, which is important to know to implicate them in allelopathic interactions where soil exposure is required.

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References

AGRELL, J., OLESZAK, W., STOCHMAL, A., OLSEN, M., and ANDERSEN, P. 2003. Herbivore-induced responses in alfalfa (*Medicago sativa*). *J. Chem. Ecol.* 29:303–320.

BAERSON, S. R., SANCHEZ-MOREIRAS, A., PEDROL-BONJONCH, N., SCHULZ, M., KAGAN, I. A., AGARWAL, A. K., EIGOSA, M. J., and DUKE, S. O. 2005. Detoxification and transcriptome response in *Arabidopsis* seedlings exposed to the allelochemical benzoxazolin-2(3H)-one. *J. Biol. Chem.* 280:21867–21881.

BAIS, H. P., VEPACHEDU, R., GILROY, S., CALLAWAY, R. M., and VIVANCO, J. M. 2003. Allelopathy and exotic plant invasion: from molecules and genes to species interactions. *Science.* 301:1377–1380.

BAIS, H. P., WALKER, T. S., STERMITZ, F. R., HUFBAUER, R. A., and VIVANCO, J. M. 2002. Enantiomeric-dependent phytotoxic and antimicrobial activity of (±)-catechin. A rhizosecreted racemic mixture from spotted knapweed. *Plant Physiol.* 128:1173–1179.

BLUM, U. 1998. Effects of microbial utilization of phenolic acids and their phenolic acid breakdown products in allelopathic interactions. *J. Chem. Ecol.* 24:685–708.

BLUM, U., and GERIG, T. M. 2006. Interrelationships between *p*-coumaric acid, evapotranspiration, soil water content, and leaf expansion. *J. Chem. Ecol.* 32:1573–1561.

BONELLO, P., and BLODGETT, J. T. 2003. *Pinus nigra-Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Pathol.* 63:249–261.

CALLAWAY, R. M., and ASCHEHOUG, E. T. 2000. Invasive plants versus their new and old neighbors: a mechanism for exotic plant invasion. *Science.* 290:521–523.

CHAVES, N., SOSA, T., and ESCUDERO, J. C. 2001. Plant growth inhibiting flavonoids in exudate of *Cistus ladanifer* and in associated soils. *J. Chem. Ecol.* 27:623–631.

COLLIER, M. H., VANKAT, J. L., and HUGHES, M. R. 2002. Diminished plant richness and abundance below *Lonicera maackii*, an invasive shrub. *Am. Mid. Nat.* 147:60–71.

CIPOLLINI, D., STEVENSON, R., and CIPOLLINI, K. 2008. Contrasting effects of allelochemicals from two invasive plants on the performance of a non-mycorrhizal plant. *Intl. J. Plant. Sci.*, in press.

CLIFFORD, M. N., WU, W. G., and KUHNERT, N. 2006. The chlorogenic acids of *Hemerocallis*. *Food Chem.* 95:574–578.

DORNING, M., and CIPOLLINI, D. 2006. Leaf and root extracts of the invasive shrub, *Lonicera maackii*, inhibit seed germination of three herbs with no autotoxic effects. *Plant Ecol.* 184:287–296.

DOWD, P. F., and VEGA, F. E. 1996. Enzymatic oxidation products of allelochemicals as a basis for resistance against insects. *Nat. Toxins.* 4:85–91.

EYLES, A., JONES, W., IEDL, K., CIPOLLINI, D., SCHWARTZ, S., CHAN, K., HERMS, D., and BONELLO, P. 2007. Comparative phloem chemistry of Manchurian (*Fraxinus mandshurica*) and two North American ash species (*F. americana* and *F. pennsylvanica*). *J. Chem. Ecol.* 33:1430–1448.

FLAMINI, G., BRACA, A., CIONI, P. J., and MORELLI, I. 1997. Three new flavonoids and other constituents from *Lonicera implexa*. *J. Nat. Prod.* 60:449–452.

GRAF, B. A., MILBURY, P. E., and BLUMBERG, J. B. 2005. Flavonols, flavones, flavanones, and human health: epidemiological evidence. *J. Med. Food.* 8:281–290.

HARIBAL, M., and ENWICK, J. A. A. 1998. Isovitexin 6″″-O-β-D-glucopyranoside: a feeding deterrent to *Pieris napi oleracea* from *Alliaria petiolata*. *Phytochemistry.* 47:1237–1240.

HARRINGTON, R. A., BROWN, B. J., and EICH, P. B. 1989. Ecophysiology of exotic and native shrubs in Southern Wisconsin. *Oecologia.* 80:356–367.

HIERRO, J. L., and CALLAWAY, R. W. 2003. Allelopathy and exotic plant invasion. *Plant Soil.* 256:29–39.

HUTCHINSON, T. F., and VANKAT, J. L. 1997. Invasibility and effects of Amur honeysuckle in southwestern Ohio forests. *Cons. Biol.* 11:1117–1124.

HVATTUM, E. 2002. Determination of phenolic compounds in rose hip (*Rosa canina*) using liquid chromatography coupled to electrospray ionisation tandem mass spectrometry and diode-array detection. *Rapid Comm. Mass Spec.* 16:655–662.

IWASHINA, T. 2003. Flavonoid function and activity to plants and other organisms. *Biol. Sci. Space.* 17:24–44.

MAATTA, K. R., KAMAL-ELDIN, A., and TORRONEN, A. R. 2003. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in berries with diode array and electrospray ionization mass spectrometric (MS) detection: *Ribes* species. *J. Agric. Food. Chem.* 51:6736–6744.

MARKHAM, K. R. 1982. Techniques of flavonoid identification. Academic, London.

OBERDORSTER, E., CLAY, M. A., COTTAM, D. M., WILMOT, F. A., MCLACHLAN, J. A., and MILNER, M. J. 2001. Common phytochemicals are ecdysteroid agonists and antagonists: a possible evolutionary link between vertebrate and invertebrate steroid hormones. *J. Steroid Biochem. Mol. Biol.* 77:229–238.

PENNACCHIO, M., JEFFERSON, L., and HAVENS, K. 2005. *Arabidopsis thaliana*: A new test species for phytotoxic bioassays. *J. Chem. Ecol.* 31:1877–1885.

RIDENOUR, W. M., and CALLAWAY, R. M. 2001. The relative importance of allelopathy in interference: the effects of an invasive weed on a native bunchgrass. *Oecologia.* 126:444–450.

RODRIGUEZ, B., ODRIGUEZ, B., de la TORRE, M. C., SIMMONDS, M. S. J., and BLANEY, W. M. 1999. From a phagostimulant natural product to semisynthetic antifeedants against *Spodoptera littoralis* larvae: chemical transformations of the neoclerodane diterpenoid scutelgalin B. *J. Nat. Prod.* 62:594–600.

SKULMAN, B. W., MATTICE, J. D., CAIN, M. D., and GBUR, E. E. 2004. Evidence for allelopathic interference of Japanese honeysuckle (*Lonicera japonica*) to loblolly and shortleaf pine regeneration. *Weed Sci.* 52:433–439.

SRIPAD, G., PRAKASH, V., and MARASING AO, M. S. 1982. Extractability of polyphenols of sunflower seed in various solvents. *J. Biosci.* 4:145–152.

TEGELBERG, R., and JULKUNEN-TITTO, R. 2001. Quantitative changes in secondary metabolites in dark-leaved willow (*Salix myrsinifolia*) exposed to enhanced ultraviolet-b radiation. *Physiol. Plant.* 113:541–547.

TRISEL, D. E. 1997. The invasive shrub, *Lonicera maackii* (Rupr.) Herder (Caprifoliaceae): Factors contributing to its success and its effect on native species. PhD Dissertation, Miami University, Oxford, USA.

USDA, NRCS. 2007. The PLANTS Database (http://plants.usda.gov, 4 December 2007). National Plant Data Center, Baton Rouge, LA., USA.

VERBEEK, R., PLOMP, A. C., VAN TOL, E. A. F., and VAN NOORT, J. M. 2004. The flavones luteolin and apigenin inhibit in vitro antigen-specific proliferation and interferon-gamma production by murine and human autoimmune T cells. *Biochem. Pharmacol.* 68:621–629.

WU, W., YAN, C. Y., LI, L., LIU, Z. Q., and LIU, S. Y. 2004. Studies on the flavones using liquid chromatography-electrospray ionization tandem mass spectrometry. *J. Chrom. A.* 1047:213–220.

ZHU, X. F., ZHANG, H. X., and LO, R. 2004. Phenolic compounds from the leaf extract of artichoke (*Cynara scolymus* L.) and their antimicrobial activities. *J. Agric. Food. Chem.* 52:7272–7278.

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Causes and Consequences of Host Expansion by *Mnesampela privata*

Fredrik Östrand · Ian R. Wallis · Noel W. Davies · Mamoru Matsuki · Martin J. Steinbauer

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Abstract The autumn gum moth, *Mnesampela privata*, utilizes several species of *Eucalyptus* planted outside regions of endemism within Australia. We investigated whether foliar monoterpene composition influenced oviposition in the field on the natural primary host (*E. globulus*) and a novel host (*E. rubida*), both characterized by nonstructural epicuticular waxes. In the laboratory, oviposition preferences of females for species and families of known host, novel hosts, and non-hosts that were characterized by both nonstructural and structural waxes but also varied in foliar concentrations of the purportedly toxic plant secondary metabolite (sideroxylonal) were studied. Although *M. privata* laid as many eggs on trees of two families of *E. rubida* as they did on trees of two families of *E. globulus*, there were significant differences in the numbers of clutches of eggs laid. When combined with data for oviposition on another five families of *E. globulus*, we found a negative relationship between mean numbers of eggs and foliar concentration of α-pinene but a positive relationship between egg numbers and the concentration of α-terpineol. The field data suggest that female *M. privata* are just as willing to lay eggs on novel hosts with comparable foliar monoterpene compositions to those of the primary host, especially if they produce

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F. Östrand
Chemical Ecology and Ecotoxicology, Department of Ecology,
Lund University,
SE-223 62 Lund, Sweden

I. R. Wallis
School of Botany and Zoology,
The Australian National University,
Canberra A.C.T. 0200, Australia

N. W. Davies
Central Science Laboratory, University of Tasmania,
Private Bag 74, Hobart, Tasmania 7001, Australia

M. Matsuki
Co-operative Research Centre (CRC) for Sustainable
Production Forestry and University of Tasmania,
c/- Department of Agriculture—Western Australia,
444 Albany Highway,
Albany, WA 6330, Australia

M. J. Steinbauer
CRC for Sustainable Production Forestry
and CSIRO Entomology,
GPO Box 1700, Canberra A.C.T. 2601, Australia

Present address:
F. Östrand
Ministervägen 4,
SE-227 62 Lund, Sweden

Present address:
M. Matsuki
Bluegum Industry Pest Management Group, CRC for Forestry and
Murdoch University, Department of Agriculture and Food
Western Australia,
444 Albany Highway,
Albany, WA 6330, Australia

Present address:
M. J. Steinbauer (✉)
Australian Plague Locust Commission,
GPO Box 858, Canberra A.C.T. 2601, Australia
e-mail: martin.steinbauer@daff.gov.au

nonstructural epicuticular waxes. Oviposition assays in the laboratory endorse this mechanism of host plant hierarchy and support the long-held assumption of the host primacy of *E. globulus*. In laboratory assays, some larvae pupated on all hosts (except *Corymbia eximia*) but the number completing larval development was greater on hosts with softer leaves. Larval survival was also reduced on hosts with high concentrations of sideroxylonal but only if those hosts also had modest to high concentrations of monoterpenes. Larval survival was high on a host (*E. macarthurii*) with a high concentration of sideroxylonal but with virtually zero monoterpene content. This suggests that the monoterpene content of a host could antagonize the effect on *M. privata* larvae of its sideroxylonal content. The larval food plant most affected the fitness of female rather than male pupae. Of the known host expansion events, all have occurred in mixed species plantations. The co-occurrence in these plantations of either the primary host or other highly ranked species probably explains the eventual expansion onto the neighboring species of *Eucalyptus* and *Corymbia*.

Keywords Myrtaceae · Autumn gum moth · Geometridae · Antagonistic chemical interactions · Exaptation · Oviposition mistakes · Lepidoptera

Introduction

The evolution of insect host specificity and host plant hierarchies depends on the suite of plant and insect species that coincided at a particular time and place (Janz and Nylin 1998; Janz et al. 2001; Braby and Trueman 2006). Typically, insect herbivores expand onto novel plant species that are taxonomically related and/or possess similar suites of plant secondary metabolites (PSMs). For example, the native Brazilian geometrid, *Thyrinteina arnorbia*, now exploits eucalypts (Grosman et al. 2005), whereas its natural hosts were a suite of endemic Myrtaceae. Hence, the expansion of *T. arnorbia* onto *Eucalyptus* provides an example of a host shift between related plant species. Perhaps rarer are instances when insects use unrelated plant families that possess similar PSMs to those of their natural hosts. Murphy and Feeny (2006) found that chemically similar extracts from both the ancestral host (family Apiaceae) as well as from three novel hosts (family Asteraceae) stimulated oviposition in two species of butterfly that belong to the *Papilio machaon* group. Steinbauer and Wanjura (2002) observed normally eucalypt-feeding species of *Anoplognathus* (Coleoptera: Scarabaeidae) eating the leaves of *Schinus molle* (family Anacardiaceae) and suggested that they were attracted by a similar monoterpene signal.

Mnesampela privata (Guenée) (Lepidoptera: Geometridae, Ennominae) is endemic to forests in south-eastern and south-western Australia. Before being called the "autumn gum moth," it was the "blue gum moth" because the only larval host then known was juvenile blue gum, *Eucalyptus globulus* (French 1900; Froggatt 1923; Evans 1943). Studies with *E. globulus* by Steinbauer (2002) and Steinbauer et al. (2004) revealed that females prefer to oviposit on the waxiest types (i.e., juvenile) and sides of leaves. The advent of plantation forestry has led to a reappraisal of the host status of *Eucalyptus* species that *M. privata* uses. One of the first cases of host expansion involved shining gum (*Eucalyptus nitens*), soon after its introduction to Tasmania (D. W. de Little, unpubl.). Other plantation and amenity eucalypts that *M. privata* now uses include *E. botryoides, E. camaldulensis, E. grandis, E. macarthurii, E. rubida, E. viminalis*, and *Corymbia maculata* (see Steinbauer and Matsuki 2004, and references cited therein). One expansion, onto *E. grandis*, in north-western Victoria, is particularly interesting because earlier records of *M. privata* that use this eucalypt are only from regions where the tree was endemic (Moore 1972). Less surprising are infestations of plantation *E. globulus* in southwest Western Australia (Loch and Floyd 2001; Hobbs et al. 2003), where *E. globulus* is far from its endemic region (Tasmania and south-eastern Australia). Because expansion and utilization of novel hosts by *M. privata* is not always documented, the phenomenon has seemed random and inexplicable to some. For example, *M. privata* had little effect on *E. macarthurii* in two mixed species plantations (Roberts and Sawtell 1981; Stone and Urquhart 1992), but it completely defoliated the species in another (E. G. Neumann and N. G. Collett, unpubl.). The events mentioned above show that this insect has successfully utilized species of eucalypt that either did not occur in a given region or were scarce in that location before plantations.

Eucalypt taxonomy is in a state of flux. In this work, the classification of Chippendale (1988) is followed, although Brooker (2000) proposes a more recent classification (but see Ladiges and Udovicic 2000). Much of the debate revolves around the status of *Corymbia*—whether it is a subgenus of *Eucalyptus* or has full genus status (Hill and Johnson 1995; Ladiges et al. 1995; Udovicic et al. 1995). Regardless of classification, *Corymbia* is still a taxon somewhat removed from the subgenera *Monocalyptus* and *Symphyomyrtus*, which explains our reason for studying it. None of the aforementioned authors disputes the subgeneric status of *Monocalyptus* or *Symphyomyrtus*. We know of no

published work that collates patterns of host tree utilization by native insects based on eucalypt PSMs.

The genus *Eucalyptus* provides an ideal group in which to study host shifts. Although there are more than 900 species, they rarely form single species forests of more than a few hectares and usually grow in close association with individuals of two, three, or more other species (Wardell-Johnson et al. 1997). Furthermore, different species of *Eucalyptus*, especially those belonging to the same subgenera, share many foliar PSMs, notably epicuticular waxes, monoterpenes, and formylated phloroglucinol compounds (FPCs; Boland et al. 1991; Li et al. 1995, 1996, 1997; Eschler et al. 2000). Hence, native eucalypt-eating insects are commonly exposed to a mosaic of volatile stimuli. Because the antennae of female *M. privata* respond to a number of ubiquitous eucalypt monoterpenes, Steinbauer et al. (2004) suggested that they are used as host location and assessment cues. These authors have also shown that the odors of the epicuticular waxes of new leaves stimulate the antennae of female moths more than do the odors of waxes from old leaves or the wax odors of glossy leaves. Steinbauer et al. (2004) have suggested that waxes provide host assessment/acceptance cues for female *M. privata*. When female *M. privata* mistakenly oviposit on *E. melliodora* or *E. sideroxylon* (both of which produce waxy leaves), their larvae suffer high neonate mortality. Steinbauer and Matsuki (2004) suggested that high neonate mortality on trees of these two species was because of high concentrations of sideroxylonal. Sideroxylonal is an FPC common to *Symphyomyrtus* species but present in some in much higher concentrations than in others. If it is possible to explain past host expansion events and oviposition mistakes by *M. privata*, then it may be possible to foresee future events.

Methods and Materials

Field Investigations of Oviposition Preferences for Novel and Natural Hosts The field investigations were conducted

Table 1 The eucalypts studied, their host utilization by *Mnesampela privata* and the characteristics of their epicuticular waxes

Species	Family Name and Code	Host Status and Wax Type (latter from Hallam and Chambers 1970)	Ontogeny and Distribution of Waxes
Field investigations GES2			
E. rubida	Boboyan Forest R28	Novel host; nonstructural waxes (tubes, compound, branching acutely)	Juvenile leaves waxiest; isobilaterally waxy
E. rubida	Glendale Crossing R22	As above	As above
E. globulus	Otways Nat. Park G25	Natural host; nonstructural waxes (tubes, compound, branching acutely)	Juvenile leaves waxiest; abaxial very waxy, adaxial less waxy
E. globulus	Jeeralang North G19	As above	As above
Field investigations GES1			
E. globulus	Geeveston G76	See details for G25	See details for G25
E. globulus	Otways Nat. Park G25	As above	As above
E. globulus	Jeeralang North G19	As above	As above
E. globulus	Badgers Creek G10	As above	As above
E. globulus	King Island G8	As above	As above
Laboratory investigations			
E. globulus	Geeveston G76	See details for G25	See details for G25
E. nitens (4[a])	Toorongo N63	Novel host; nonstructural waxes (tubes, compound, branching acutely)	Juvenile leaves waxiest; isobilaterally waxy
E. nitens (3[a])	Southern N.S.W. N64	As above	As above
E. nitens (2[a])	Macalister N65	As above	As above
E. nitens (1[a])	Rubicon N66	As above	As above
Corymbia eximia	Black Mountain Cor	Unknown status; structural waxes[b]	Leaf types comparable; isobilaterally dull matt
E. macarthurii	Black Mountain Mac	Unknown status; structural waxes[b]	Juvenile leaves waxiest; abaxial dull matt, adaxial sub-glossy

[a] Visual ranking of waxiness where 1=most waxy and 4=least waxy
[b] Waxes not studied by Hallam and Chambers (1970).

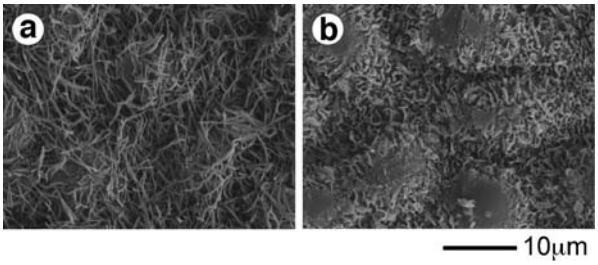

Fig. 1 Scanning electron micrographs of the epicuticular waxes of two hosts used in the laboratory investigations. **a** Abaxial surface of leaf of *E. nitens* family N66 showing nonstructural waxes and **b** leaf of *C. eximia* showing structural waxes. Nonstructural waxes can be removed by abrasion, whereas structural waxes cannot

in two common arboreta, i.e., Ginninderra Experiment Stations 2 and 1, hereafter referred to as GES2 and GES1. Our objective was to compare the oviposition preferences of *M. privata* for trees belonging to five families of *E. globulus* with those for two families of *E. rubida* (a novel host). Both species have waxy leaves, the chemical composition of which relates them to other species that possess nonstructural waxes (Table 1. Fig. 1). Because of their close taxonomic affinity (both subgenus *Symphyomyrtus*, series Viminales), it was thought that differences in oviposition according to species and family would indicate the influence of foliar monoterpenes on female preference. We surveyed 54 trees of each species in GES2 and 100 of each in GES1 for egg clutches for 1 min on at least 11 separate occasions that spanned two moth seasons. At the end of a survey, the numbers of eggs in each previously located clutch was counted. Data are expressed as the mean number of clutches or eggs per target tree per minute per survey.

Our interest was the influence of tree genotype on oviposition rather than the timescale of oviposition, so we ignored the repeated measures aspect of the data. Observations of "nil oviposition" were also ignored, which caused the oviposition data to be unbalanced. Therefore, general linear modeling (GLM) using the data for clutches and square root transformed egg data were used to assess oviposition preferences according to species and families of trees. The number of leaves examined during each 1-min survey and the number of clutches found were included as covariates, but were dropped from later models if statistically insignificant. A post hoc one-way analysis of variance (ANOVA) of transformed data was used to compare individual means.

Laboratory Investigations of Oviposition Preferences for Novel and Natural Hosts The attractiveness of natural and novel hosts was compared by using binary choice assays with groups of females and also by using multiple choice assays with individual females (details given in Table 2). With the exception of *C. eximia*, the two novel host species (*E. nitens* and *E. macarthurii*) belong to the subgenus *Symphyomyrtus* (series Viminales). Furthermore, the four *E. nitens* showed considerable variation in the waxiness of their leaves, all of which have nonstructural waxes, whereas *C. eximia* and *E. macarthurii* have structural epicuticular waxes that cannot be abraded. *Eucalyptus globulus* had waxier leaves than the waxiest *E. nitens* studied. The foliage of the four *E. nitens* came from another common arboretum, i.e., GES3. Of the other two trees, leaves of *C. eximia* were taken from a specimen growing in the grounds of Commonwealth Scientific and Industrial Research Organisation, whereas leaves of *E. globulus* came from a specimen growing in the garden of MJS (specimen belonged to same family as that planted in GES1).

Table 2 Laboratory investigations used to assay responses of *Mnesampela privata* to novel and natural hosts as well as hosts of unknown host status (F = female and M = male)

Assay	Branchlets (moths) and Leaves (larvae) Per Assay	Number of Insects Per Assay	Number of Cages (moths) or Dishes (larvae) Per Assay
Oviposition preference			
Binary choice	N66, G76	3–4 f, 3–6 m	9
As above	N64, N66	4 f, 4–5 m	7
As above	Mac, N64	4 f, 3–5 m	5
As above	Cor, N64	4 f, 3 m	5
As above	N64, N66	1 f, 1–3 m	11
Multiple choice	N63, N64, N65, N66	1 f, 1–3 m	13
Larval survival and performance			
	Cor, Mac, N66, G76	5 eggs per leaf	6 per tree
	N63, N64, N65, N66	5 eggs per leaf	6 per tree

To ensure that test females were host novices, male and female pupae were allowed to ecdyse in the cages that housed the test foliage. Assays were conducted in cages (54×37×28 cm) with gauze sides housed in a controlled temperature room (range 20–21°C) under a 12:12 h reverse-cycle lighting regimen with a fan circulating air. Branchlets were pruned so that they had either the same number of leaves or about the same leaf area before placing them upright in containers of moistened floral foam in diagonally opposite corners of cages. Moths were given 5% sugar water for sustenance. Branchlets were left for 7 days before removing them, counting the eggs, and measuring the total leaf area (i.e., leaf area multiplied by 2), with an AM100 portable leaf area meter (ADC Bioscientific Ltd., Herts).

Two-tailed paired-sample *t*-tests were used to analyze the results from binary choice assays after first transforming the data—square root (eggs+0.5)—to account for zero values. Paired *t*-tests cannot incorporate covariates, such as leaf area, and so these were compared separately. There were significant differences in total leaf area per cage only for *E. macarthurii* vs. *E. nitens* N64 (P=0.001). The juvenile leaves of *E. macarthurii* were a third the size of those of *E. nitens* N64, so it was difficult to match leaf area per cage. Nevertheless, the preference of females for *E. nitens* N64 over *E. macarthurii* could be because of differences in the leaf areas.

A GLM was used to analyze the results of the multiple choice assays, with leaf area as a covariate, after transforming the data [square root (eggs+0.5)]. The total leaf area did not explain significant variation (P=0.57) and was dropped from later models. A post hoc one-way ANOVA of the transformed data was used to separate the treatment means.

Laboratory Investigations of Larval Survival and Performance on Novel and Natural Hosts In two sets of assays, the survival and performance of larvae, from hatching to pupation, was assayed by using leaves of all the trees used in the oviposition experiments. In the first set of assays, the survival of six groups of five larvae on each of *E. globulus*, *E. nitens* N66, *E. macarthurii*, and *C. eximia* was followed by recording the number of dead larvae twice weekly. The second set of assays was the same, except larvae were reared on leaves of the four *E. nitens*. Groups of larvae were reared in individual 15-cm Petri dishes (same temperature and lighting regime as used for the oviposition assays) with saturated plaster of Paris bases until the third instar and then transferred them to 750-ml plastic containers each half filled with moist vermiculite as a pupation substrate. Fresh leaves were supplied to all groups of larvae at the same time when needed. Individuals that pupated were sexed, oven dried at 40°C, and weighed.

Log-rank tests were used to compare pairs of Kaplan–Meier survival curves. Because there were six pairs of comparisons per survival assay, the test statistic was compared against critical values of the chi-square distribution with df=1, but adjusted P values used the Bonferroni method because there were more comparisons than there were trees in each assay. This adjustment was also applied to the regression results of female pupal weight vs. leaf traits because they met the same condition. The dry weights of male and female pupae were log transformed and compared with one-way ANOVA.

Analyses of Foliage The concentration [given as milligrams tridecane (i.e., the internal-standard)] equivalents per gram of leaf dry mass (DM) of α-pinene, limonene, 1,8-cineole, γ-terpinene, and α-terpineol in fresh leaves from two representative trees of each family in GES2 and GES1 were measured according to Steinbauer et al. (2004). The extraction procedure entails immersion of 100 mg of fresh leaf slivers in 400 μl of high-performance liquid chromatography grade hexane (containing 100 ppm tridecane as an internal standard) in sealed glass tubes followed by heating to 100°C for 1 h. One microliter volume of the solvent extract was subjected to gas chromatography/mass spectrometry analysis, and quantities of each monoterpene were calculated based on reference to the response factor for the internal standard.

FÖ and MJS independently examined the same eight branchlets (each with six leaves) of each of the four *E. nitens* and ranked them on abundance of epicuticular waxes. The two sets of rankings were the same, i.e., *E. nitens* N66, N65, N64, to N63, with N66 the waxiest and N63 the least waxy.

Freeze-dried leaves were used in chemical analyses of nitrogen and sideroxylonal (an FPC), respectively. Leaves were ground in a Tecator Cyclotec 1093 mill to pass through a 1-mm sieve, and nitrogen content was measured by using 0.35 g of leaf powder and a semi-micro Kjeldahl technique with a Tecator 2012 digester, selenium catalyst (3.5 g of K_2SO_4, 3.5 mg Se) and a Gerhardt Vapodest-5 distillation and titration apparatus. The method was standardized by using ammonium sulfate. The sideroxylonal content of ten individual leaves of each host used in the laboratory investigations was measured following Wallis et al. (2003). However, to ensure the extraction of all the sideroxylonal, the leaves were finely chopped, sonicated in solvent, and then extracted in solvent for 8 rather than 5 h.

Specific leaf weight (SLW) was measured as an indicator of leaf toughness because leaf toughness is the only foliar trait known to link oviposition preference and neonate performance in *M. privata* (Steinbauer 2002; Steinbauer and Matsuki 2004). Foliar water content is inversely correlated with SLW (as is nitrogen; Steinbauer and Matsuki 2004) and can be measured coincident with SLW. Consequently, it was also measured (as a percentage of leaf mass) and arcsine transformed for use in statistical tests.

The physical and chemical characteristics of the trees were also characterized to help differentiate them from one another. Global nonmetric multidimensional scaling (GNMDS), an ordination procedure, using PC-ORD was used to compare the traits of the trees. The settings used for the analyses were 40 runs with real data, 100 runs with randomized data, 500 maximum iterations, 5 (for GES2 and GES1 data; i.e., 5 monoterpenes) or 9 (for laboratory data; i.e., 5 monoterpenes, sideroxylonal A and C, toughness and water content) starting dimensions, and <0.001 minimum instability threshold. The results of these analyses are provided as Supplementary Material.

Results

Field Investigations of Oviposition Preferences for Novel and Natural Hosts In GES2, there was no significant difference in the mean numbers of eggs on the families of *E. globulus* and *E. rubida*. There were, however, different

Table 3 Results of statistical analyses of oviposition in field and laboratory investigations

Source or Comparison	*df* and/or Significance-level and Tail	SS or Critical *t* value	*F* or *t* Value	*P* Value
Field investigations GES2 (GLM)				
Clutches	1	3316.02	256.72	< 0.001
Species	1	111.28	8.61	0.004
Family (species)	2	10.31	5.15	0.671
Error	259	3345.43	12.92	
Total	263			
GES2 (post hoc one-way ANOVA)				
Clutches by family	3, 260	24.60	3.89	0.010
Eggs by species	1, 262	22.70	0.87	0.351
Eggs by family	3, 260	162.10	2.11	0.099
Field investigations GES1 (GLM)				
Clutches	1	770.63	61.92	< 0.001
Family	4	125.46	2.52	0.044
Error	135	1680.04		
Total	140			
GES1 (post hoc one-way ANOVA)				
Clutches by family	4, 136	3.44	1.93	0.109
Eggs by family	4, 136	319.30	4.43	0.002
Laboratory investigations, binary choice assays (paired-sample *t* tests)				
N66 cf. G76 (3–4 f)	0.05 (1), 8	1.86	2.45	0.020
N64 cf. N66 (3–4 f)	0.05 (2), 6	2.45	8.49	< 0.001
Mac cf. N64 (3–4 f)	0.05 (1), 4	2.13	0.71	0.259
Cor cf. N64 (3–4 f)	0.05 (1), 4	2.13	4.23	0.007
N64 cf. N66 (1 f)	0.05 (2), 9	2.09	3.38	0.008
Laboratory investigations, multiple choice assays (GLM)				
Family	3	62.62	3.40	0.028
Female	12	218.44	2.96	0.006
Error	36	221.19		
Total	51			
Laboratory investigations, multiple choice assays (post hoc one-way ANOVA)				
Eggs by family	3, 48	62.62	2.28	0.091
Eggs by female	12, 39	218.44	2.50	0.015

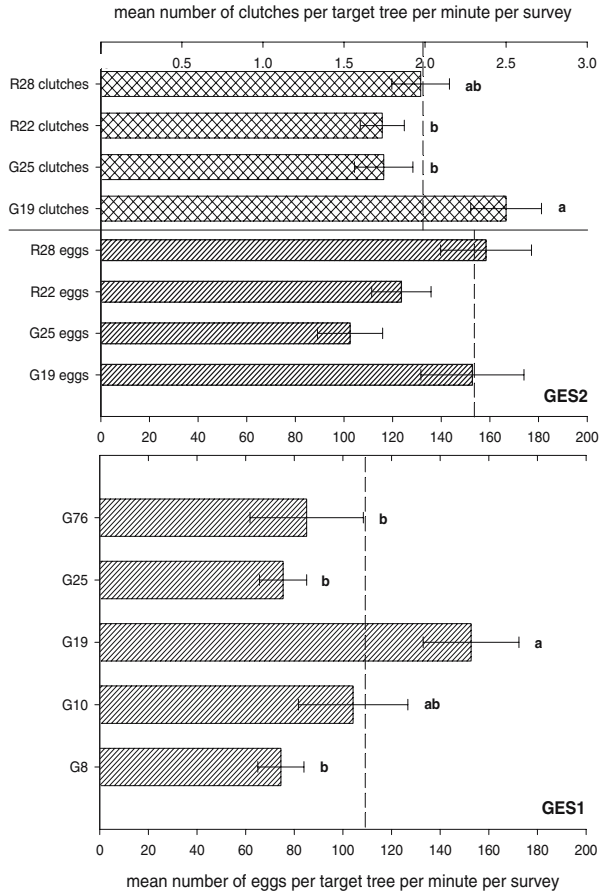

Fig. 2 Field observations of oviposition by *M. privata* on novel and natural hosts. (*GES2*) oviposition (mean number of clutches as well as mean number of eggs) on two families of *E. globulus* (*G19* and *G25*) and two families of *E. rubida* (*R28* and *R22*). *GES1* oviposition on five families of *E. globulus* (*G8*, *G10*, *G19*, *G25*, and *G76*). Data are means±SE *Dashed vertical lines* indicate mean overall oviposition. *Letters alongside bars* indicate statistical similarity of means

numbers of clutches laid on the different families of *E. globulus* and *E. rubida* (Table 3, Fig. 2). Specifically, *M. privata* laid most clutches on *E. globulus* family G19 with only slightly fewer clutches on *E. rubida* family R28. Female moths laid almost identical numbers of clutches on *E. globulus* family G25 and *E. rubida* family R22, but the numbers did not differ significantly from the number laid on *E. rubida* family R28.

In GES1, the family of tree as well as the number of clutches significantly influenced the number of eggs on individual *E. globulus* (Table 3). Of the five families of primary hosts, *M. privata* preferred to lay on *E. globulus* family G19, followed by family G10 with equal preference for the remaining three families (Fig. 2).

There were two statistically significant relationships between oviposition on the nine genotypes of tree in both arboreta and the concentrations of monoterpenes in their leaves. Oviposition was negatively related to the concentration of α-pinene but positively related to the concentration of α-terpineol (Fig. 3). Appendix 1 shows the monoterpene concentrations. An ordination of the genotypes from both arboreta is provided in Supplementary Material, Fig. 1.

Laboratory Investigations of Oviposition Preferences for Novel and Natural Hosts Despite the leaves of *E. nitens* N66 being equally waxy on both surfaces, whereas those of *E. globulus* are most waxy on the abaxial surface, *M. privata* preferred laying on branchlets of *E. globulus* than on branchlets of *E. nitens* N66 (Table 3, Fig. 4a). The moths preferred ovipositing on leaves of the waxiest *E.*

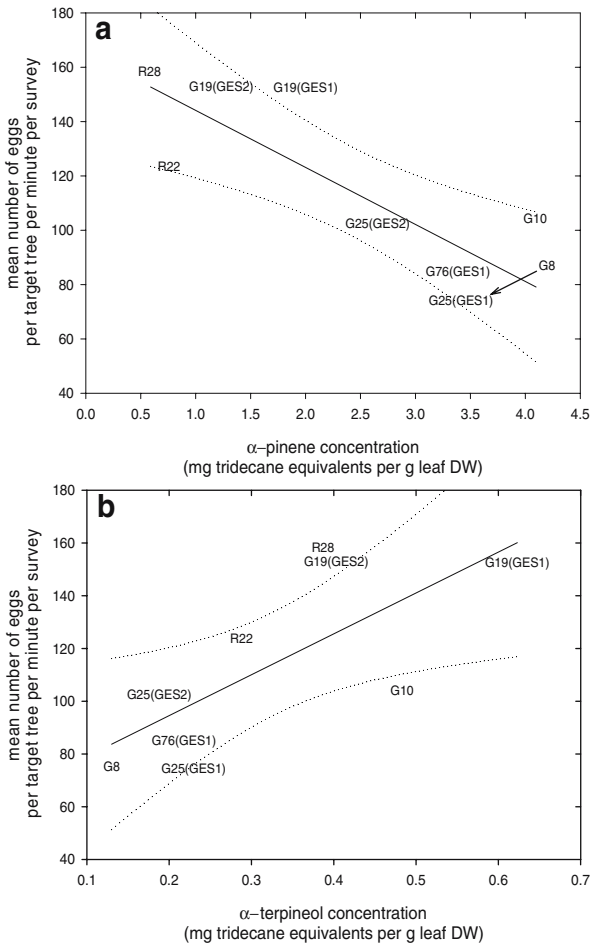

Fig. 3 Oviposition on families of *E. globulus* and *E. rubida* growing in GES2 and GES1 relative to monoterpene content. **a** Oviposition in relation to the concentration of α-pinene (location of G8 offset for clarity; number of eggs=−20.987×α-pinene concentration+165.092; r^2=66.8%, $F_{1,\ 7}$ =14.109, P=0.007; SE slope=5.6, P=0.007; SE intercept=15.2, P=0.001). **b** Oviposition in relation to the concentration of α-terpineol (number of eggs=154.984×α-terpineol concentration+63.560; r^2=52.9%, $F_{1,\ 7}$=7.866, P=0.026; SE slope=55.3, P=0.026; SE intercept=19.9, P=0.015)

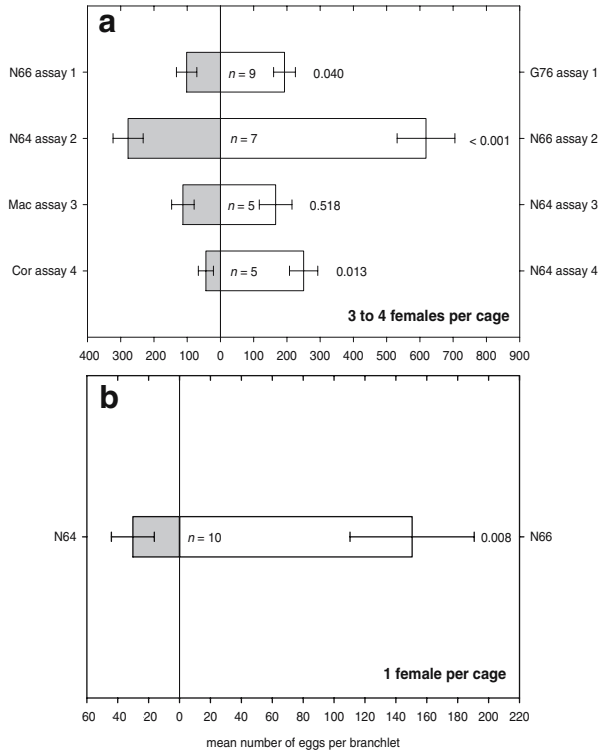

Fig. 4 Results of binary choice assays. **a** Oviposition by groups of three to four females per cage on branchlets of *E. nitens* N66 or *E. globulus* G76, *E. nitens* N64 or *E. nitens* N66, *E. macarthurii* (Mac) or *E. nitens* N64, and *C. eximia* (Cor) or *E. nitens* N64. **b** Oviposition by individual females on branchlets of *E. nitens* N64 or *E. nitens* N66. Data are means±SE. Two-tail probabilities, from paired-sample *t*-tests, given at RHS of bars

nitens, namely N66, to the third waxiest leaves, *E. nitens* N64. There was no difference in oviposition rates on the leaves of *E. macarthurii*, which possess structural waxes, compared to those of *E. nitens* N64 that produce nonstructural waxes. Least preferred were the leaves of *C. eximia*. Assays with 11 individual female moths offered *E. nitens* N64 and N66 tended to confirm the preferences obtained with groups of females. Ten females laid more eggs on leaves of *E. nitens* N66 than on leaves of N64 (P=0.008; Fig. 4b), but the other female laid 337 eggs on *E. nitens* N64 and only 51 eggs on N66 and reduced the probability of the preference response to P=0.061.

When individual *M. privata* were given a choice of four *E. nitens* on which to lay, they tended to choose them from most (N66) to least waxy (N63 then N64) (P=0.028; Fig. 5). Not surprisingly, the post hoc ANOVA indicated there were significant differences in the fecundities of the 13 females used in these assays.

Laboratory Investigations of Larval Survival and Performance on Novel and Natural Hosts All larvae reared on *C. eximia* died before completing the second instar (Fig. 6a). Not surprisingly, the survival curves for larvae on *C. eximia* differed significantly from those chosen for comparison (*E. globulus, E. nitens* N66, and *E. macarthurii*). Survival curves for these latter three did not differ (Table 4). Interestingly, larval survival was high and time to pupation short on *E. macarthurii*, although its leaves had the highest concentration of sideroxylonal (Fig. 6a and Appendix 1). In contrast, the leaves of *C. eximia* were devoid of sideroxylonal. The leaves of *C. eximia*, however, were the toughest of those in this set of assays, i.e., mean SLW of 0.124 mg per mm^2 compared to 0.079, 0.082, and 0.104 mg per mm^2 for the leaves of *E. nitens* N66, *E. globulus*, and *E. macarthurii*, respectively (Appendix 2).

Larvae survived significantly better on the leaves of *E. nitens* N66 and N63 than on those of N65 (Fig. 6b and

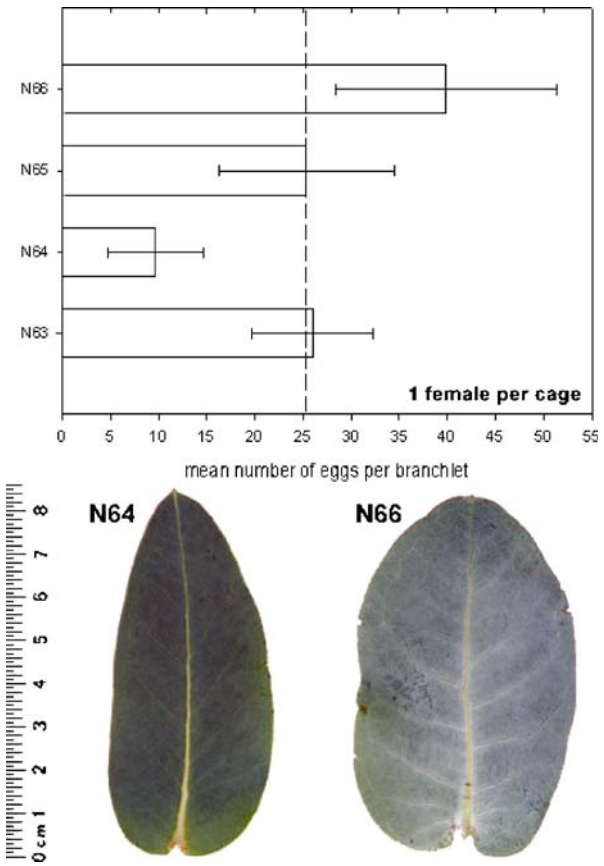

Fig. 5 Results of multiple choice assays with four *E. nitens*. Data are means±SE. *Dashed vertical line* indicates mean overall oviposition. The *bottom two images* are of the leaves of *E. nitens* N64 (least preferred) and of *E. nitens* N66 (most preferred) illustrating the relative abundance of epicuticular waxes. Epicuticular waxes are evenly distributed on both sides of juvenile *E. nitens* leaves

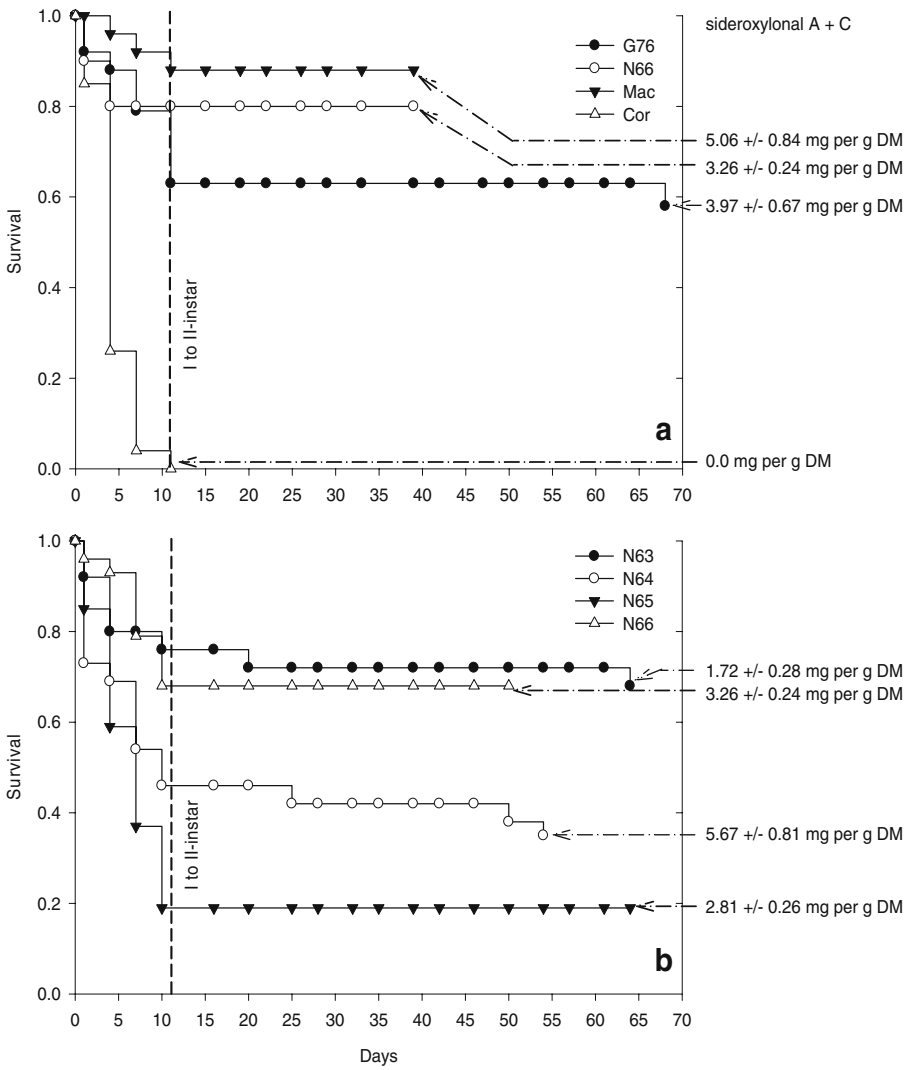

Fig. 6 Survival of larvae on novel and natural species of *Eucalyptus* and *Corymbia*. **a** Survival on leaves of *E. globulus* G76, *E. nitens* N66, *E. macarthurii* (Mac), and *C. eximia* (Cor). **b** Survival on leaves of *E. nitens* N63, N64, N65, and N66. Larval survival is shown relative to the total sideroxylonal content of leaves. Period to day 11 (indicated by *dashed vertical line*) encompasses larval development to end of the second instar

Table 4). As in the case of *C. eximia*, the higher mortality on leaves of *E. nitens* N65 was likely due in part to leaf toughness (Appendix 2). It is interesting to note that final larval survival on *E. nitens* N66 and N63 (both 0.68) as well as N64 (0.35) roughly matched the total sideroxylonal contents of the leaves of those trees (see Fig. 6b).

The dry weights of male pupae were less variable than those of female pupae (Fig. 7). With the exception of individuals fed *E. nitens* N63, male pupae tended to weigh the same irrespective of their larval diet (Table 4). In contrast, the weight of female pupae was less predictable. For example, there was little variation in the weight of female pupae reared on *E. globulus, E. macarthurii*, and *E. nitens* N66 in the first set of assays. There was, however, more variation in the weight of pupae reared on the four *E. nitens*. In particular, pupae reared on *E. nitens* N66 were significantly heavier than pupae reared on the leaves of *E. nitens* N64, whereas those fed *E. nitens* N63 were of intermediate weight. Thus, the results for larval performance exhibit a linkage to female oviposition preferences on these four *E. nitens*.

After a Bonferroni adjustment, there was only a single significant relationship ($P \leq 0.003$) between female pupal weight and one of the eight leaf traits measured. Female pupal weight was negatively related to the toughness of the leaves used to rear larvae (pupal weight=−1069.5×leaf toughness+354.6; r^2=95.1%, $F_{1,\ 6}$=76.9, P<0.001; SE slope=122.0, P<0.001; SE intercept=14.8, P<0.001). There was a trend toward a positive relationship between the weight of female pupae and foliar nitrogen (P=0.010). Leaf toughness and nitrogen were negatively related to one another (nitrogen content=−101.5×leaf toughness+25.7;

Table 4 Results of statistical analyses of larval survival and performance

Comparison or Source	θ or *df*	Variance Associated with θ or SS	Log-rank Statistic or *F* Value	*P* Value
Larval survival on G76, N66, Mac and Cor (log-rank tests)[a]				
G76 cf. N66	1.6	3.26	0.80	0.371
G76 cf. Mac	−3.5	2.85	4.41	0.036
G76 cf. Cor	12.9	5.35	31.05	<0.001
N66 cf. Mac	−1.5	1.93	1.21	0.271
N66 cf. Cor	12.1	5.11	28.44	<0.001
Mac cf. Cor	15.4	4.84	48.77	<0.001
Larval survival on N63, N64, N65 and N66 (log-rank tests)[a]				
N63 cf. N64	−5.9	5.35	6.62	0.010
N63 cf. N65	−9.0	5.89	13.78	0.002
N63 cf. N66	−0.4	3.74	0.04	0.84
N64 cf. N65	−3.5	7.58	1.57	0.21
N64 cf. N66	6.0	5.57	6.47	0.011
N65 cf. N66	9.6	6.03	15.14	<0.001
Larval performance on G76, N63, N64, N66 and Mac (one-way ANOVA; N65 not included, only two pupae of each gender; no larvae pupated on Cor)				
male pupal weight by species and family	4, 38	0.06	4.25	0.006
female pupal weight by species and family	4, 50	0.13	7.33	<0.001

[a] Bonferroni adjustment requires $P \leq 0.004$ for statistical significance.

r^2=73.7%, $F_{1,\ 6}$=11.23, P=0.029; SE slope=30.3, P=0.029; SE intercept=3.7, P=0.002).

Discussion

There were three main findings of this study. First, the laboratory assays confirmed the long-held assumption that the juvenile leaves of *E. globulus* are the primary host of *M. privata*. Second, the field observations and laboratory assays showed that female *M. privata* base their oviposition decisions on both nonstructural epicuticular wax and foliar monoterpene cues. Thus, they are as likely to lay their eggs on novel hosts as on a known host if the foliar chemistry resembles that of the primary host. Finally, tough leaves and high concentrations of PSMs decrease larval survival, with the fitness of female survivors apparently more likely to be adversely affected by natal host than male fitness. Importantly, these conclusions were reached by studying female oviposition preference and larval performance simultaneously and by using closely related species and families of eucalypts and a less closely related taxon within Myrtaceae, namely *C. eximia*.

By studying the oviposition preferences of *M. privata* for two members of the series Viminales (*E. macarthurii* with structural waxes and *E. nitens* with nonstructural waxes), our assays are the first to show that wax structural type can influence insect host assessment and oviposition. For some unknown reason, female *M. privata* preferred to lay more eggs on the least waxy family of *E. nitens* than on *E. macarthurii* that is characterized by structural waxes. The identity of the waxes that influence female oviposition remains unknown. However, because the chemical composition of a species' epicuticular waxes, as well as their method of crystallization, determines the physical structure of its wax layer, we can narrow the potential suite of biologically active wax compounds (Hallam and Chambers 1970; Carr et al. 1985). Specifically, Hallam and Chambers (1970) suggested that the presence or absence of β-diketones determined whether a eucalypt had tube or platelet type waxes, the presence of β-diketones being associated with the formation of tubes (nonstructural waxes) and the absence of β-diketones with the formation of platelets (structural waxes). Jones et al. (2002) and Rapley et al. (2004) found that reduced defoliation of certain families of *E. globulus* was correlated with high concentrations of benzyl *n*-tetracosanoate in the epicuticular waxes of those trees and suggested that this wax ester was an oviposition repellent. Steinbauer et al. (2004) argued that the epicuticular waxes of eucalypts were likely to be oviposition stimulants. Because, according to Hallam and Chambers (1970), wax esters are associated with both tube and platelet waxes, benzyl *n*-tetracosanoate seems even less likely to be the biologically active component responsible

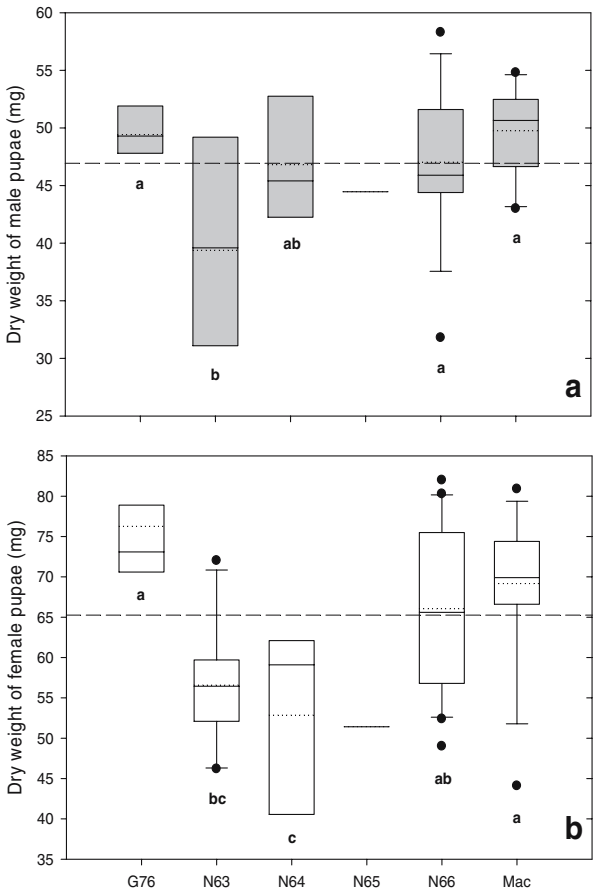

Fig. 7 Dry weights of pupae reared during survival assays. **a** Male pupae. **b** Female pupae. No larvae survived to pupation on *C. eximia*. *Figures* are box plots with standard error bars (if $N \geq 10$); the boundary of the *box nearest the x-axis* represents the 25th percentile, the *solid line within the box* marks the median, the *dotted line within the box* marks the mean, the *boundary of the box furthest from the x-axis* represents the 75th percentile and *solid circles* are outlying pupae. The *dashed horizontal lines* extending across each individual figure mark mean overall pupal weight. *Letters beneath boxes* indicate statistical similarity of means. Two male and two female pupae only were reared on *E. nitens* N65

for the repellence of oviposition by female *M. privata*. Final elucidation of the biological activity of particular wax compounds will require "boutique" synthesis and assay in choice experiments, perhaps with supplementary electrophysiological studies.

The possibility that host attractiveness for oviposition may be influenced by the concentrations of α-pinene and α-terpineol is of interest because there is apparently only one other parallel in insect–Myrtaceae relationships. Specifically, Wheeler and Ordung (2005) reported that the psyllid *Boreioglycaspis melaleuca* (Hemiptera: Psyllidae) laid more than twice as many eggs on a viridiflorol chemotype of its host (*Melaleuca quinquenervia*) than it did on a *E*-nerolidol chemotype. The families of *E. globulus* and *E. rubida* in GES2 and GES1 were best differentiated by their concentrations of 1,8-cineole (100%), followed by α-pinene (98.0%), and least differentiated by γ-terpinene (38.0%; see GNMDS analysis in Supplementary Material, Fig. 1). The leaves of *E. globulus* had two to three times more 1,8-cineole than did those of *E. rubida*, but *M. privata* laid a similar number of eggs on each species or family. Therefore, if female *M. privata* prefer hosts with high concentrations of 1,8-cineole, they should not choose *E. rubida* when there are ample *E. globulus* nearby. Our results, however, suggest that *M. privata* do not discriminate hosts so simply. In exploratory analyses not presented here, there was a negative relationship (r^2=94.6%) between oviposition and the ratio of the concentrations of α-pinene to 1,8-cineole. There was, however, a close positive correlation between the concentrations of α-pinene and 1,8-cineole (r^2=65.4%), which illustrates how difficult it is to separate the influences on oviposition of individual monoterpenes. Finally, terpenes that occur in lower concentrations than 1,8-cineole or α-pinene should not be ignored because the antennae of female moths responded to several of these aromatics, e.g., terpinolene, α-campholene aldehyde, α-terpineol, and possibly also *trans*-pinocarveol (see Steinbauer et al. 2004).

Exaptation of *M. privata* larvae enables them to eat the leaves of more species of eucalypt (notably those with glossy leaves) than females would choose to oviposit upon. Irrespective of this, high leaf toughness is universally detrimental to neonates because their small size and gape constrains the physical forces they can exert on leaf surfaces. Steinbauer and Matsuki (2004) reported that small groups of neonate *M. privata* larvae (e.g., ≤5 larvae) were unable to create a feeding scar when leaf toughness exceeded 0.20 mg per mm^2. High leaf toughness may partly explain the deaths of all the larvae on *C. eximia* before the completion of the second instar. However, there were probably additional reasons for the demise of the larvae fed *Corymbia*, although sideroxylonal could not have been among them because the species is devoid of this compound. For example, *C. eximia* differed notably from others used in the laboratory investigations by its high concentrations of α-pinene and γ-terpinene (Supplementary Material, Fig. 2). Exactly how sideroxylonal might be detrimental to neonate *M. privata* is a mystery. Research by Moore and Evertz (2006, unpubl.) has shown that sideroxylonal is most likely synthesized by the cells surrounding the oil glands and may be stored within the oil glands. However, neonate and second instars typically

feed on young, expanding leaves and, even then, around oil glands (Steinbauer and Matsuki 2004). Because oil glands are not fully formed until leaf expansion is complete (Carr and Carr 1970), it seems probable that young larvae ingest sideroxylonal by rupturing or swallowing oil gland progenitor cells. Oil gland progenitor cells synthesize sideroxylonal and monoterpenes before the maturation of the oil glands and are densely packed in expanding leaves (Moore, pers. comm.). When larvae are large enough to ingest whole leaf fragments (i.e., from the third instar onward), they are exposed to the sideroxylonal in the leaves of their hosts because they disrupt and swallow mature oil glands.

The lower larval survival on hosts that produce both sideroxylonal and modest to high concentrations of monoterpenes vs. the higher survival of larvae on a host that produces high concentrations of sideroxylonal but virtually no monoterpenes (namely *E. macarthurii*) could suggest that variations in sideroxylonal concentration alone do not determine survival. [According to Boland et al. (1991), *E. macarthurii* has a total oil content between 0.8 and 1.1% (fresh weight), but its main component is geranyl acetate, followed by β-, α-, and γ-eudesmol, respectively]. The severity of defoliation by adult Christmas beetles of trees of six species of eucalypt was negatively correlated with the concentration of 1,8-cineole in leaves (Edwards et al. 1993). Edwards et al. (1993) did not attribute this reduction in feeding solely to cineole. It has subsequently been shown in folivorous marsupials of eucalypts only (although a similar interaction is considered likely to occur in insects also) that high cineole odors act as a cue for the presence of high concentrations of sideroxylonal, which is actually the compound that the animals appear to avoid (Lawler et al. 2000). McLean et al. (2004) have shown that the biological activity of jensenone and sideroxylonal arises because the functional aldehyde groups of both compounds bind with the natural amines on the animal's gut wall (again, a similar mechanism is thought likely to also occur in insect herbivores). Hence, there is some evidence for interactive effects between monoterpenes and FPCs—albeit pre-ingestion. Because a number of species of insects metabolize certain foliar monoterpenes (Ohmart and Larsson 1989; Fletcher et al. 2000; Schmidt et al. 2000; Southwell et al. 1995, 2003), perhaps, the addition of high concentrations of sideroxylonal to the ingesta overwhelms the capacity of the insect gut to degrade all the compounds, and it is via this mode of action that sideroxylonal affects larval survival. If high oil content alone antagonized the toxicity of moderate concentrations of sideroxylonal, such an interaction should have been noted sooner than now because the relationship would hold across different *Eucalyptus* species that synthesize it. Because *E. nitens* N64 (the family least preferred by female and least suitable for larvae) can be only partially differentiated from trees of the three other families of *E. nitens* by its concentrations of α-terpineol, 1,8-cineole, and limonene (Supplementary Material, Fig. 3), perhaps attention should be given to the interactions of these monoterpenes and sideroxylonal.

The phenomenon surrounding the occurrence of outbreaks of *M. privata* in mixed plantations of *E. globulus* and other eucalypts may be a consequence of the insect experiencing a variety of primary host and other eucalypt monoterpene odors. Liu et al. (2005) reported that *Plutella xylostella* (Lepidoptera: Plutellidae) could be attracted to non-host volatiles on a preferred host plant if they had previously experienced the odor. By this scenario, *M. privata* that develop on *E. globulus* might acquire an induced preference for the neighboring species of eucalypt, thus increasing the chance that they might later oviposit on them. The results of Linn et al. (2005) that showed that most *Rhagoletis pomonella* (Diptera: Tephritidae) fly toward the odor of their developmental host suggest that such a change would happen slowly. Likewise, the similarity of the natal host and the non-host is likely to influence the extent to which slight alterations of preference result in expansion onto the novel species (Bengtsson et al. 2006). Perhaps, given this background, outbreaks of *M. privata* in single species plantations of novel species of eucalypt are as yet unknown.

We suggest that our findings demonstrate how similarities between novel and preferred eucalypt species, both in terms of their epicuticular wax and monoterpene compositions, may have facilitated the host expansions by *M. privata* that have been observed in the past couple of decades. From an applied perspective, an important question to address would be whether host races of *M. privata* now exist. Future studies should examine whether females prefer to oviposit on the species on which they develop and whether they prefer to mate with males that have developed on that same host. It would also be important to know whether the limits to the detoxification capacity of larvae have prevented the occurrence of a greater number of host expansion events than have already been witnessed.

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Appendix 1

Table 5 Results of foliar chemical analyses

Family	α-Pinene	Limonene	1,8-Cineole	γ-Terpinene	α-Terpineol	Sideroxylonal A	Sideroxylonal C	Nitrogen
Field investigations GES2 n=4 leaves (SE are per tree estimates [leaves from two trees per family])								
R28	0.60^{b}±0.033	0.92^{a}±0.163	5.92^{ab}±0.029	0	0.39±0.004			
R22	0.76^{b}±0.055	1.66^{a}±0.299	4.88^{b}±0.794	0.007±0.010	0.29±0.033			
G25	2.66^{a}±0.322	0.01^{b}±0.004	12.02^{ab}±4.571	0.005±0.007	0.19±0.180			
G19	1.23^{b}±0.025	0.05^{b}±0.004	14.50^{a}±1.294	0.88±0.127	0.40±0.088			
Field investigations GES1 n=4 leaves (SE are per tree estimates [leaves from two trees per family])								
G76	3.38±0.831	0.05±0.025	12.22^{b}±1.025	0.04±0.021	0.22^{b}±0.067			
G25	3.42±1.128	0.02±0.007	12.21^{b}±0.286	0	0.23^{b}±0.007			
G19	2.00±0.124	0.04±0.004	18.43^{ab}±0.877	0.07±0.064	0.62^{a}±0.088			
G10	4.10±0.103	0.27±0.343	19.68^{a}±1.439	0	0.48^{a}±0.060			
G8	3.62±0.332	0.03±0.000	15.20^{ab}±0.735	0.01±0.014	0.13^{b}±0.078			
Laboratory investigations								
	n=12 leaves					n=10		n=4
G76	$0.84±0.044^{b}$	$0.69±0.053^{a}$	$9.14±0.577^{a}$	$0.02±0.002^{b}$	$0.30±0.027^{a}$	$2.82±0.465^{ab}$	$1.15±0.203^{ab}$	$15.4±0.81^{a}$
N63	$0.45±0.043^{c}$	$0.06±0.007^{c}$	$1.07±0.093^{c}$	$0.01±0.002^{b}$	$0.02±0.008^{c}$	$1.19±0.204^{b}$	$0.53±0.078^{b}$	$13.6±0.78^{ab}$
N64	$0.60±0.032^{bc}$	$0.13±0.007^{b}$	$1.77±0.085^{b}$	0	$0.28±0.015^{a}$	$4.17±0.642^{a}$	$1.50±0.241^{a}$	$12.3±0.41^{b}$
N65	$0.46±0.029^{c}$	$0.07±0.005^{c}$	$1.52±0.073^{bc}$	0	$0.09±0.008^{b}$	$1.98±0.185^{b}$	$0.83±0.078^{ab}$	$8.4±0.46^{c}$
N66	$0.74±0.050^{bc}$	$0.10±0.005^{bc}$	$1.32±0.061^{c}$	0	$0.06±0.005^{bc}$	$2.29±0.167^{ab}$	$0.97±0.072^{ab}$	$15.0±0.77^{a}$
Cor	$2.26±0.166^{a}$	$0.03±0.002^{c}$	$0.03±0.002^{d}$	$0.34±0.028^{a}$	0	0	0	$14.5±0.29^{a}$
Mac	0	0	0	0	0	$3.72±0.617^{a}$	$1.34±0.227^{a}$	$17.0±0.89^{a}$

Data are Means±SE. Superscripted letters down columns for same investigation indicate mean difference at $P \leq 0.05$. Concentrations of monoterpenes are as milligrams tridecane equivalents per gram of leaf dry weight. All other concentrations are as actual milligrams per gram leaf dry weight.

Appendix 2

Table 6 Leaf toughnesses (in milligrams per square millimeter) and water contents (in percentages) of leaves representative of those used in laboratory investigations

Assay	Host 1	Host 2	Host 3	Host 4
Oviposition preference				
Binary 1	N66	G76		
Toughness	$0.151±0.0062^{a}$	$0.111±0.0049^{b}$		
Water	$45.8±0.46^{b}$	$54.9±0.54^{a}$		
Binary 2	N64	N66		
Toughness	$0.147±0.0065^{a}$	$0.119±0.0042^{b}$		
water	50.7±0.78	49.6±0.67		
Binary 3	Mac	N64		
Toughness	$0.104±0.0037^{b}$	$0.144±0.0054^{a}$		
Water	47.2±1.35	47.5±0.37		
Binary 4	Cor	*N64*		
Toughness	0.124±0.0034	0.135±0.0050		
Water	$59.1±0.94^{a}$	$52.6±0.62^{b}$		
Multiple	N63 n=20 leaves	N64	N65	N66
Toughness	$0.125±0.0057^{b}$	$0.131±0.0027^{b}$	$0.154±0.0051^{a}$	$0.118±0.0030^{b}$
Water	52.4±0.75	54.4±0.70	46.8±0.94	52.1±0.48
Larval survival and performance				
	Mac[†]	Cor[†]	N66	G76
Toughness	0.104	0.124	0.079±0.0021	0.082±0.0033
Water	47.2	59.1	$61.8±0.66^{b}$	$65.3±1.03^{a}$
	N63	N64	N65	N66
Toughness	$0.137±0.0041^{a}$	$0.132±0.0034^{ab}$	$0.150±0.0078^{a}$	$0.115±0.0045^{b}$
Water	$49.5±0.34^{a}$	$48.1±0.40^{b}$	$46.0±0.27^{c}$	$50.3±0.30^{a}$

[†] These means are the same as those measured for leaves used in binary choice assays 3 and 4. The leaves used in the choice assays were harvested on 31 January and 7 February, respectively, whereas the survival assay was begun on 6 March. Therefore, the toughness of the leaves of *C. eximia* and *E. macarthurii* that were used in this assay was not thought necessary to measure because they were likely to be comparable to those of leaves harvested earlier

Data are means±SE, N=10 leaves unless otherwise stated. Superscripted letters along rows indicate mean difference at $P \leq 0.05$

References

BENGTSSON, M., JAASTAD, G., KNUDSEN, G., KOBRO, S., BÄCKMAN, A.-C., PETTERSSON, E., and WITZGALL, P. 2006. Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella. Entomol. Exp. Appl.* 118:77–85.

BOLAND, D. J., BROPHY, J. J., and HOUSE, A. P. N. 1991. *Eucalyptus* Leaf Oils: Use, Chemistry, Distillation and Marketing. Inkata Press, Melbourne, Australia.

BRABY, M. F. and TRUEMAN, J. W. H. 2006. Evolution of larval host plant associations and adaptive radiation in pierid butterflies. *J. Evol. Biol.* 19:1677–1690.

BROOKER, M. I. H. 2000. A new classification of the genus *Eucalyptus* L'Hér. (Myrtaceae). *Aust. Syst. Bot.* 13:79–148.

CARR, D. J. and CARR, S. G. M. 1970. Oil glands and ducts in *Eucalyptus* L'Hérit. II. Development and structure of oil glands in the embryo. *Aust. J. Bot.* 18:191–212.

CARR, D. J., CARR, S. G. M., and LENZ, J. R. 1985. Oriented arrays of epicuticular wax plates in *Eucalyptus* species. *Protoplasma* 124:205–212.

CHIPPENDALE, G. M. 1988. Flora of Australia, Volume 19. Myrtaceae—*Eucalyptus*, *Angophora*. Australian Government Publishing Service, Canberra, Australia.

EDWARDS, P. B., WANJURA, W. J., and BROWN, W. V. 1993. Selective herbivory by Christmas beetles in response to intraspecific variation in *Eucalyptus* terpenoids. *Oecologia* 95:551–557.

ESCHLER, B. M., PASS, D. M., WILLIS, R., and FOLEY, W. J. 2000. Distribution of foliar formylated phloroglucinol derivatives amongst *Eucalyptus* species. *Biochem. Syst. Ecol.* 28:813–824.

EVANS, J. W. 1943. Insect Pests and Their Control. Government Printer, Hobart, Australia.

FLETCHER, M. T., LOWE, L. M., KITCHING, W., and KONIG, W. A. 2000. Chemistry of Leichhardt's grasshopper, *Petasida ephippigera*, and its host plants, *Pityrodia jamesii*, *P. ternifolia* and *P. pungens*. *J. Chem. Ecol.* 26:2275–2290.

FRENCH, C. 1900. A Handbook of the Destructive Insects of Victoria, With Notes on the Methods to be Adopted to Check and Extirpate Them. Part III. Government Printer, Melbourne, Australia.

FROGGATT, W. W. 1923. Forest Insects of Australia. Government Printer, Sydney, Australia.

GROSMAN, A. M., VAN BREEMEN, M., HOLTZ, A., PALLINI, A., RUGAMA, A. M., PENGEL, H., VENZON, M., ZANUNCIO, J. C., SABELIS, M. W., and JANSSEN, A. 2005. Searching behaviour of an omnivorous predator for novel and native host plants of its herbivores: a study on arthropod colonisation of *Eucalyptus* in Brazil. *Entomol. Exp. Appl.* 116:135–142.

HALLAM, N. D. and CHAMBERS, T. C. 1970. The leaf waxes of the genus *Eucalyptus* L'Héritier. *Aust. J. Bot.* 18:335–386.

HILL, K. D. and JOHNSON, L. A. S. 1995. Systematic studies in the eucalypts 7. A revision of the bloodwoods, genus *Corymbia* (Myrtacaeae). *Telopea* 6:185–504.

HOBBS, R. J., FLOYD, R. B., CUNNINGHAM, S. A., CATLING, P., and IVE, J. 2003. Hardwood Plantations: Quantifying Conservation and Environmental Service Benefits. Rural Industries Research and Development Corporation, Canberra, Australia. http://www.rirdc.gov.au/reports/AFT/02-83.pdf

JANZ, N. and NYLIN, S. 1998. Butterflies and plants: a phylogenetic study. *Evolution* 52:486–502.

JANZ, N., NYBLOM, K., and NYLIN, S. 2001. Evolutionary dynamics of host-plant specialisation: a case study of the tribe Nymphalini. *Evolution* 55:783–796.

JONES, T. H., POTTS, B. M., VAILLANCOURT R. E., and DAVIES, N. W. 2002. Genetic resistance of *Eucalyptus globulus* to autumn gum moth defoliation and the role of cuticular waxes. *Can. J. For. Res.* 32:1961–1969.

LADIGES, P. Y. and UDOVICIC, F. 2000. Comment on a new classification of the eucalypts. *Aust. Syst. Bot.* 13:149–152.

LADIGES, P. Y., UDOVICIC, F., and DRINNAN, A. N. 1995. Eucalypt phylogeny—molecules and morphology. *Aust. Syst. Bot.* 8:483–497.

LAWLER, I. R., FOLEY, W. J., and ESCHLER, B. M. 2000. Foliar concentrations of a single toxin creates habitat patchiness for a marsupial folivore. *Ecology* 81:1327–1338.

LI, H., MADDEN, J. L., and POTTS, B. M. 1995. Variation in volatile leaf oils of the Tasmanian *Eucalyptus* species I. Subgenus *Monocalyptus*. *Biochem. Syst. Ecol.* 23:299–318.

LI, H., MADDEN, J. L., and POTTS, B. M. 1996. Variation in volatile leaf oils of the Tasmanian *Eucalyptus* species II. Subgenus *Symphyomyrtus*. *Biochem. Syst. Ecol.* 24:547–569.

LI, H., MADDEN, J. L., and POTTS, B. M. 1997. Variation in leaf waxes of the Tasmanian *Eucalyptus* species I. Subgenus *Symphyomyrtus*. *Biochem. Syst. Ecol.* 25:631–657.

LINN Jr., C. E., DAMBROSKI, H., NOJIMA, S., FEDER, J. L., BERLOCHER, S. H., and ROELOFS, W. L. 2005. Variability in response specificity of apple, hawthorn, and flowering dogwood-infesting *Rhagoletis* flies to host fruit volatile blends: implications for sympatric host shifts. *Entomol. Exp. Appl.* 116:55–64.

LIU, S.-S., LI, Y.-H., LIU, Y.-Q., and ZALUCKI, M. P. 2005. Experience-induced preference for oviposition repellents derived from a non-host plant by a specialist herbivore. *Ecol. Letters* 8:722–729.

LOCH, A. D. and FLOYD, R. B. 2001. Insect pests of Tasmanian blue gum, *Eucalyptus globulus globulus*, in south-western Australia: History, current perspectives and future prospects. *Austral Ecol.* 26:458–466.

MCLEAN, S., BRANDON, S., DAVIES, N. W., FOLEY, W. J., and MULLER, H. K. 2004. Jensenone: biological reactivity of a marsupial antifeedant from *Eucalyptus*. *J. Chem. Ecol.* 30:19–36.

MOORE, K. M. 1972. Observations on some Australian forest insects 26. Some insects attacking three important tree species. *Aust. Zool.* 17:30–39.

MURPHY, S. M. and FEENY, P. 2006. Chemical facilitation of a naturally occurring host shift by *Papilio machaon* butterflies (Papilionidae). *Ecol. Mono.* 76:399–414.

OHMART, C. P. and LARSSON, S. 1989. Evidence for absorption of eucalypt essential oils by *Paropsis atomaria* Olivier (Coleoptera: Chrysomelidae). *J. Aust. Ent. Soc.* 28:201–205.

RAPLEY, L. P., ALLEN, G. R., and POTTS, B. M. 2004. Susceptibility of *Eucalyptus globulus* to *Mnesampela privata* defoliation in relation to a specific foliar wax compound. *Chemoecology* 14:157–163.

ROBERTS, R. J. and SAWTELL, N. L. 1981. Survival and growth of local and other eucalypts planted on the Northern Tablelands, pp. 87–94, in K. M. Old, G. A. Kile, and C. P. Ohmart (eds.). Eucalypt Dieback in Forests and Woodlands. CSIRO, Melbourne, Australia.

SCHMIDT, S., WALTER, G. H., and MOORE, C. J. 2000. Host plant adaptations in myrtaceous-feeding pergid sawflies: essential oils and the morphology and behaviour of *Pergagrapta* larvae (Hymenoptera, Symphyta, Pergidae). *Biol. J. Linn. Soc.* 70:15–26.

SOUTHWELL, I. A., MADDOX, C. D. A., and ZALUCKI, M. P. 1995. Metabolism of 1,8-cineole in tea tree (*Melaleuca alternifolia* and *M. linariifolia*) by pyrgo beetle (*Paropsisterna tigrina*). *J. Chem. Ecol.* 21:439–453.

SOUTHWELL, I. A., RUSSELL, M. F., MADDOX, C. D. A., and WHEELER, G. S. 2003. Differential metabolism of 1,8-cineole in insects. *J. Chem. Ecol.* 29:83–94.

STEINBAUER, M. J. 2002. Oviposition preference and neonate performance of *Mnesampela privata* in relation to heterophylly

in *Eucalyptus dunnii* and *E. globulus*. *Agric. For. Entomol.* 4:245–253.

STEINBAUER, M. J. and MATSUKI, M. 2004. Suitability of *Eucalyptus* and *Corymbia* for *Mnesampela privata* (Guenée) (Lepidoptera: Geometridae) larvae. *Agric. For. Entomol.* 6:323–332.

STEINBAUER, M. J. and WANJURA, W. J. 2002. Christmas beetles (*Anoplognathus* spp., Coleoptera: Scarabaeidae) mistake peppercorn trees for eucalypts. *J. Nat. Hist.* 36:119–125.

STEINBAUER, M. J., SCHIESTL, F. P., and DAVIES, N. W. 2004. Monoterpenes and epicuticular waxes help female autumn gum moth differentiate between waxy and glossy *Eucalyptus* and leaves of different ages. *J. Chem. Ecol.* 30:1117–1142.

STONE, C. and URQUHART, C. A. 1992. Effects of treating young hand planted eucalypts with selected systemic insecticides, and identification of associated foliage insects. *Gen. Appl. Entomol.* 24:53–63.

UDOVICIC, F., MCFADDEN, G. I., and LADIGES, P. Y. 1995. Phylogeny of *Eucalyptus* and *Angophora* based on 5S rDNA spacer sequence data. *Mol. Phylogen. Evol.* 4:247–256.

WALLIS, I. R., HERLT, A. J., ESCHLER, B. M., TAKASAKI, M., and FOLEY, W. J. 2003. Quantification of sideroxylonals in *Eucalyptus* foliage by high-performance liquid chromatography. *Phytochem. Anal.* 14:360–365.

WARDELL-JOHNSON, G. W., WILLIAMS, J. E., HILL, K. D., and CUMMING, R. 1997. Evolutionary biogeography and contemporary distribution of eucalypts, pp. 92–128, in J. E. Williams and J. C. Woinarski (eds.). Eucalypt ecology: Individuals to Ecosystems. Cambridge University Press, Cambridge, UK.

WHEELER, G. S. and ORDUNG, K. M. 2005. Secondary metabolite variation affects the oviposition preference but has little effect on the performance of *Boreioglycaspis melaleucae*: A biological control agent of *Melaleuca quinquenervia*. *Biol. Cont.* 35:115–123.

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Host Recognition by the Specialist Hoverfly *Microdon mutabilis*, a Social Parasite of the Ant *Formica lemani*

Karsten Schönrogge · Emma K. V. Napper · Michael A. Birkett · Christine M. Woodcock · John A. Pickett · Lester J. Wadhams · Jeremy A. Thomas

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Abstract The larva of the hoverfly *Microdon mutabilis* is a specialist social parasite of the ant *Formica lemani* that is adapted to local groups of *F. lemani* colonies but maladapted to colonies of the same species situated only a few hundred meters away. At a study site in Ireland, *F. lemani* shares its habitat with four other ant species. All nest under stones, making the oviposition choice by *M. mutabilis* females crucial to offspring survival. In this study, we tested the hypothesis that, as an extreme specialist, *M. mutabilis* should respond to cues derived from its host rather than from its microenvironment, a phenomenon that has hitherto only been addressed in the context of herbivorous insects and their parasitoids. In behavioral assays, *M. mutabilis* females reacted to volatiles from *F. lemani* colonies by extending their ovipositors, presumably probing for an oviposition substrate. This behavior was not observed toward negative controls or volatiles from colonies of *Myrmica scabrinodis*, the host ant of the closely related *Microdon myrmicae*. Coupled gas chromatography-electroantennography (GC-EAG) that used antennal preparations of *M. mutabilis* located a single physiologically active compound within an extract of heads of *F. lemani* workers. Coupled GC-mass spectrometry (GC-MS) tentatively identified the compound as a methylated methylsalicylate. GC co-injection of the extract with authentic samples showed that of the four possible isomers (methyl 3-, 4-, 5-, and 6-methylsalicylate), only methyl 6-methylsalicylate co-eluted with the EAG-active peak. Furthermore, the response to methyl 6-methylsalicylate was four times higher than to those of the other isomers. Coupled GC-EAG and GC-MS also revealed physiological responses to two constituents, 3-octanone and 3-octanol, of the *M. scabrinodis* alarm pheromone. However, the behavioral trials did not reveal any behavior that could be attributed to these compounds. Results are discussed in the context of four phases of host location behavior, and of the characteristics, which volatile cues should provide to be useful for an extreme specialist such as *M. mutabilis*.

Keywords Host/parasite interactions · Host recognition behavior · Syrphidae · Formicidae · EAG · GC · Methyl 6-methylsalicylate

K. Schönrogge (✉) · E. K. V. Napper
Centre for Ecology and Hydrology, CEH Wallingford,
Maclean Building, Benson Lane,
Wallingford OX10 8BB, UK
e-mail: ksc@ceh.ac.uk

E. K. V. Napper · M. A. Birkett · C. M. Woodcock · J. A. Pickett · L. J. Wadhams
Biological Chemistry Division, Rothamsted Research,
Harpenden AL5 2JQ, UK

J. A. Thomas
Department of Zoology, University of Oxford,
Tinbergen Building, South Parks Road,
Oxford OX1 3PS, UK

Introduction

Host localization and recognition is crucial in the lives of female parasites and parasitoids (Godfray 1994), particularly when the larval stages do not disperse and are unlikely to detect a host themselves (Sadeghi and Gilbert 2000). In these situations, an ovipositing female has to locate suitable hosts, which may be hidden in a complex environment, widely dispersed or rare, making detection difficult (Morehead and Feener 2000; Morehead et al. 2001).

There are typically four stages to host localization: (1) finding a suitable habitat for the host, (2) locating the host

within that habitat, (3) accepting the host, and finally (4) ovipositing (Godfray 1994; Sutherland et al. 2001). Throughout these stages, parasites rely on a range of cues, including olfactory, visual, and/or acoustic signals. These may come directly from the host animal or plant, or in combination from the host and the microenvironment (Godfray 1994). The response of females to cues can be genetically fixed, although an increasing number of studies show that cues can also be acquired by conditioning and possibly by learning (Godfray 1994).

Whether parasites and parasitoids use habitat or host-derived cues is thought to reflect their level of host specificity (Vet and Dicke 1992; Vet et al. 1993; Cortesero et al. 1997; De Moraes and Mescher 2005). Although habitat-derived cues may be adaptive for generalists, host-derived cues are used for host location and recognition by parasitoids that attack one or a few species (van Alphen and Visser 1990; Godfray 1994). However, aphid attack also can cause host plants to produce different chemical signals, depending on the species of aphid, and thereby attract a specialized parasitoid (Du et al. 1998). Host pheromones provide useful host recognition cues. Because of their consistency and volatility, they provide easily detectable and reliable cues both for intraspecific communication and for use as kairomones (Feener et al. 1996; Orr et al. 2003; Powell and Pickett 2003). As the compounds are fundamental to host communication, there may be a cost in reducing pheromone production to avoid parasitoid attack (Godfray 1994). Of course, different cues may be utilized through the four stages of host location (Dicke et al. 1985). Although volatile chemicals are long-range indicators of the presence of a host, these may be shared by several co-existing species; less volatile, species-specific compounds might then be used for host acceptance (Morehead 2001).

The larvae of the syrphid fly *Microdon mutabilis* live as predators of ant brood in colonies of their host ant *Formica lemani* (Elmes et al. 1999; Schönrogge et al. 2006). Like many hosts of social parasites, *F. lemani* is moderately polygyne, at least in populations infested by *Microdon* (Gardner et al. 2007). *M. mutabilis* has a short free-living adult stage of up to 10 d in the laboratory but probably <5 d in the wild, and females oviposit in the environment of *F. lemani* (Schönrogge et al. 2006). The larvae apparently develop for 2 yr before pupation in the ant nest, from which the emerging adult has to escape (Schönrogge et al. 2000). The distinctive feature of *M. mutabilis*' relationship with *F. lemani* is its extreme host specificity, in which a hoverfly population is specific not only to *F. lemani* as a species, but to certain *F. lemani* nests within a host ant population (Elmes et al. 1999; Schönrogge et al. 2006). The exact mechanism by which *M. mutabilis* infiltrates host colonies is unknown, although there are indications that it relates to chemical compounds deposited by the females on the eggs (Elmes et al. 1999; Schönrogge et al. 2006). One consequence of host specificity at this level is the limited dispersal by *M. mutabilis* females of just a few meters from the natal nest (Schönrogge et al. 2006). Even over such short distances, however, the female would improve survival chances of her offspring if she could recognize colonies of *F. lemani* for oviposition.

This study was carried out on The Burren, Ireland, the largest outcrop of limestone pavement in Europe where *F. lemani*, like other ant species, builds nests under loose stones. We characterized the environment in which *M. mutabilis* females identify suitable ant nests for oviposition. Then, by using a combination of behavioral assays, electrophysiological studies, and chemical analysis, we identified olfactory host location and/or oviposition cues, thus testing the hypothesis that the extreme specificity of *M. mutabilis* originates from the host directly, rather than from the environment.

Methods and Materials

Distribution of Ant Nests within Sites All species of ants were mapped in three sites (1,000–1,500 m^2 and 6–24 km apart) on the Burren, Co. Claire, Ireland in May 2000, and the position of every nest was determined by triangulation by using a Laser Range Finder (Laser Technologies, Inc.). The Burren represents a roughly circular area of limestone, about 25 km in diameter. Both *F. lemani* and *M. mutabilis* were found locally throughout the Burren, although valleys with dense hazel undergrowth may be barriers to dispersal, and little is known about the population structure across this large area (Schönrogge et al. 2006). At three sites, all ant species were recorded.

Collection and Maintenance of Microdon and Host Ants One hundred *M. mutabilis* pupae were collected from *F. lemani* nests on Mull, Scotland, in 2001 and on the Burren in 2003. Emerging males and females were mated and the females left at 16°C for 24–72 hr, and then acclimatized at 20°C for 1 hr before being used in experiments. Five females from Mull were used in electrophysiological studies, and 44 females from the Burren were used in the olfactometer experiments.

Samples of at least 150 workers were taken from each of six *F. lemani* nests on the Burren and from six colonies of *Myrmica scabrinodis*, in Dorset, UK, which until recently was thought to be an alternative host for *M. mutabilis* and is host to the closely related hoverfly *Microdon myrmicae* (Schönrogge et al. 2002). Laboratory colonies of 100 workers from each colony were established in nest boxes comprising two small plastic arenas, with a 3-cm^2 piece of

sponge cloth covered in glass and secured with an elastic band (Fig. 1) (Elmes et al. 1991). All workers were introduced to the laboratory colonies at least 24 hr before olfactometer experiments began. A second set of nest boxes without ants was used as a control. All experimental and control nest boxes contained 0.1 g of *Drosophila melanogaster* larvae and 0.2 g sugar granules as ant food, and the sponge cloth was kept moist.

Olfactometer Set-up and Behaviors Scored A four-way olfactometer, comprising a square Perspex arena with treatment arms at each corner, was used (Fig. 1). This design was chosen because *M. mutabilis* females did not acclimatize well in the traditional Pettersson four-way olfactometer (Al Abassi et al. 2001), and the additional depth of our olfactometer allowed females to display a larger range of behaviors (Fig. 1). Four test areas of equal size were designated, each containing a treatment arm at the center. A nest box was connected to each treatment arm, containing either *F. lemani* or *M. scabrinodis* workers (treatment), or no ants (control). Olfactometers were set up with two treatment and two control arms, with like arms opposite.

All *M. mutabilis* females were mated to ensure that they were motivated to find ant colonies in which to lay eggs (virgin females were not tested). Also, the females had no contact with ants after pupation. Females were placed individually into the olfactometer, which was closed with a glass lid. An airflow of 500 ml/min was established by using a pump attached to a hole in the base of the olfactometer. The airflow drew air from the treatment and control arms into the arena, but was low enough to ensure that the fly was not disturbed. Ant nests were obscured from the females' view by using black screens to eliminate visual cues. All trials were conducted under natural light.

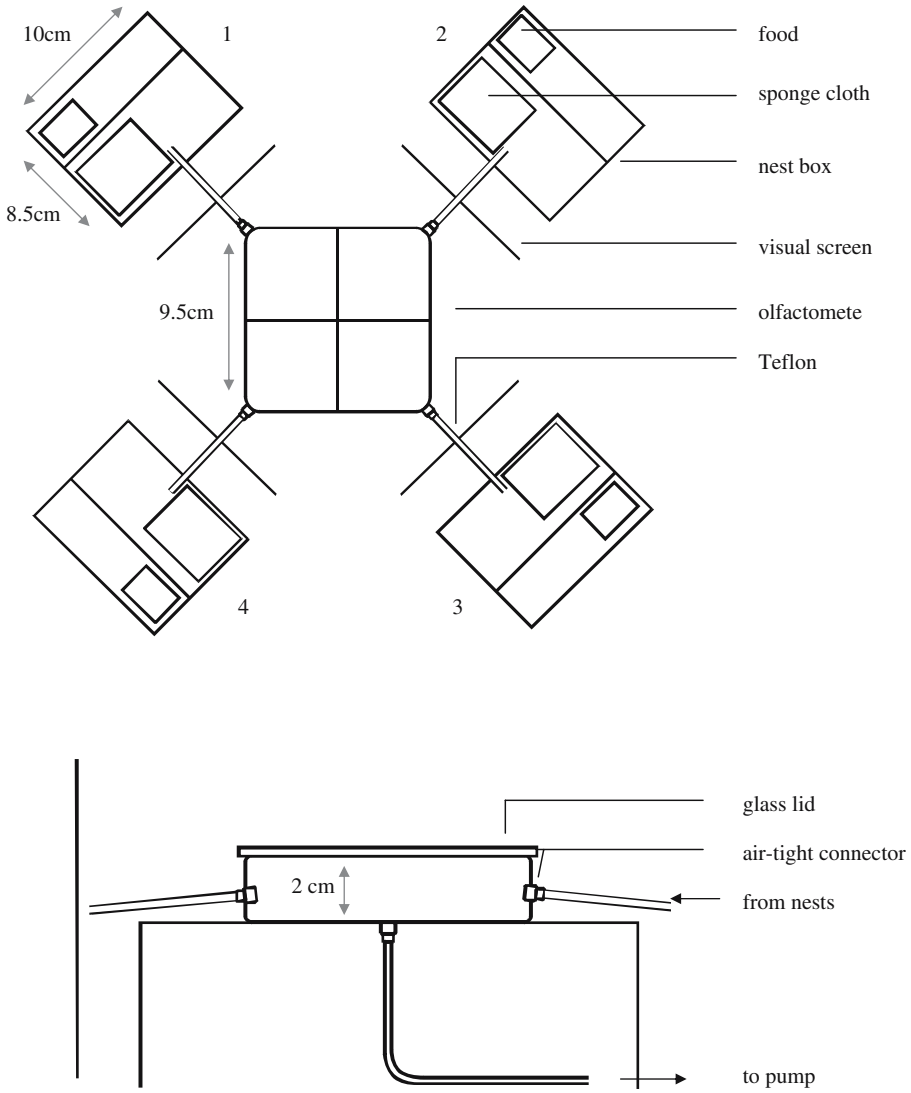

Fig. 1 Olfactometer used to examine the response of mated *M. mutabilis* females to nest boxes containing test ant colonies (100 workers of either their host ant or a non-host ant) compared with control nest boxes containing no ants. Two test ant colonies and two control boxes were used in each experiment with like arms opposite (e.g., test colony on *1* and *3*, control boxes on *2* and *4*)

We recorded the amount of time the *M. mutabilis* female spent in each of the four sections and how often and where she rested, cleaned herself, or displayed oviposition behavior, i.e., the extension of the ovipositor (although no eggs were laid in any of the trials). The latter was scored when the female extended her ovipositor and curled it under her abdomen, as observed when females oviposit onto soil (see also Sutherland et al. 2001). Resting periods were recorded when females remained motionless for more than 10 sec.

Olfactometer Trials Fifteen *M. mutabilis* females were used to assess the response to *F. lemani* workers (host ant) relative to negative controls. Two *F. lemani* colonies were randomly chosen from the six test nests before the start of each bioassay (nests were numbered 1–6 and lots were drawn). Females were allowed to acclimatize for 15 min before recording began. After 30 min, trials were terminated and the female removed. The whole chamber was cleaned with warm water and detergent (Decon) and then rinsed with distilled water and allowed to dry. The olfactometer was rotated 90° before the start of the next trial to eliminate any effect of the direction of light. Each *M. mutabilis* female was used only once. Another 16 *M. mutabilis* females were used to assess their response to the non-host *M. scabrinodis* by using the same procedure described above, but replacing the *F. lemani* nests with those of randomly chosen *M. scabrinodis* nests.

To test for significance, we calculated the difference in frequency of the behaviors in the treatment and control areas of the arena and tested for a difference from 0 by using the Exact Wilcoxon signed-rank test. Similarly, the time spent in the treatment sections of the arena was tested for significant differences from 50%.

Identification of Olfactory Cues

Insects Two hundred and fifty *M. scabrinodis* workers and 20 *F. lemani* frozen workers (−70°C for 5 min) were extracted in diethylether by submerging them for 2 min. The *M. scabrinodis* extract was vacuum distilled to remove high molecular weight impurities (Griffiths and Pickett 1980). In addition, 20 *F. lemani* workers were snap-frozen and the cadavers separated into head, thorax, and abdomen. Three samples that contained heads, thoraxes, and abdomens, respectively, were extracted in diethylether. All ant samples were concentrated under N_2 to 0.1 ant/μl equivalents.

Gas Chromatography (GC) Volatiles were analyzed on a Hewlett-Packard 5890A gas chromatograph equipped with a cold on-column injector, a flame ionization detector (FID), and a 50 m×0.32 mm i.d. HP-1 bonded phase-fused silica capillary column. The oven temperature was maintained at 40°C for 2 min and then programmed at 10°/min to 250°C. The carrier gas was hydrogen.

Electrophysiology Electroantennogram (EAG) recordings were made by using Ag-AgCl glass electrodes filled with saline solution (composition as in, but without glucose; Maddrell 1969). Mated *M. mutabilis* females were immobilized by chilling, and one antenna was removed as close to the head as possible. The cut base of the antenna was inserted into the indifferent electrode. The tip of the arista was excised, and the recording electrode was slipped over the cut surface. The signals produced by the antenna were passed through a high impedance amplifier (UN-06, Syntech, The Netherlands) and recorded by using a customized software package (EAG for Windows, Syntech).

Stimulus Delivery The delivery system employed a filter paper in a disposable Pasteur pipette cartridge, as described by Wadhams et al. (1982). The stimulus (2 sec duration) was delivered into a purified air stream (1 l/min) flowing continuously over the preparation. Samples (10 μl) of extracts or standard solutions of test compounds (10^{-6} g/10 μl were applied to filter paper strips, and the solvent was allowed to evaporate (30 sec) before the strip was placed in the cartridge. The control stimulus was hexane (10 μl). Fresh cartridges were prepared immediately before each stimulation.

M. mutabilis female antennae were tested for responses to *F. lemani* workers (whole), *F. lemani* worker heads, abdomens, and thoraxes, and *M. scabrinodis* whole workers before coupled GC-EAG experiments (see below). Synthetic standards of compounds tentatively identified by GC-MS (see below) were tested for EAG activity. Responses to a solvent control and solutions of the synthetic standards (N=5–6) were normalized to the response to (4a*S*,7*S*,7a*R*)-nepetalactone, a compound known to have activity in other Syrphidae (C. Woodcock, pers. obs.) but not found in either *F. lemani* or *M. scabrinodis* workers. The responses were compared for significant differences by using Student's t test.

Coupled Gas Chromatography-Electroantennography (GC-EAG) The coupled GC-electrophysiology system in which the effluent from the GC column is simultaneously directed to the antennal preparation and the GC detector has been described previously (Wadhams 1990). Separation of the volatiles was achieved on an AI 93 GC equipped with a cold on-column injector and an FID. Two columns were used, a 50 m×0.32 mm i.d. HP-1 column and a 30 m×0.3 mm i.d. HP-WAX column. For the HP-1 column, the oven temperature was maintained at 30°C for 2 min and then programmed at 5°/min to 100°C and then at 10°/min to

Table 1 Geographical location of three sites on the Burren, Co. Clare, Ireland, the area sampled and number of nests found for each of five ant species recorded

	Coordinates	Area sampled (m^2)	*F. lemani*	*L. flavus*	*L. niger*	*M. sabuleti*	*M. schencki*
Site 1	53°03′72/ 9°21′09	1056	43(8)	11	19	7	1
Site 2	53°02′05/ 9°00′09	1375	50(17)	11	1	2	1
Site 3	53°03′72/ 8°59′99	1641	58(8)	11	–	12	1

Numbers in parenthesis for *F. lemani* indicate the number of nests infested with *M. mutabilis.*

250°C. For the HP-WAX column, the oven temperature was maintained at 30°C for 1 min and then programmed at 10°/min to 220°C. The carrier gas was hydrogen. The outputs from the EAG amplifier and the FID were monitored simultaneously and recorded with a customized software package (GC/EAD for Windows, Syntech, The Netherlands).

Coupled Gas Chromatography-Mass Spectrometry (GC-MS) A capillary GC column (50 m×0.32 mm i.d. HP-1) fitted with an on-column injector was directly coupled to a mass spectrometer (VG Autospec, Fisons Instruments). Ionization was by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°/min to 250°C. Tentative identification by GC-MS was confirmed by peak enhancement with authentic samples (Pickett 1990).

Chemicals Methyl 5-methylsalicylate, 3-octanone, and (±)-3-octanol were purchased from Sigma-Aldrich (Gillingham, UK). Methyl 3-methylsalicylate and methyl 4-methylsalicylate were synthesized in high purity (>99% pure by GC) by esterification of 3-methylsalicylic acid (Aldrich) and 4-methylsalicylic acid (Aldrich), respectively. Methyl 6-methylsalicylate was synthesized in high purity (>99% pure by GC) in two steps, via diazotization of 2-amino-6-methylbenzoic acid (Aldrich) to generate 6-methylsalicylic acid, and subsequent esterification. Confirmation of the identity of synthesized materials was obtained by ^{1}H and ^{13}C NMR spectroscopy (Bruker 500 MH_z).

Results

Ant Communities on the Burren We identified nests of five ant species on three *M. mutabilis* sites in the Burren: *Lasius flavus*, *L. niger*, *F. lemani*, *Myrmica sabuleti*, and *M. schencki*. Table 1 gives the location and area of each site and the number of nests of each ant species. We found no evidence of *M. mutabilis* in the nests of any species other than *F. lemani*. Figure 2 shows, as an example, the distribution of ant nests occupied by the five species at site 1 (Table 1). *F. lemani* was found throughout the site, although most were concentrated toward the bottom parts of the square. Nests of the other ant species are found mostly to the top and left of the plot, although there is much overlap. There was no obvious visual difference between the stones covering *F. lemani* nests and those of the other ant species.

Olfactometer Trials Ovipositor extensions were observed only in trials where *M. mutabilis* females were exposed to

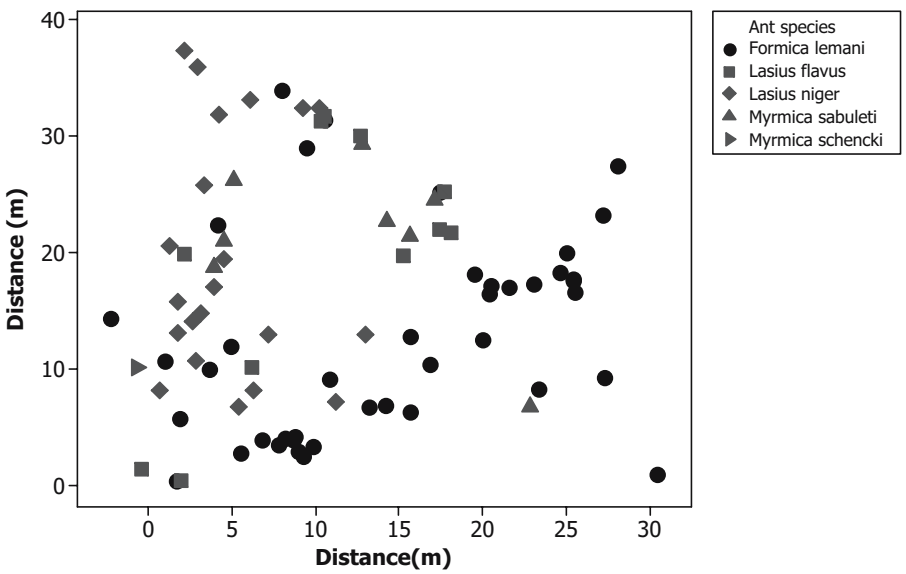

Fig. 2 Spatial distribution of colonies of five ant species recorded at site 1 (Table 1) on the Burren, Ireland

odors from their host ant, *F. lemani*. There were no ovipositor extensions in trials with the non-host *M. scabrinodis*, although cleaning and resting were observed in both sets. In the 16 trials of *M. mutabilis* females with *F. lemani* nests vs. empty control nests, ovipositor extension was observed in nine, cleaning in six, and resting in 12 trials.

There were no significant differences in the frequency of cleaning or resting behavior in either sets of trials with treatments of either the host *F. lemani* (cleaning: v=12, N=6, P=0.834; resting: v=45, N=12, P=0.666), or the non-host *M. scabrinodis* (cleaning events were too few to be analyzed; resting: v=23, N=10, P=0.683) (Fig. 3). The average percentage time spent in front of the ant inlet in *F. lemani* trials was 51.3%±5.73% (Exact Wilcoxon: v=77, N=16, P=0.66) and in *M. scabrinodis* trials 52.9%±4.69% (Exact Wilcoxon: v=62, N=15, P=0.932). However, females extended the ovipositor more often in front of the inlet from *F. lemani* colonies compared to the controls (v=45, N=9, P< 0.01; Fig. 3a), whereas no oviposition attempts were recorded in trials with the non-host *M. scabrinodis* (Fig. 3b).

Electrophysiology and Identification of Active Compounds In contrast to the behavioral trials, *M. mutabilis* responded to extracts of both *F. lemani* and *M. scabrinodis* workers in the EAG. With the extracts of *F. lemani* heads, thoraxes, and abdomens, *M. mutabilis* responded only to the head extracts (Fig. 4).

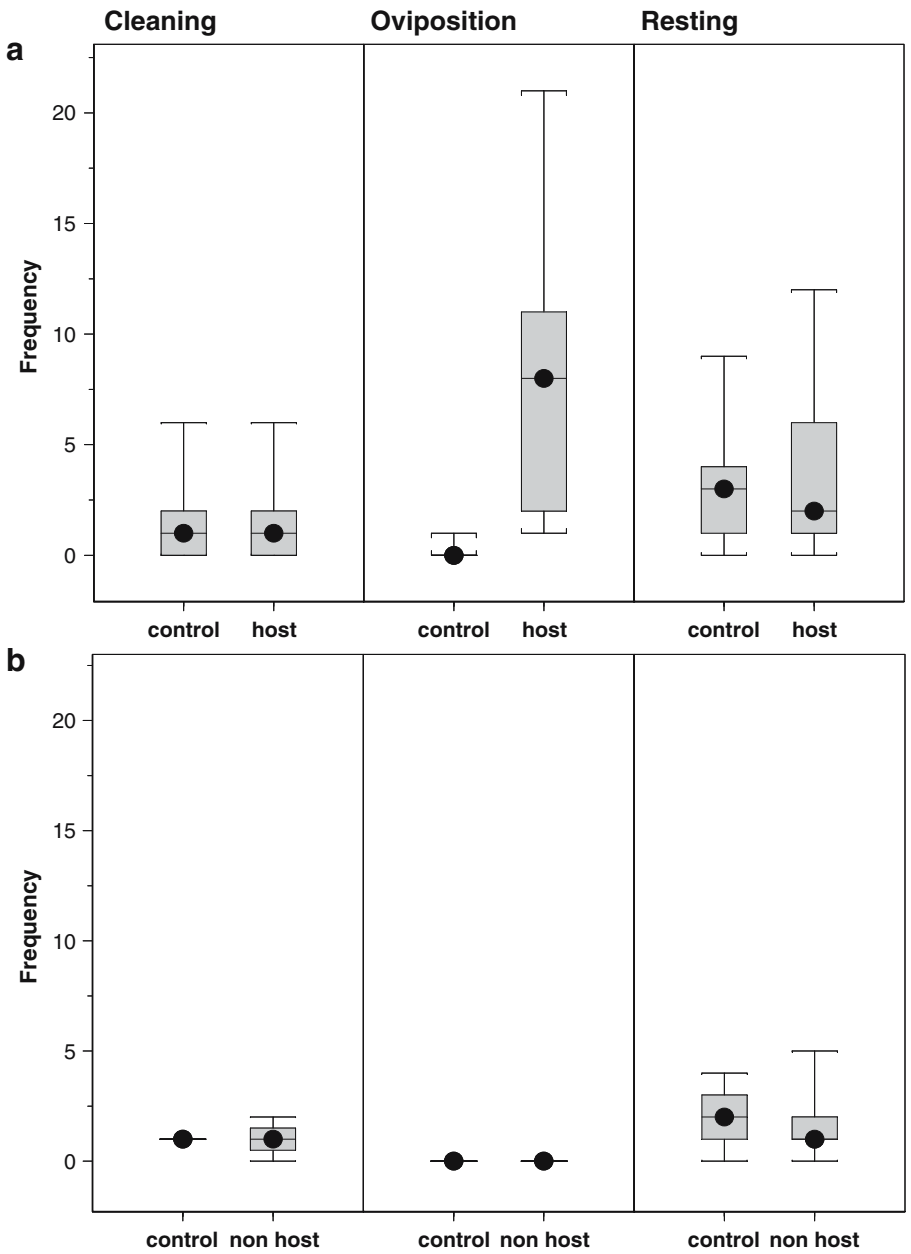

Fig. 3 Frequencies of cleaning, oviposition, and resting behavior using (**a**) the host ant *F. lemani* and (**b**) the non-host *M. scabrinodis* as treatments against negative controls. The dot and horizontal bar marks the median, the top and bottom of the box the 25% and 75% quartiles, and the whiskers indicate the range. The frequency in oviposition behavior in front of *F. lemani* nests differed significantly (Exact Wilcoxon sign-rank test: v=45, N=9, P<0.01)

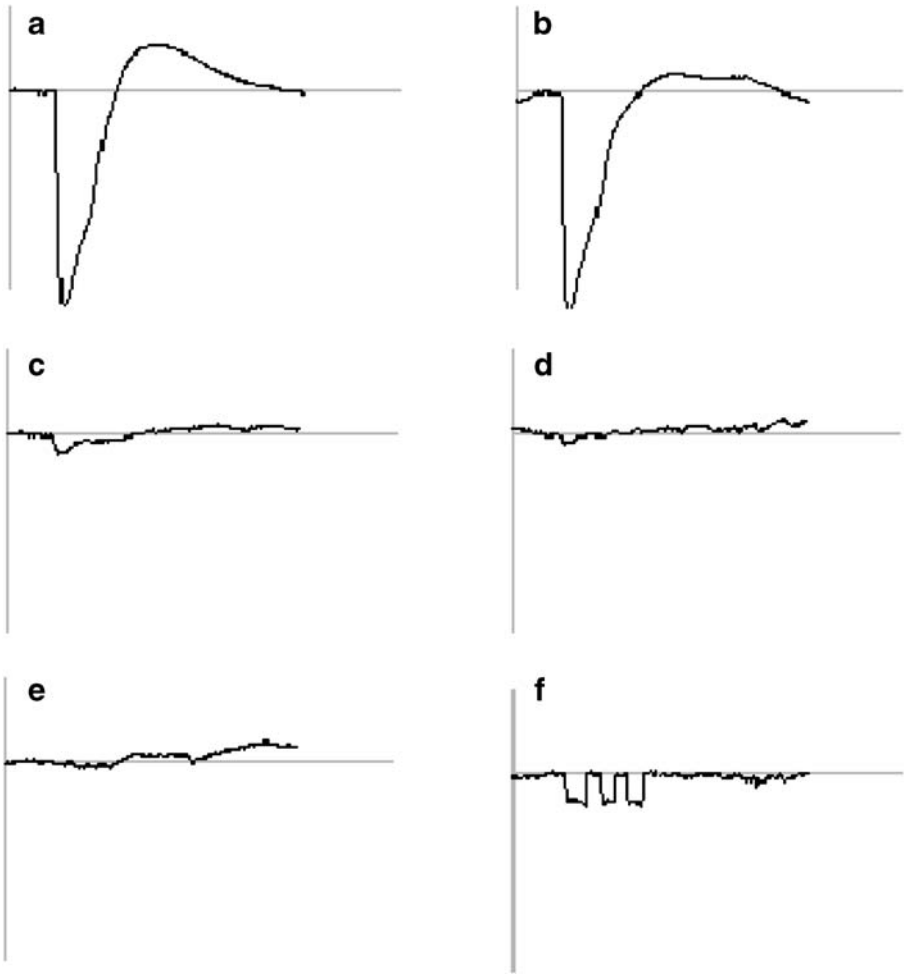

Fig. 4 EAG responses of female *Microdon mutabilis* females to ant samples. **a**=*Formica lemani* heads; **b**=*Myrmica scabrinodis* whole bodies; **c**=*F. lemani* thoraxes; **d**=*F. lemani* abdomens; **e**=hexane; **f**= 0.1 mV signal

Coupled GC-EAG analysis located a single active component in the *F. lemani* whole workers extract (Fig. 5a). Further GC-EAG on *M. mutabilis* confirmed that this compound was found only in the heads of *F. lemani* workers. The active compound was tentatively identified by coupled GC-MS as a methylated analogue of methyl salicylate. The identity of the peak as methyl 6-methylsalicylate was confirmed by GC coinjection of the *F. lemani* extract with an authentic sample, resulting in successful peak enhancement. GC coinjection of the extract with the other isomers (methyl 3-methylsalicylate, methyl 4-methylsalicylate, methyl 5-methylsalicylate) showed no peak enhancement. All four synthetic isomers were presented to five *M. mutabilis* antennae, and gave significantly larger EAG responses than that of the solvent (Table 2). However, the response to methyl 6-methylsalicylate was significantly (four times) higher than the other isomers (Table 2)

There were also two compounds in the sample of *M. scabrinodis* workers to which *M. mutabilis* antennae responded (Fig. 5b). Mass spectra indicated that the active compounds were 3-octanone and (±)-3-octanol, and their identities were confirmed with GC by coinjecting the synthetic compounds with ant extract. The stereochemistry of the 3-octanol was not determined. Synthetic 3-octanone and 3-octanol (racemic) were presented to the antennae of *M. mutabilis*, and both gave a significant response relative to the solvent (Table 2).

Discussion

Female *M. mutabilis* hoverflies exposed to volatile odors from colonies of the ant species *F. lemani* extend the ovipositor, suggesting that they use a volatile cue to identify nest sites of suitable hosts. Subsequent electrophysiological studies identified only a single compound in the mixture of volatiles that is recognized by *M. mutabilis*. MS and additional GC and EAG studies confirm that this is methyl 6-methylsalicylate. However, by also responding to 3-octanol and 3-octanone from *M. scabrinodis*, *M. mutabilis* may also be able to avoid nests of this non-host species.

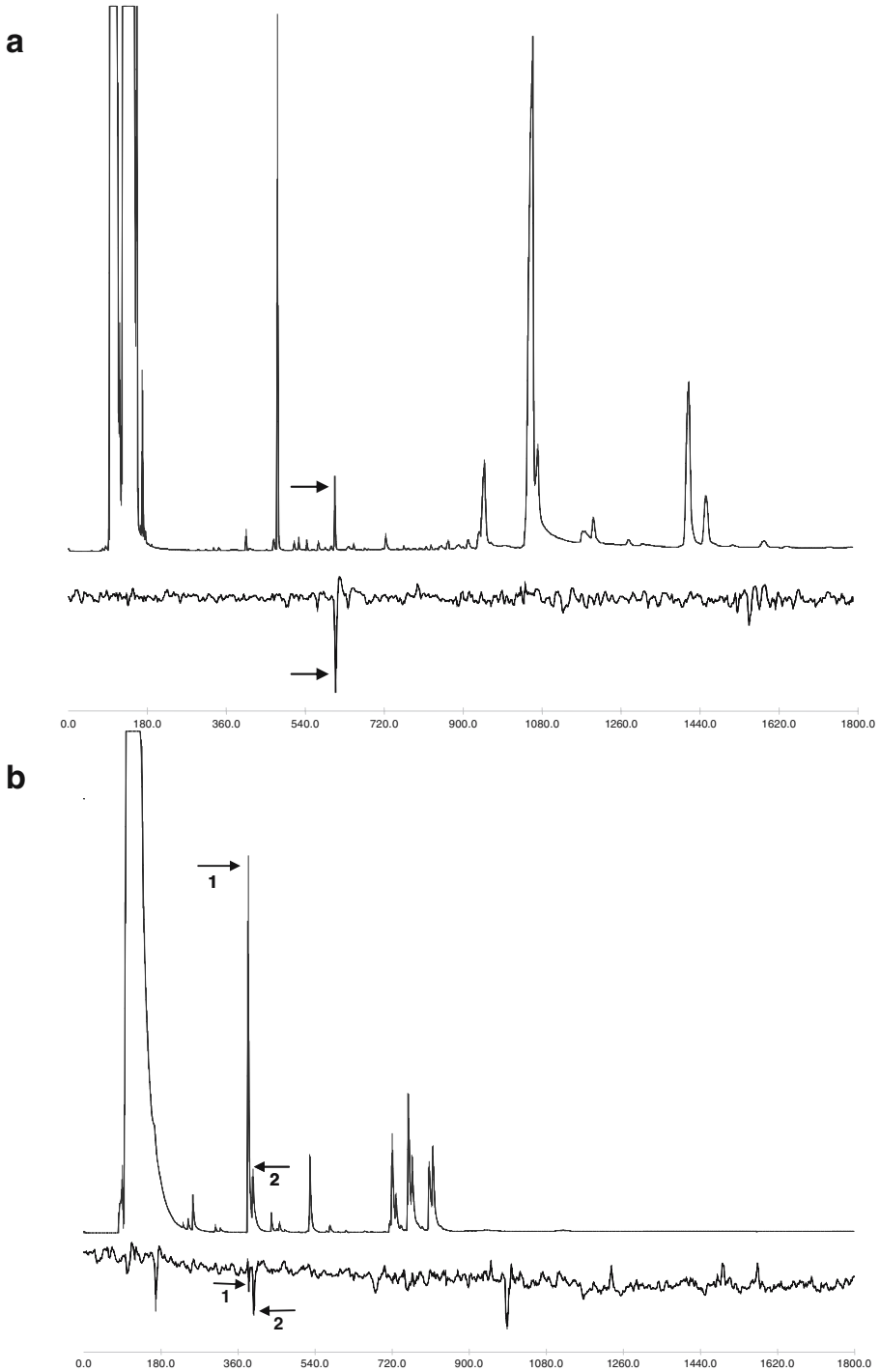

Fig. 5 *Microdon mutabilis* female excised antennae tested in coupled GC-electroantennograms with (**a**) extract of whole *F. lemani* workers and (**b**) extract of whole *M. scabrinodis* workers (vacuum distilled, 0.1 worker equivalents). The upper lines in (**a**) and (**b**) show the output from the GC detector and the lower lines that from the excised antennae. Active compounds are indicated by arrows. In (**a**), the peaks refer to methyl 6-methylsalicylate and in (**b**), to *1*) 3-octanone and *2*) 3-octanol

The use of volatile cues that originate from host ant colonies has been suggested as a mechanism for oviposition patterns in other social parasites such as the myrmecophilous butterflies in the genus *Maculinea* (van Dyck et al. 2000). However, in this system, eggs are laid on host plants and not in ant colonies directly, and subsequent analyses indicate that the developmental stage and niche of the plants determine the spatial patterns of butterfly oviposition, rather than ant-derived cues (Thomas and Elmes 2001; Musche et al. 2006). The myrmecophilous beetle *Lomechusa* (=*Atemeles*) *pubicollis*, however, has been shown to use volatile cues for orientation when changing from the summer host (*Formica* spp. ants) to the winter host (*Myrmica* spp. ants) (Hölldobler 1969, 1970; Hölldobler and Wilson 1990). Whereas the orientation behavior of *L. pubicollis* has been established, the nature of the volatile cue(s) has not been identified. Also, in contrast to the observed behavior by *L. pubicollis*, the extension of the

Table 2 EAG responses of female *Microdon mutabilis* to synthetic compounds (10^{-6} g/10 μl)

Compound	Percent response (±S.E.)	*t*—(against solvent)	*t*—(isomers against methyl 6−methylsalicylate)
Methyl 3-methylsalicylate	52.4 (±9.99)	6.77	50.95
Methyl 4-methyl salicylate	69.0 (±10.66)	32.00	46.02
Methyl 5-methyl salicylate	63.4 (±6.52)	11.11	43.25
Methyl 6-methyl salicylate	199.0 (±28.5)	55.48	
3-octanone	57.0 (±10.6)	15.32	
(±) 3-octanol	79.0 (±4.00)	17.54	
Hexane	46.6 (±11.63)		

Responses expressed as a percentage of the response to a standard application of (4aS,7S, 7aR)-nepetalactone at the same level ($N = 5$; t tests at 8 degrees of freedom; all tests are significant: $P < 0.01$)

ovipositor in *M. mutabilis* is likely to reflect host acceptance in reference to the four stages of host location (Godfray 1994; Sutherland et al. 2001). In our studies, the time spent in front of the ant colonies (host or non-host) or the empty control nests did not differ significantly. It is possible that the olfactometer was too small to investigate orientation behavior in *M. mutabilis* females. Casual observations in the field, when a stone is lifted from above a *F. lemani* nest, suggest that escaping odors are attractive to adult females in the vicinity (G.W. Elmes, pers. obs.). As methyl 6-methylsalicylate was the only physiologically active volatile compound in the *F. lemani* extracts, it could also be responsible for attraction; additional studies are needed to establish whether *M. mutabilis* uses methyl 6-methylsalicylate for orientation.

To be useful as a cue for either orientation or host acceptance, a volatile compound or mixture needs to distinguish the correct host from a non-host. We identified methyl 6-methylsalicylate as a constituent of the *F. lemani* head extract. The same compound has been reported as a constituent of the mandibular gland in other Formicidae, including males of several *Camponotus* species, where it acts as a swarming pheromone (Torres et al. 2001), and in the ponerine ant *Gnaptogenys pleurodon*, where it functions as an alarm pheromone (Duffield and Blum 1975). It has also been identified in the poison gland of *Tetramorium impurum,* where it acts as a major component of the trail pheromone (Attygalle et al. 1990). The function of this compound in intraspecific communication of *F. lemani* is as yet unclear, but while it has been found in several other ant species, none of them lives sympatrically with *F. lemani*. Although our knowledge about the occurrence of methyl 6-methylsalicylate in other ant species is limited, it appears that within the communities where *F. lemani*, *M. scabrinodis*, and *M. mutabilis* co-occur, methyl 6-methylsalicylate provides a mechanism for the hoverfly to identify *F. lemani* reliably. The purity of methyl 6-methylsalicylate could be a criterion associated with *F. lemani.*

3-Octanone and 3-octanol, found in the extracts of *M. scabrinodis* ants, elicited a response from *M. mutabilis* antennae. Both compounds are known constituents of the alarm pheromone of most European *Myrmica* species (Morgan et al. 1978; Cammerts et al. 1981, 1982, 1983, 1985). In the olfactometer experiments, we observed no behavior of *M. mutabilis* females toward *M. scabrinodis* that could be attributed to the recognition of volatile cues, but the same argument regarding the limitations of our experimental set-up to study orientation might apply. Nevertheless, we suggest that these chemicals may provide cues that allow female *M. mutabilis* to identify and reject this unsuitable species of host ant in the field.

As predicted by Cortesero et al. (1997), it appears that *M. mutabilis*, as an extreme specialist, may use a host-derived, rather than a microenvironment-derived cue to identify *F. lemani* colonies. Whereas the hypothesis was formulated in the context of parasitoid host specificity, it appears to be applicable equally to parasites and social parasites. It seems unlikely that the extreme host specificity shown by *M. mutabilis* to particular groups of *F. lemani* colonies could be based on a single compound (Elmes et al. 1999; Schönrogge et al. 2006), although this work suggests a mechanism by which *M. scabrinodis* nests might be avoided. The contents of ant mandibular glands, which are often involved in alarm responses, have also been suggested as having a role in nest mate recognition (Jaffe and Sanchez 1984). However, the argument generally refers to mixtures of chemicals, where colony-specific ratios of compounds might confer sufficient information to distinguish among colonies. It is difficult to imagine how this could be achieved with one compound. However, colonies in the behavioral trials included only workers, and it is possible that other members of the colony (brood, queens) could be sources of additional volatile cues.

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References

Al Abassi, S., Birkett, M. A., Petterson, J., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. 2001. Response of the ladybird parasitoid Dinocampus coccinellae to toxic alkaloids from the seven-spot ladybird, Coccinella septempunctata. *J. Chem. Ecol.* 27:33–43.

Attygalle, A. B., Billen, J. P. J., Jackson, B. D., and Morgan, E. D. 1990. Morphology and Chemical Contents of Dufour Glands of Pseudomyrmex Ants (Hymenoptera, Formicidae). *Z. Naturforsch.* 45:691–697.

Cammerts, M. C., Attygalle, A. B., Evershed, R. P., and Morgan, E. D. 1985. The pheromonal activity of chiral 3-Octanol for *Myrmica* ants. *Physiol. Ent.* 10:33–36.

Cammerts, M. C., Evershed, R. P., and Morgan, E. D. 1981. Comparative study of the mandibular gland secretion of 4 species of *Myrmica* ants. *J. Insect Physiol.* 27:225–231.

Cammerts, M. C., Evershed, R. P., and Morgan, E. D. 1982. Mandibular gland secretions of workers of *Myrmica rugulosa* and *Myrmica schencki*—Comparison with 4 other *Myrmica* species. *Physiol. Ent.* 7:119–125.

Cammerts, M. C., Evershed, R. P., and Morgan, E. D. 1983. The volatile components of the mandibular gland secretion of workers of the ants *Myrmica lobicornis* and *Myrmica sulcinodis*. *J. Insect Physiol.* 29:659–664.

Cortesero, A. M., De Moraes, C. M., Stapel, J. O., Tumlinson, J. H., and Lewis, W. J. 1997. Comparisons and contrasts in host-foraging strategies of two larval parasitoids with different degrees of host specificity. *J. Chem. Ecol.* 23:1589–1606.

De Moraes, C. M., and Mescher, M. C. 2005. Intrinsic competition between larval parasitoids with different degrees of host specificity. *Ecol. Ent.* 30:564–570.

Dicke, M., Lenteren, J. C., Boskamp, G. J. F., and Voorst, R. 1985. Intensification and prolongation of host searching in *Leptopilina heterotoma* (Thomson)(Hymenoptera: Eucoilidae) through a kairomone produced by *Drosophila melanogaster*. *J. Chem. Ecol.* 11:125–136.

Du, Y., Poppy, G. M., Powell, W., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. 1998. Identification of semiochemicals released during aphid feeding that attract the parasitoid *Aphidius ervi*. *J. Chem. Ecol.* 24:1355–1368.

Duffield, R. M., and Blum, M. S. 1975. Methyl 6-methyl salicylate: Identification and function in a ponerine ant (*Gnamptogenys pleurodon*). *CMLS.* 31:466–466.

Elmes, G. W., Barr, B., Thomas, J. A., and Clarke, R. T. 1999. Extreme host specificity by *Microdon mutabilis* (Diptera: Syrphidae), a social parasite of ants. *Proc. R. Soc. Lond. B.* 266:447–453.

Elmes, G. W., Thomas, J. A., and Wardlaw, J. C. 1991. Larvae of *Maculinea rebeli*, a Large Blue Butterfly, and their *Myrmica* host ants—Wild adoption and behavior in ant nests. *J. Zool.* 223:447–460.

Feener, D. H., Jacobs, L. F., and Schmidt, J. O. 1996. Specialized parasitoid attracted to a pheromone of ants. *Anim. Behav.* 51:61–66.

Gardner, M. G., Schönrogge, K., Elmes, G. W., and Thomas, J. A. 2007. Increased genetic diversity as a defence against parasites is undermined by social parasites: *Microdon mutabilis* hoverflies infesting *Formica lemani* ant colonies. *Proc. R. Soc. Lond.B.* 274:103–110.

Godfray, H. C. J. 1994. Parasitoids: Behavioral and Evolutionary Ecology. Princeton University Press, New Jersey.

Griffith, D. C., and Pickett, J. A. 1980. A potential application of aphid alarm pheromones. *Entomol. Exp. Appl.* 27:199–201.

Hölldobler, B. 1969. Host Finding by odor in the myrmecophilic beetle *Atemeles pubicollis* Bris.(Staphylinidae). *Science.* 166:757–758.

Hölldobler, B. 1970. Zur Physiologie der Gast-Wirt-Beziehungen (Myrmecophilie) bei Ameisen. II. Das Gastverhäiltnis des imaginalen *Atemeles pubicollis* Bris.(Col, Staphylinidae) zu *Myrmica* und *Formica* (Hym. Formicidae). *Z. Vergl. Physiol.* 66:176–189.

Hölldobler, B., and Wilson, E. O. 1990. The ants. Springer Verlag, Berlin.

Jaffe, K., and Sanchez, C. 1984. On the nestmate recognition system and territorial marking behavior in the ant *Camponotus rufipes*. *Insectes Soc.* 31:302–315.

Maddrell, S. H. P. 1969. Secretion by the malpighian tubules of *Rhodnius*. The movements of ions and water. *J. Exp. Biol.* 51:71–97.

Morehead, S. A. 2001. Geographic variation in host location cues for a dipteran parasitoid of *Paraponera clavata*. *Biotropica.* 33:495–501.

Morehead, S. A., and Feener, D. H. 2000. Visual and chemical cues used in host location and acceptance by a dipteran parasitoid. *J. Insect Behav.* 13:613–625.

Morehead, S. A., Seger, J., Feener, D. H., and Brown, B. V. 2001. Evidence for a cryptic species complex in the ant parasitoid *Apocephalus paraponerae* (Diptera: Phoridae). *Evol. Ecol. Res.* 3:273–284.

Morgan, E. D., Inwood, M. R., and Cammerts, M. C. 1978. Mandibular gland secretion of the ant *Myrmica scabrinodis*. *Physiol. Entomol.* 3::107–114.

Musche, M., Anton, C., Worgan, A., and Settele, J. 2006. No experimental evidence for host ant related oviposition in a parasitic butterfly. *J. Insect Behav.* 19:631–643.

Orr, M. R., De Camargo, R. X., and Benson, W. W. 2003. Interactions between ant species increase arrival rates of an ant parasitoid. *Anim. Behav.* 65:1187–1193.

Pickett, J. A. 1990. GC-MS in insect pheromone identification: three extreme case histories, pp. 299–309, in A. R. McCaffery, and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and PheromonesPlenum Press, New York and London.

Powell, W., and Pickett, J. A. 2003. Manipulation of parasitoids for aphid pest management: progress and prospects. *Pest Manag. Sci.* 59:149–155.

Sadeghi, H., and Gilbert, F. 2000. Oviposition preferences of aphidophagous hoverflies. *Ecol. Entomol.* 25:91–100.

Schönrogge, K., Wardlaw, J. C., Thomas, J. A., and Elmes, G. W. 2000. Polymorphic growth rates in myrmecophilous insects. *Proc. R. Soc. Lond. B.* 267:771–777.

Schönrogge, K., Barr, B., Wardlaw, J. C., Napper, E. K. V., Gardner, M. G., Breen, J., Elmes, G. W., and Thomas, J. A. 2002. When rare species become endangered: cryptic speciation in myrmecophilous hoverflies. *Biol. J. Linn. Soc.* 75:291–300.

Schönrogge, K., Gardner, M. G., Elmes, G. W., Napper, E. K. V., Simcox, D. J., Wardlaw, J. C., Breen, J., Knapp, J. J., Pickett, J. A., and Thomas, J. A. 2006. Host propagation permits extreme local adaptation in a social parasite of ants. *Ecol. Lett.* 9:1032–1040.

Sutherland, J. P., Sullivan, M. S., and Poppy, G. M. 2001. Oviposition behavior and host colony size discrimination in *Episyrphus balteatus* (Diptera: Syrphidae). *Bull. Entomol. Res.* 91:411–417.

Thomas, J. A., and Elmes, G. W. 2001. Food-plant niche selection rather than the presence of ant nests explains oviposition patterns in the myrmecophilous butterfly genus *Maculinea*. *Proc. R. Soc. Lond. B.* 268:471–477.

Torres, J. A., Snelling, R. R., Blum, M. S., Flournoy, R. C., Jones, T. H., and Duffield, R. M. 2001. Mandibular gland

chemistry of four Caribbean species of *Camponotus* (Hymenoptera: Formicidae). *Biochem. Syst. Ecol.* 29:673–680.

VAN ALPHEN, J. J. M., and VISSER, M. E. 1990. Superparasitism as an adaptive strategy for insect parasitoids. *Annu. Rev. Entomol.* 35:59–79.

VAN DYCK, H., OOSTERMEIJER, J. G. B., TALLOEN, W., FEENSTRA, V., VAN DER HIDDEA., and WYNHOFF, I. 2000. Does the presence of ant nests matter for oviposition to a specialized myrmecophilous *Maculinea* butterfly. *Proc. R. Soc. Lond. B.* 267:861–866.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VET, L. E. M., SOKOLOWSKI, M. B., MACDONALD, D. E., and SNELLEN, H. 1993. Responses of a generalist and a specialist parasitoid (Hymenoptera, Eucoilidae) to drosophilid larval kairomones. *J. Insect Behav.* 6:615–624.

WADHAMS, L. J., ANGST, M. E., and BLIGHT, M. M. 1982. Responses of the olfactory receptors of *Scolytus scolytus* to the stereoisomeres of 4-methyl-3-heptanol. *J. Chem. Ecol.* 8:477–492.

WADHAMS, L. J. 1990. The use of coupled gas chromatography: electrophysiological techniques in the identification of insect pheromones, pp, pp. 289–298, in A.R. McCaffery, and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and PheromonesPlenum Press, New York and London.

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Protected by Fumigants: Beetle Perfumes in Antimicrobial Defense

Jürgen Gross · Kerstin Schumacher · Henrike Schmidtberg · Andreas Vilcinskas

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Abstract Beetles share with other eukaryotes an innate immune system that mediates endogenous defense against pathogens. In addition, larvae of some taxa produce fluid exocrine secretions that contain antimicrobial compounds. In this paper, we provide evidence that larvae of the brassy willow leaf beetle *Phratora vitellinae* constitutively release volatile glandular secretions that combat pathogens in their microenvironment. We identified salicylaldehyde as the major component of their enveloping perfume cloud, which is emitted by furrow-shaped openings of larval glandular reservoirs and which inhibits *in vitro* the growth of the bacterial entomopathogen *Bacillus thuringiensis*. The suggested role of salicylaldehyde as a fumigant in exogenous antimicrobial defense was confirmed *in vivo* by its removal from glandular reservoirs. This resulted in an enhanced susceptibility of the larvae to infection with the fungal entomopathogens *Beauveria bassiana* and *Metarhizium anisopliae.* Consequently, we established the hypothesis that antimicrobial defense in beetles can be expanded beyond innate immunity to include external disinfection of their microenvironment, and we report for the first time the contribution of fumigants to antimicrobial defense in animals.

J. Gross (✉) · H. Schmidtberg · A. Vilcinskas
Institute of Phytopathology and Applied Zoology,
Justus-Liebig University Giessen,
Heinrich-Buff-Ring 26-32,
35392 Giessen, Germany
e-mail: juergen.gross@agrar.uni-giessen.de

K. Schumacher
Institute for Integrated Plant Protection, Federal Biological Research Centre for Agriculture and Forestry,
Stahnsdorfer Damm 81,
14532 Kleinmachnow, Germany

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Introduction

Larvae of the leaf beetle taxon Chrysomelina possess nine pairs of dorsal exocrine glands, which are inserted in the body surface and contain reservoirs of glandular secretions. In case of predator attack, the larvae evert their glandular reservoirs to present the fluid secretions toward the exterior. The secreted toxins vary both in structure and biosynthetic origin. The major components secreted by leaf beetle larvae belonging to the taxa *Phaedon*, *Gastrophysa*, *Linaeidea*, and most *Phratora* species are iridoid monoterpenes, which are produced *de novo* via the acetate-mevalonate pathway. In contrast, larvae of *Chrysomela* spp. and *Phratora vitellinae* emit secretions in which salicylaldehyde is the major component (Wain 1943; Oldham et al. 1996; Pasteels et al. 1982, 1988). When feeding upon willows (Salicaceae), larvae of the latter species sequester phenolic glycosides (e.g., salicin) from their host plants as precursors to produce salicylaldehyde (Wallace and Blum 1969; Pasteels et al. 1988; Gross and Hilker 1995). Different biological functions have been reported for larval glandular secretions from beetles: Some prevent intraspecific competition (Gross and Hilker 1995), whereas others show insecticidal activities (Dettner et al. 1992) or act as allomones in defense against predators. Although salicylaldehyde has repellent activity against some generalist predators (Blum et al. 1972; Hilker and Schulz 1994; Pasteels et al. 1986; Gross et al. 2004), it also has detrimental effects for the larvae by attracting specialized predators and parasitoids (Köpf et al. 1997;

Gross et al. 2004; Zvereva and Rank 2004). This raises the question: Why do leaf beetle larvae permanently produce salicylaldehyde?

Beetles are of the order with the highest number of species among eukaryotes. Like other eukaryotes, they have evolved an innate immune system that provides protection against intruding pathogens. The first representative genome, that of the red flour beetle *Tribolium castaneum*, has recently been sequenced (Tribolium Genome Sequencing Consortium 2008). The innate immunity of this species bears similarities to that of other insects. For example, numerous homologous molecules have been identified in *Tribolium* that are known to mediate perception of infection and immune-related signaling, or represent antimicrobial peptides such as defensins or thaumatins (Altincicek et al. 2007). Studies provide evidence that the larval secretions of some Chrysomelina species possess antimicrobial activity against bacteria and entomopathogenic fungi *in vitro* (Gross et al. 1998, 2002). However, the biological impact of these secretions was unclear, because the contact of the larval surface with fungal conidia did not elicit secretion discharge.

Hence, in the present study, we addressed the following questions to find out how an antimicrobial secretion can protect its producer against infection with entomopathogenic microorganisms: (1) Do larvae of the brassy willow beetle *P. vitellinae* permanently emit volatile compounds in their secretions, and by means of which morphological structure? (2) Do larvae benefit from these fumigants in case of an infestation by conidia of entomopathogenic fungi? To answer these questions, we collected the headspace of undisturbed larvae, analyzed it by GC-MS, and examined the surface of the glandular reservoirs by scanning electron microscopy. Further, we artificially emptied the glandular reservoirs of larvae and applied conidia of several entomopathogenous fungi onto their body surface, followed by monitoring the mortality of larvae with full and empty glandular reservoirs. Finally, the effect of the main component of larval headspace, salicylaldehyde, was tested against entomopathogenic bacteria in bioassays.

Methods and Materials

Insects Larvae of *P. vitellinae* were collected on willows (mainly *Salix fragilis* and *S. rubens*) at Berlin, Germany, transferred to a climate chamber, and reared on the willow *S. fragilis* at 20°C with a natural light/dark cycle.

Fungi Four strains of entomopathogenous fungi, isolated and provided by the Biological Research Centre for Agriculture and Forestry (BBA), Darmstadt, Germany, were used: *Beauveria bassiana* strain 135 (isolated from *Ips typographus* [Coleoptera], 1994) and strain 138 (isolated from Boverol© [Fytovita, Czech Republic], 1995); *Metarhizium anisopliae* strain 140 (isolated from *Ips typographus* [Coleoptera], 1994) and strain 79 (isolated from *Agrotis segetum* [Lepidoptera], 1982).

Bacteria Two subspecies of *Bacillus thuringiensis* were used: *B. thuringiensis tenebrionis* strain 10 BI 256-82, isolated from *Tenebrio molitor* (Coleoptera: Tenebrionidae), and *B. thuringiensis kurstaki* strain HD 1.

Analysis of Larval Headspace

Sampling. Five larvae with filled glandular reservoirs were cooled to 4 °C and transferred to a closed silanisized glass vial, taking care that they did not evert their glandular reservoirs. A continuous flow of purified air was passed for 3 hr through the vial and subsequently through a small carbon filter (5 mg; Graenicher/Quartero, Daumazan, France). The airflow was regulated by a flowmeter (Supelco, Bellefonte, PA, USA) and sucked through the system by a membrane vacuum pump (Vakuubrand, Germany). The filter was then eluted with 20 µl dichloromethane that contained tridecane as internal standard (50 ng/µl; Aldrich, Germany), and the sample was analyzed by gas chromatography mass spectrometry (GC-MS) as described below. For sampling the emission of larvae that had everted their glandular reservoirs, the same procedure was used; however, two artificial stimuli were given during the sampling period: a magnet inside the glass vial was gently moved by a second one outside the system, until all larvae had everted their glandular reservoirs. This was performed after 1 and after 2 hr during the sampling period. For measuring the time-dependent regeneration of the volatiles, the same procedure as described above was carried out with minor modifications: During the sampling period, a leaf of *S. fragilis* was added as food. After sampling of untreated larvae, their glandular reservoirs were artificially emptied (as described below) and the sampling procedure was repeated after 3, 6, 9, 24, and 48 hr. For each treatment, six replications were conducted.

Identification and Quantification of Salicylaldehyde in Larval Headspace One microliter of each sample was injected splitless into a gas chromatograph (Fisons GC 8060, Thermoquest, Germany, injector temperature 240°C) equipped with a 30 m×0.32 mm×0.25 µm db-5 column (Fisons). The temperature program started at 40°C, was held for 4 min, then raised by 10°C/min to 280°C. Helium (Air Liquide, Germany) was used as carrier gas (inlet pressure 10 kPa). The gas chromatograph was coupled to a quadrupole mass spectrometer (Fisons MD 800, Thermoquest, Germany), and EI mass spectra were recorded at

70 eV. The antimicrobial active compound (salicylaldehyde) was identified as described earlier (Gross et al. 2002). The relative amount of salicylaldehyde (ng/µl solvent) compared to the internal standard was calculated with the following formula:

$$\frac{\text{peak area of salicylaldehyde} \times 50\,[\text{ng}/\mu\text{l}]}{\text{peak area of internal standard}}$$

Scanning Electron Microscopy (SEM) Larvae were fixed with 2.5% glutardialdehyde in 0.1 M phosphate-buffered saline (PBS; pH 7.4) for 1 hr at room temperature under moderate shaking. After washing several times in PBS, the animals were dehydrated in a graded series of ethanol. Subsequently, they were dried at critical point with CO_2 in a Balzers CPD 030 and sputtered with gold with a sputter coater 0712 B (Balzer/Leitz). Microscopic investigations were carried out on a Philips XL 20 scanning electron microscope.

Bioassay for Testing Antifungal Activity *In Vivo*

Cultivation of Fungi and Preparation of Conidia Suspensions Conidia from the stock cultures were inoculated into nutrient broth (25 g Standard I, 20 g glucose (Merck, Germany), dissolved in 1,000 ml distilled water) on a gyratory shaker (200 rpm). After 6 d of incubation, 1 ml of the suspension was transferred to malt peptone agar plates (90 mm diam; MPA, LAB 37, UK) and spread with a Drygalsky spatula. After complete sporulation at room temperature for a number of days, the conidia were harvested and mixed for 1 min on a vortex (Vibrofix VF1, Germany) with 2 ml of sterile saline, adjusted to pH 6.1, and 1 µl Tween 20. The conidia were counted in a Neubauer chamber and adjusted to different concentrations (5×10^3, 10^4, 10^5, 10^6 conidia/ml). Fresh spore suspensions were prepared for every experiment.

Removal of Larval Secretion Larvae of the third stage (and in one experiment also of the second stage) were randomly selected and fixed on an ice-block. Under gentle pressure from a needle, the glandular reservoirs were everted by the larva, and the secretion from every individual reservoir was sucked onto a small disk of filter paper (5-mm diameter). After this, the larva was washed in ice-cold deionized water for 2 sec and placed on a filter paper for drying. No food was supplied until the larvae were used for headspace sampling, or until conidia were applied.

Application of Conidia Four groups of larvae were used in the biotests. The first and second groups were "milked" as described above, whereas the third and fourth groups retained their full glandular reservoirs. One test larva each from the first and third group was set on an ice-cold Petri dish to prevent eversion of its glandular reservoirs, and 1 µl of the corresponding spore suspension (1×10^4, 1×10^5, 1×10^6 conidia/ml) was applied to its back. For comparison of the temporal dependent survival of two larval stages, 0.5 µl of spore suspension (1×10^4 conidia/ml; L_2) or 1 µl (5×10^3 conidia/ml; L_3) was applied, corresponding to an application of five conidia/larva. The control larvae for the second and fourth groups were treated with 1 µl of sterile saline. After a short drying period, all 10 larvae from the same treatment were put in a Petri dish (53-mm diameter) lined with moistened filter paper and supplied with leaves of *S. fragilis*. The mortality was controlled every 24 hr until all surviving beetles had hatched to adults. Each experiment was repeated at least 40 times.

Inhibition Zone Assays Suspensions of *Bacillus thuringiensis tenebrionis* and *B. thuringiensis kurstaki* were grown up at 29°C in sterile nutrient bouillon (Standard I, Merck, Germany) to a density of 0.50–0.54, measured at a wavelength of 546 nm. Two hundred and twenty microliters of this suspension were transferred to a 45°C temperate agar suspension comprising 4.07 g Standard I nutrient agar (Merck, Germany) dissolved in 110 ml deionized water. Sterile Petri dishes (Falcon, 8.7-cm diameter) were filled with 5 ml each of this agar suspension and stored at 6 °C for a maximum of 4 d until use. Salicylaldehyde (Roth, Germany) was added either undiluted, with one sample per test plate (located in the middle of the test plate), or diluted (1:10 and 1:100), with one or six samples evenly spaced on the same test plate. Three microliters of each salicylaldehyde sample were applied directly into a hole (2.3-mm diameter), which was punched into the agar beforehand, or indirectly on a piece of filter paper fixed with glue to the middle of the Petri dish lid. For control, the solvent dichloromethane was also tested in the same manner.

Statistical Analysis Comparisons between the amounts of salicylaldehyde emitted by the larvae before and after artificial emptying of their glandular reservoirs was performed by *Wilcoxon matched pairs tests*. The time-dependent headspace sampling of emptied glandular reservoirs was compared to controls (filled reservoirs) by *Friedman* tests. Differences in survival between the controls and the test groups in the bioassays were evaluated by χ^2 *tests*. Comparison of time-dependent survival between two larval stages was analyzed by χ^2 *tests* followed by *sequential Bonferroni correction* (Holm 1979; Bärlocher 1999). Differences between one and six samples per test plate in the inhibition zone tests with *Bt* were analyzed by Mann–Whitney *U* test. All statistical tests were done by using the Statistica 5.5 software (StatSoft 1999).

Results

Headspace samples taken from *P. vitellinae* larvae that did not evert their glandular reservoirs were analyzed by GC-MS, revealing that volatile salicylaldehyde was permanently present as the main component in headspace (average relative concentration compared to internal standard 29.7±28 ng/µl, N=6). When two artificial stimuli were given to trigger an eversion of the glandular reservoirs during the sampling period, the concentration of salicylaldehyde increased to 119.2±99 ng/µl (N=6). Permanent emission of salicylaldehyde was measured during a 48-hr experiment in which the larvae were provided with food. Between 3 and 24 hr after artificial removal of glandular secretion, the content of salicylaldehyde in the larval headspace was reduced. After 48 hr, the relative amount of salicylaldehyde was as high as before the removal (Fig. 1). Typical GC-MS total ion chromatograms before and 6 hr after removal of the secretion are provided in Fig. 2. The peaks of internal standard and salicylaldehyde are indicated. The other peaks represent volatiles emitted from plant leaf, larval frass, and feces.

We examined the body surface of third instars of *P. vitellinae* by SEM (Fig. 3). When the glandular reservoirs are everted (Fig. 3a,c), the secretion is presented on top of the reservoirs. In case the reservoirs are not everted, furrow-shaped openings of glandular reservoirs are visible above the glandular reservoirs (Fig 3b, indicated by arrow).

Artificial draining of glandular secretions did not affect the survival of untreated larvae, but significantly reduced survival after treatment with conidia from both strains of *B. bassiana* or *M. anisopliae*, respectively (conidia concentration 10^4 conidia/ml, Table 1, χ^2 test, P<0.05). By increasing the concentration of the applied conidia, statistically significant differences in survival were measured in one *M. anisopliae* strain (Ma 140) and both *B. bassiana* strains at 10^5 conidia/ml, and in *B. bassiana* strain 135 at 10^6 conidia/ml (Table 1, χ^2 *test*, P<0.05). Detailed analysis of the time-dependent survival, i.e., survival of larvae treated with *M. anisopliae* strain 79 conidia (Fig. 4), revealed a significant reduction in larvae with emptied reservoirs compared to larvae with full reservoirs from the 15th day until the end of the experiment with 5×10^3 conidia/ml (P<0.05), and from the 5th day with 10^4 conidia/ml (P<0.001), respectively. Further increase of conidia dose (10^5 conidia/ml) broke the fumigant defense line, and more than 80% larvae of both test groups died 16 d after the treatment (Fig. 4).

To investigate the differences between two different larval stages, another experiment was conducted that used a concentration corresponding to an application of 5 *M. anisopliae* (Ma 79) conidia on each larva (Fig. 5). While there were no statistically significant differences between the two groups of both larval stages after 5 d, 95% of the second-stage larvae with full glandular reservoirs were still surviving after 10 d compared to only 55% of those with artificially drained reservoirs. These differences lasted until the end of experiment (χ^2 *test* followed by *sequential Bonferroni correction*, P<0.01). In contrast, differences between groups of third-stage larvae were detected only at the end of the experiment (85% vs. 57.5% for the two groups, respectively; P<0.01). Comparison of time-dependent survival between the two larval stages revealed differences between 5 and 10 d (χ^2 tests followed by *sequential Bonferroni correction*, P<0.01).

Results of the inhibition zone assays with salicylaldehyde treatment of two different strains of *Bacillus thuringiensis*

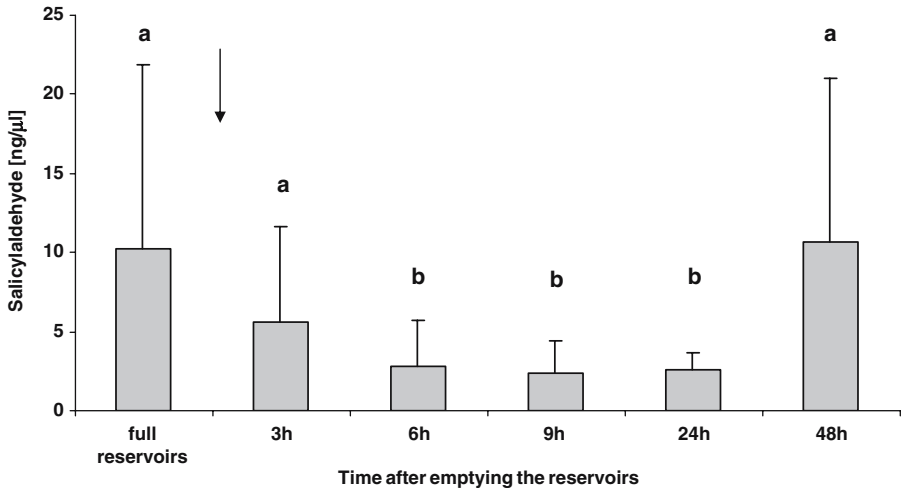

Fig. 1 Permanent emission of relative amounts of salicylaldehyde from headspace of *Phratora vitellinae* (L_3) and its regeneration after artificial emptying (arrow) of the reservoirs: mean values and standard deviations (N=6) from GC-MS analysis of headspace samples (five larvae each for 3 hr) are given. Different letters indicate significant differences of treatments compared to control (*Friedman ANOVA*, 6 hr, 9 hr, P<0.01; 24 hr, P<0.05)

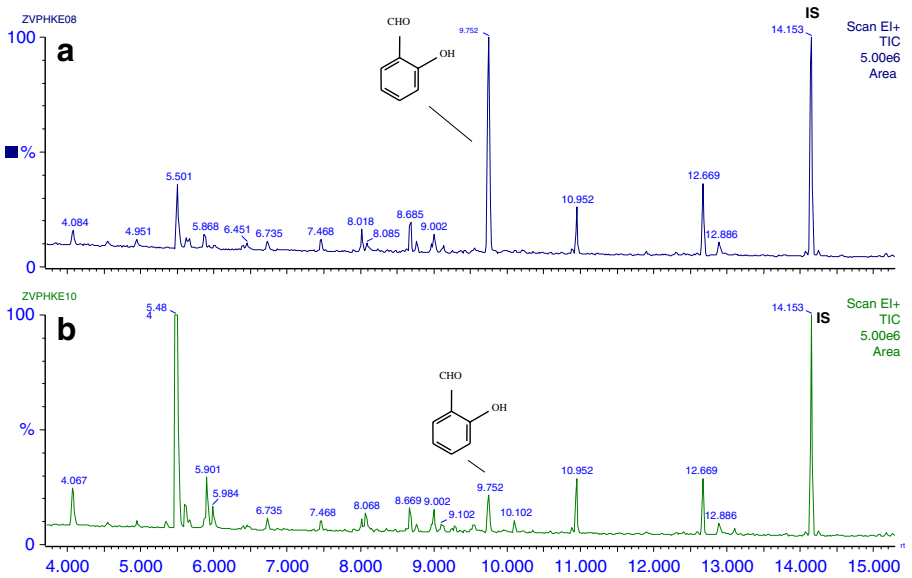

Fig. 2 Typical GC-MS total ion chromatograms of headspace samples (five larvae each for 3 hr, feeding on leaves of *Salix fragilis*) of *Phratora vitellinae* (L_3). The main component of larval secretion (salicylaldehyde) is indicated by its formula. (**a**) Untreated larvae with full reservoirs, (**b**) larvae 6 hr after artificial emptying of the reservoirs. IS: Internal standard (tridecane). Unassigned peaks represent plant odors

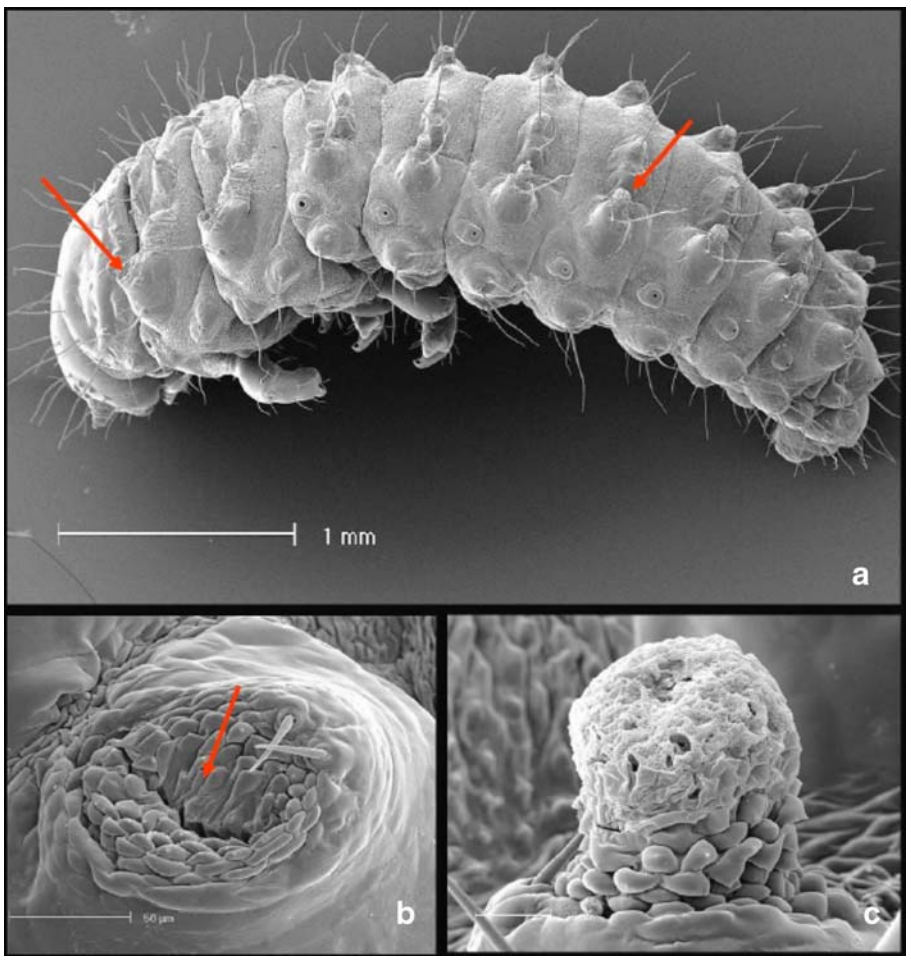

Fig. 3 Larva of the leaf beetle *Phratora vitellinae* with nine pairs of dorsally located exocrine glands inserted into the body surface on top of small tubercles. (**a**) Whole larva with everted abdominal (right arrow) and inserted thoracal glandular reservoirs (left arrow). (**b**) Retracted reservoir with its furrow-shaped opening (arrow). (**c**) Everted glandular reservoir presenting its secretion at the top

Table 1 Mean percentage of surviving larvae of *Phratora vitellinae* (± standard deviation) with full or artificial emptied glandular reservoirs after dorsal application of different conidia concentrations (conidia/ml) of *Beauveria bassiana* (*Bb*) strains 135 and 138 or *Metarhizium anisopliae* (*Ma*) strains 79 and 140 at the end of the experiment

Fungus Strain	Control			1×10^4			1×10^5			1×10^6		
	Full	Empty	*P*	Full	Empty	*P*	Full	Empty	*P*	Full	Empty	*P*
Bb 135	90.0±8.2	90.0±8.2	n.s.	87.5±5.0	57.5±9.6	**	87.5±9.6	52.5±9.6	***	77.5±5.0	47.5±20.6	**
Bb 138	87.5±12.6	80.0±8.2	n.s.	75.0±10.0	50.0±24.5	*	72.5±9.6	47.5±25.0	*	59.0±22.6	45.0±17.3	n.s.
Ma 79	85.0±5.8	87.5±12.6	n.s.	62.5±25.0	17.5±17.1	***	10.0±14.1	15.0±12.9	n.s.	5.0±5.8	0	n.s.
Ma 140	87.5±5.0	84.6±5.5	n.s.	55.0±5.8	10.0±11.5	***	10.0±8.2	0	*	2.5±5.0	0	n.s.

Number of replications: Each $N=40$, except *M. anisopliae* strain 79: 1×10^4 und 1×10^5 $N=70$). Statistical analysis by $\chi 2$ *test*; n.s. not significant; $P>0.05$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$.

showed that growth of this entomopathogenous bacterium was inhibited, regardless of whether the main component of the beetle's perfume was applied undiluted or in a 1:10 dilution. The inhibition increased when six samples were tested simultaneously on the same plate, indicating that salicylaldehyde samples act additionally over the gas phase. A dilution of 1:100 showed no inhibition of bacterial growth. When salicylaldehyde was applied on the Petri dish lids, it also inhibited the growth of *B. thuringiensis tenebrionis* in both the undiluted and 1:10 dilution treatments (five samples simultaneously tested). The solvent dichloromethane did not affect the growth of *B. thuringiensis* (Table 2).

Discussion

Insects are threatened by pathogenous microorganisms throughout their whole life. Their sclerotized chitinous exoskeleton provides an efficient primary barrier against microbial invasion, which can only be directly penetrated by entomopathogenic fungi such as *B. bassiana* or *M. anisopliae.* On the other hand, viral and bacterial pathogens usually gain entry into their insect hosts via the intestinal tract (Clarkson and Charnley 1996; Gillespie et al. 2000). Once microorganisms have entered the insect hemocoel they are exposed to the host innate immune system, which is responsible for sensing of infections (Royet et al. 2005)

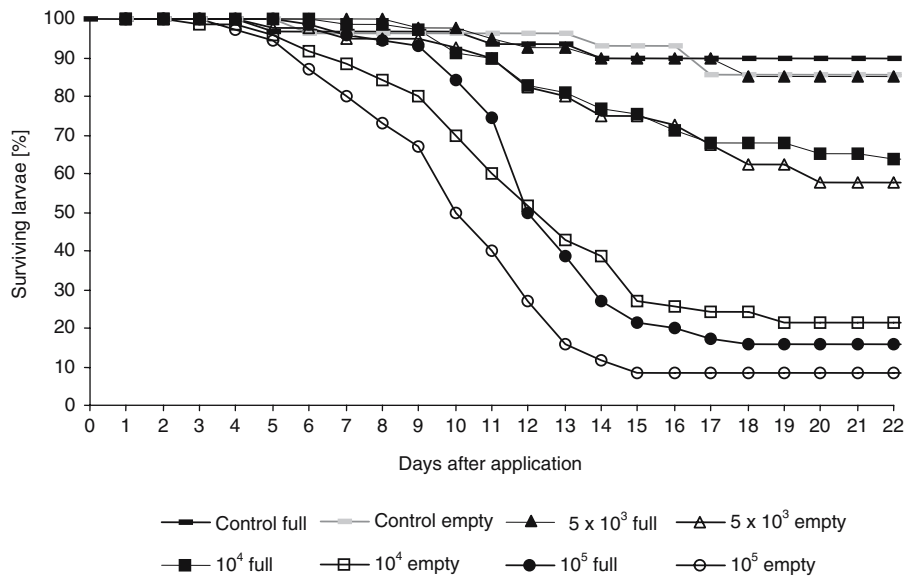

Fig. 4 Temporally dependent cumulative mortality of *Phratora vitellinae* larvae (L_3) with full or emptied glandular reservoirs after application of different conidia concentrations from *Metarhizium anisopliae* strain 79 (controls and 5×103 conidia/ml: $N=40$; 1×10^4 and 1×10^5 conidia/ml: $N=70$)

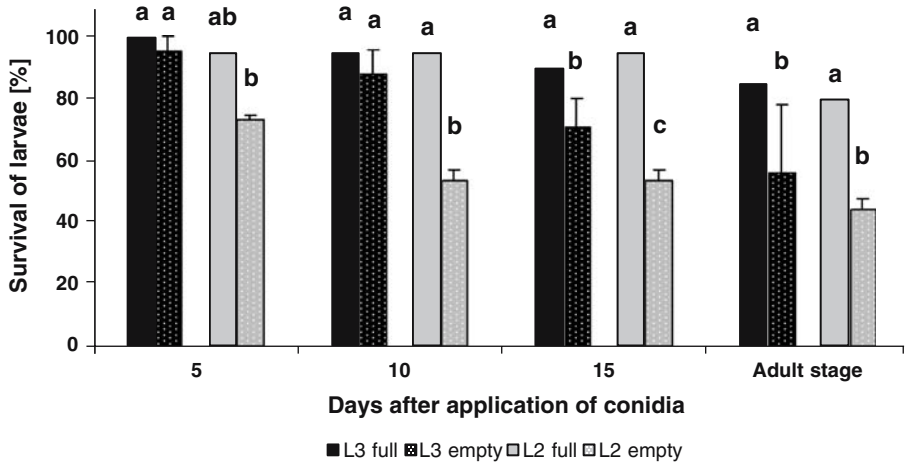

Fig. 5 Comparison of temporally dependent survival of two larval stages (L_2 gray bars, N=20; L_3 black bars, N=40) after dorsal application of five conidia (*M. anisopliae*, strain 79) each. The glandular reservoirs of two groups (dotted bars) were emptied artificially. Different letters indicate statistically significant differences ($\chi 2$ *test* followed by *sequential Bonferroni correction*, $P<0.025$)

and mounting of complex cellular and humoral defense reactions (Vilcinskas and Götz 1999). The latter encompass the rapid synthesis of a diverse array of powerful antimicrobial peptides (Hoffmann 2003).

Although the endogenous innate immunity in insects has been intensively studied for decades, our knowledge about exogenous antimicrobial defense mechanisms nevertheless remains fragmentary. The contribution of fluid secretions in antimicrobial defense was studied in some insect species. For example, water beetles have glandular secretions that inhibit the growth of bacteria on their integument (Dettner 1985; Kovac and Maschwitz 1990), whereas in other insects they are effective at combating microorganisms in their nests or stored food (Rosengaus et al. 2000; Traniello et al. 2002; Herzner et al. 2007; Herzner and Strohm 2007). However, the role of insect antimicrobial secretions in defense against entomopathogens has not been established. Based on our observation that main components of leaf beetles' glandular secretions exert *in vitro* antimicrobial activity against bacteria and entomopathogenic fungi (Gross et al. 1998, 2002), we hypothesized that antimicrobial defense in beetles can be expanded beyond innate immunity to include external disinfection of the microenvironment. In support of this, we discovered in this study that leaf beetles also produce volatiles that exhibit antimicrobial activity. These were shown to form a disinfecting cloud that prevents germination of entomopathogenic fungi on their cuticle. Consequently, we analyzed whether the leaf beetle fumigants are permanently produced and whether this perfume provides protection against entomopathogenic fungi *in vivo*.

The larvae of the leaf beetle *P. vitellinae*, like others of taxon Chrysomelina, possess nine pairs of exocrine glands, which are everted in case of a predator attack, presenting their secretion at the top of the everted reservoir (Fig. 3c). After 1–2 sec, they are subsequently reinverted with the help of specialized retractor muscles (Garb 1915; Hinton 1951). The reservoirs are filled with different chemical compounds, which are at least partly volatiles (Pasteels et al. 1988). In the present study, investigations of the

Table 2 Results of inhibition zone assays with salicylaldehyde against different strains of *Bacillus thuringiensis*

Dilution	No of Samples/Test Plate	Application Mode	Inhibition Zone [mm]	
			Bt.t.	*Bt.k.*
Undiluted	1	Directly	Complete	Complete
1:10	1	Directly	3.1±0.37[a]	4.8±1.11[a]
1:10	6	Directly	Complete[b]	12.7±0.38[b]
Undiluted	1	Indirectly	Complete	Not tested
1:10	5	indirectly	Complete	Not tested

Mean values and standard deviations of inhibition zones are provided. Different letters indicate significant differences between one sample and six samples per test plate (*Mann–Whitney U* test, $P<0.05$). The solvent (DCM) and higher dilutions (1:100) caused no inhibition of bacterial growth.

morphological structure of the cuticle of *P. vitellinae* larvae revealed furrow-shaped openings above glandular reservoirs, allowing the volatile components to emit continuously (Fig. 3b). The openings are distributed segmentally and pairwise on the larval body from the mesothorax to the seventh abdominal segment (Fig. 3a), allowing a perfect distribution of the fumigant cloud all over the larval body. By analyzing the chemical composition of the headspace of undisturbed larvae of *P. vitellinae*, salicylaldehyde was revealed as the main component (Figs. 1 and 2). This component is known to show antimicrobial activity against both bacteria and fungi (Gross et al. 2002). After detecting the conditions required for protection by a permanently produced fumigant, we conducted *in vivo* bioassays to test our hypothesis.

The removal of glandular secretion from larval reservoirs of *P. vitellinae* decreased their survival after external exposure with conidia of the fungal entomopathogens *B. bassiana* and *M. anisopliae* compared to that of untreated larvae (Table 1, Fig. 4). These two species are known as the most frequently occurring fungal pathogens of Chrysomelidae (Humber 1996). Successful removal of the fumigants was confirmed by GC-MS analysis of headspace samples from larvae with full or emptied glandular reservoirs (Figs. 1 and 2). Beyond this, we determined that *P. vitellinae* larvae need approximately 48 hr to emit the same amount of salicylaldehyde from their glandular reservoirs (Fig. 1). The higher amount of salicylaldehyde in the headspace 3 hr after emptying the reservoirs compared to 6 hr later was caused by an unavoidable contamination of the beetles' surface with secretion during the draining, which continued to emit during the first 3 hr. The reduced amounts of salicylaldehyde in the headspace seen in the second experiment compared to the first may be caused by addition of a leaf during headspace sampling, on whose surface a portion of the volatile salicylaldehyde may have been adsorbed. Such an effect was shown previously for volatile secretion of the closely related leaf beetle *Phaedon cochleariae*, which adsorbed to the surface of Chinese cabbage leaves (Rostás and Hilker 2002). Emptying the glandular reservoirs 48 hr after application of conidia, no significant differences in mortality were revealed compared to the mortality of larvae with full reservoirs (data not shown). Thus, transient reduction in fumigant concentration around the larvae by a single removal of the content from their dorsal glands is sufficient to result in increased mortality when exposed to fungal pathogens. The determined *in vivo* effect, in combination with reported morphological and chemical structures, lends some support to our hypothesis that leaf beetles use volatile glandular secretions that prevent germination of conidia from fungal pathogens on their cuticle. Fungal pathogens can directly infect their insect hosts via the integument (Gillespie et al. 2000). Consequently, fumigation of fungal conidia before germination on the exoskeleton is a plausible explanation for the prevention of infection.

Until now, the activity of salicylaldehyde has not been tested for the detrimental effects against entomopathogenic bacteria. Thus, we tested the effects of this compound against two different subspecies of *Bacillus thuringiensis* in concentrations that occur naturally in leaf beetle secretions (Gross et al. 2002). When the bacteria were treated directly or indirectly (via the gas phase) with salicylaldehyde, their growth and development was inhibited (Table 2). Pathogenic bacteria invade the larval body by oral uptake or via injuries. Hence, salicylaldehyde may act additionally against entomopathogenic bacteria, if the fumigant cloud also exerts its activity at contaminated feeding sites, killing the bacteria before they are ingested by food.

Recently, it has been reported that phytophagous insects that consume bacteria within their diet suffer immune responses that negatively affect their fitness costs in terms of pupation time and pupal mass (Freitak et al. 2007). Hence, activation of the indigenous immune system causes costly life history trade-offs. The synthesis of salicylaldehyde takes place by sequestration and catabolization of the plant-borne precursors salicin and saligenin (Kuhn et al. 2004), causing no additional costs for the larvae (Rowell-Rahier and Pasteels 1986). The investigated cloud of salicylaldehyde putatively reduces such immune-related fitness costs, if fumigation of the microenvironment prevents both ingestion of living bacteria and infection with parasitic fungi, such as *B. bassiana* and *M. anisopliae*, that can directly enter the insect host via the cuticle.

This effect could be strengthened by a special behavior known as cycloalexy that is displayed by many leaf beetle larvae (Grégoire 1988), whereby Chrysomelina larvae feed close together in groups of 10 to 30 larvae. This behavior is explained as better protection against enemy attack, but in leaf feeding insects it is also related to overcoming plant defenses, especially with respect to feeding establishment (Nahrung et al. 2001; Wilson et al. 2003). We modeled this behavior by applying different numbers of volatile secretions on test plates, and detected a statistically significant increase in the diameter of every inhibition zone when six samples were applied per test plate instead of only one sample (Table 2, Mann–Whitney U test, $P<0.05$). Whereas predator attack will cause a loss of fluid and volatile secretions and deteriorate the individual antimicrobial protection, cycloalexy may increase the efficacy of antimicrobial defense through synergistic enlargement of the mutual fumigant cloud. Further studies will be necessary to prove this hypothesis.

Insect bodily fluids represent a promising reservoir for new antimicrobial active compounds (Vilcinskas and Gross 2005). If disinfection of the microenvironment by insect perfumes is widespread in nature, a targeted analysis of

volatile glandular secretions from other insects may result in identification of novel fumigants that could be used as a source of innovative pesticides for pathogen control, e.g., for disinfection of stored food or decontamination in hospitals. Overall, our results provide evidence that beetles made use of volatiles that disinfect their microenvironment. As far as we are aware, this is the first report on the principle of fumigation in antimicrobial defense of animals.

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References

ALTINCICEK B., KNORR E., and VILCINSKAS A. 2007. Beetle immunity: Identification of immune-inducible genes from the model insect *Tribolium castaneum. Dev. Comp. Immunol.,* DOI 10.1016/j.dci.2007.09.005.

BÄRLOCHER, F. 1999. Biostatistik. Thieme Publ., Stuttgart, Germany.

BLUM, M. S., BRAND, J. M., WALLACE, J. B., and FALES, H. M. 1972. Chemical characterisation of the defensive secretion of a chrysomelid larva. *Life Sci.* 11:525–531.

CLARKSON, J. M., and CHARNLEY, A. K. 1996. New insights into the mechanisms of fungal pathogenesis in insects. *Trends Microbiol.* 4:197–203.

DETTNER, K. 1985. Ecological and phylogenetic significance of defensive compounds from pygidial glands of Hydradephaga (Coleoptera). *Proc. Acad. Nat. Sci. Philadelphia* 137:156–171.

DETTNER, K., FETTKÖTHER, R., ANSTEEG, O., DEML, R., LIEPERT, C., PETERSEN, B., HASLINGER, E., and FRANCKE, W. 1992. Insecticidal fumigants from defensive glands of insects—a fumigant test with adults of *Drosophila melanogaster*. *J. Appl. Entomol.* 113:128–137.

FREITAK, D., WHEAT, C. W., HECKEL, D. G., and VOGEL, H. 2007. Immune system responses and fitness costs associated with consumption of bacteria in larvae of *Trichoplusia ni*. *BMC Biology*, DOI 10.1186/1741-7007-5-56.

GARB, G. 1915. The eversible glands of a chrysomelid larva, *Melasoma lapponica*. *J. Entomol. Zool.* 7:87–97.

GILLESPIE, J. P., BAILEY, A. M., COBB, B., and VILCINSKAS, A. 2000. Fungi as elicitors of insect immune responses. *Arch. Insect Biochem. Physiol.* 44:49–68.

GRÉGOIRE, J.-C. 1988. Larval gregariousness in the Chrysomelidae, pp. 253–260, in P. H., Jolivet, E., Petitpierre, T. H., and Hsiao (eds.). Biology of ChrysomelidaeKluwer Academic Publisher, Dordrecht, The Netherlands.

GROSS, J., and HILKER, M. 1995. Chemoecological studies of the exocrine glandular larval secretions of two chrysomelid species (Coleoptera): *Phaedon cochleariae* and *Chrysomela lapponica*. *Chemoecology* 5/6:185–189.

GROSS, J., MÜLLER, C., VILCINSKAS, A., and HILKER, M. 1998. Antimicrobial activity of the exocrine glandular secretions, hemolymph and larval regurgitate of the mustard leaf beetle *Phaedon cochleariae*. *J. Invertebr. Pathol.* 72:296–303.

GROSS, J., PODSIADLOWSKI, L., and HILKER, M. 2002. Antimicrobial activity of exocrine glandular secretion of *Chrysomela* larvae. *J. Chem. Ecol.* 28:317–331.

GROSS, J., FATOUROS, N. E., NEUVONEN, S., and HILKER, M. 2004. The importance of specialist natural enemies for *Chrysomela lapponica* in pioneering a new hostplant. *Ecol. Entomol.* 29:584–593.

HERZNER, G., and STROHM, E. 2007. Fighting fungi with physics: Food wrapping by a solitary wasp prevents water condensation. *Curr. Biol.* 17:R46–R47.

HERZNER, G., SCHMITT, T., PESCHKE, K., HILPERT, A., and STROHM, E. 2007. Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *J. Chem. Ecol.* 33:849–859.

HILKER, M., and SCHULZ, S. 1994. Composition of larval secretion of *Chrysomela lapponica* (Coleoptera, Chrysomelidae) and its dependence on host plant. *J. Chem. Ecol.* 20:1075–1093.

HINTON, H. E. 1951. On a little-known protective device of some chrysomelid pupae (Coleoptera). *Proc. R. Entomol. Soc. Lond.* 26:67–73.

HOFFMANN, J. A. 2003. The immune response of *Drosophila*. *Nature* 426:33–38.

HOLM, S. 1979. A simple sequentially rejective multiple test procedure. *Scand. J. Stat.* 6:65–70.

HUMBER, R. A. 1996. Fungal pathogens of the Chrysomelidae and prospects of their use in biological control, pp. 93–115, in P. H., Jolivet, M. L., and Cox (eds.). Chrysomelidae Biology, SPB Academic Publ., Amsterdam, The Netherlands.

KÖPF, A., RANK, N. E., ROININEN, H., and TAHVANAINEN, J. 1997. Defensive larval secretions of leaf beetles attract a specialist predator *Parasyrphus nigritarsis*. *Ecol. Entomol.* 22:176–183.

KOVAC, D., and MASCHWITZ, U. 1990. Secretion-grooming in aquatic beetles (Hydradephaga): A chemical protection against contamination of the hydrofuge respiratory region. *Chemoecology* 1:131–138.

KUHN, J., PETTERSSON, E. M., FELD, B., BURSE, A., TERMONIA, A., PASTEELS, J. M., and BOLAND, W. 2004. Selective transport systems mediate sequestration of plant glucosides in leaf beetles: A molecular basis for adaptation and evolution. *Proc. Natl. Acad. Sci. U. S. A.* 101:13808–13813.

NAHRUNG, H. F., DUNSTAN, P. K., and ALLEN, G. R. 2001. Larval gregariousness and neonate establishment of the eucalypt-feeding beetle *Chrysophtharta agricola* (Coleoptera: Chrysomelidae: Paropsini). *Oikos* 94:358–364.

OLDHAM, N. J., VEITH, M., and BOLAND, W. 1996. Iridoid monoterpene biosynthesis in insects: evidence for a *de novo* pathway occurring in the defensive glands of *Phaedon armoraciae* (Chrysomelidae) leaf beetle larvae. *Naturwissenschaften* 83:470–473.

PASTEELS, J. M., BRAEKMAN, J.-C., DALOZE, D., and OTTINGER, R. 1982. Chemical defence in chrysomelid larvae and adults. *Tetrahedron* 38:1891–1897.

PASTEELS, J. M., DALOZE, D., and ROWELL-RAHIER, M. 1986. Chemical defence in chrysomelid eggs and neonate larvae. *Physiol. Entomol.* 11:29–37.

PASTEELS, J. M., BRAEKMAN, J.-C., and DALOZE, D. 1988. Chemical defence in the Chrysomelidae, pp. 233–252, in P. H. JOLIVET, E. PETITPIERRE, and T. H. HSIAO (eds.). Biology of ChrysomelidaeKluwer Academic Publ., Dordrecht, The Netherlands.

ROSENGAUS, R. B., LEFEBVRE, M. L., and TRANIELLO, J. F. A. 2000. Inhibition of fungal spore germination by Nasutitermes: Evidence for a possible antiseptic role of soldier defensive secretions. *J. Chem. Ecol.* 26:21–39.

ROSTÁS, M., and HILKER, M. 2002. Feeding damage by larvae of the mustard leaf beetle deters conspecific females from oviposition and feeding. *Entomol. Exp. Appl.* 103:267–277.

ROWELL-RAHIER, M., and PASTEELS, J. M. 1986. Economics of chemical defense in Chrysomelinae. *J. Chem. Ecol.* 12:1189–1203.

ROYET, J., REICHHART, J. M., and HOFFMANN, J. A. 2005. Sensing and signaling during infection in *Drosophila*. *Curr. Opin. Immunol.* 17:11–17.

StatSoft I. 1999. STATISTICA for Windows users manual, version 5.5.

TRANIELLO, J. F., ROSENGAUS, R. B., and SAVOIE, K. 2002. The development of immunity in a social insect: Evidence for the group facilitation of disease resistance. *Proc. Natl. Acad. Sci. U. S. A.* 99:6838–6842.

Tribolium Genome Sequencing Consortium 2008. The genome of the developmental model beetle and pest *Tribolium castaneum*. *Nature: in press*

VILCINSKAS, A., and GÖTZ, P. 1999. Parasitic fungi and their interactions with the insect immune system. *Adv. Parasitol.* 43:268–313.

VILCINSKAS, A., and GROSS, J. 2005. Drugs from bugs: The use of insects as a valuable source of transgenes with potential in modern plant protection strategies. *Journal of Pest Science* 78:187–191.

WAIN, R. L. 1943. The secretion of salicylaldehyde by the larvae of the brassy willow beetle (*Phyllodecta vitellinae L.*). *Ann. Rep. Agric. Horticult. Res. Stat*. 108–110.

WALLACE, J. B., and BLUM, M. S. 1969. Refined defensive mechanisms in *Chrysomela scripta*. *Ann. Entomol. Soc. Am.* 62:503–506.

WILSON, K., KNELL, R., BOOTS, M., and KOCH-OSBORNE, J. 2003. Group living and investment in immune defence: an interspecific analysis. *J. Anim. Ecol.* 72:133–143.

ZVEREVA, E. L., and RANK, N. E. 2004. Fly parasitoid *Megaselia opacicornis* uses defensive secretions of the leaf beetle *Chrysomela lapponica* to locate its host. *Oecologia* 140:516–522.

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Enantiomeric Specificity in a Pheromone–Kairomone System of Two Threatened Saproxylic Beetles, *Osmoderma eremita* and *Elater ferrugineus*

Glenn P. Svensson · Mattias C. Larsson

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Abstract The scarab beetle *Osmoderma eremita* and its larval predator, the click beetle *Elater ferrugineus*, are threatened saproxylic beetles regarded as indicators of the species-richness of insect fauna of hollow deciduous trees. Male *O. eremita* produce the pheromone (*R*)-(+)-γ-decalactone to attract conspecific females, and this compound is also utilized by *E. ferrugineus* as a kairomone, presumably for detection of tree hollows containing prey. We have investigated enantiomeric specificity to γ-decalactone in this pheromone–kairomone system by electrophysiological and field trapping experiments. In single-sensillum recordings from male and female *O. eremita*, which used the (*R*)-enantiomer and the racemic mixture of γ-decalactone as odor stimuli, numerous olfactory receptor neurons (ORNs) responding to both stimuli were found. No neurons responded preferentially to the racemic mixture, showing that these beetles seem to lack receptors specific for the (*S*)-enantiomer. The enantiomeric specificity of ORNs was confirmed by gas chromatography-linked single-sensillum recordings where the two enantiomers in a racemic mixture were separated on a chiral column. Furthermore, in field experiments that used the (*R*)-enantiomer and the racemic mixture as lures, the attraction of *O. eremita* females corresponded to the amount of (*R*)-enantiomer released from lures with the (*S*)-enantiomer displaying no antagonistic effects. Trap catch data also suggested that the (*S*)-enantiomer is not a behavioral antagonist for *E. ferrugineus*. The odor-based system can be highly efficient in attracting the larval predator where trap catch in 1 yr almost equaled the total number of specimens collected in Sweden until 1993. Our study shows that racemic γ-decalactone could be used for cost-effective monitoring of both beetles.

Keywords *Osmoderma eremita* · *Elater ferrugineus* · Scarabaeidae · Elateridae · γ-Decalactone · Sex pheromone · Kairomone · Predator–prey interaction · Single-sensillum recording · Olfactory receptor neuron · Conservation

G. P. Svensson (✉)
Department of Ecology, Ecology Building, Lund University,
SE-223 62 Lund, Sweden
e-mail: glenn.svensson@ekol.lu.se

M. C. Larsson
Department of Crop Protection Biology,
Swedish University of Agricultural Sciences,
P.O. Box 44, SE-230 53 Alnarp, Sweden

Introduction

Ever since the first insect pheromone was identified half a century ago (Butenandt et al. 1959), the ultimate practical goal of research on insect chemical ecology has been the development of semiochemical-based strategies to control populations of agricultural and forest pests (e.g., Cardé and Minks 1995; Borden 1997; Foster and Harris 1997). In contrast, surprisingly little effort has gone into the exploration of the chemical ecology of threatened insects and the potential to use infochemicals in conservation biology. Monitoring with volatile attractants could be more efficient than traditional survey methods in gathering ecological data on focal species for conservation and in assessing species diversity of threatened insect communities. Priority should be given to assemblages that are difficult to detect and monitor with currently available methods. One such group is saproxylic beetles that live in hollow deciduous trees. The severe fragmentation and isolation of this habitat in recent centuries (Hannah et al. 1995) has resulted in the associated insect fauna being endangered all over Europe. Surveys of beetles that live inside tree trunks are often both

labor-intensive and time-consuming, and in many cases, only a small fraction of suitable trees in an area can be surveyed.

We recently initiated a research program to integrate chemical ecology and conservation biology of saproxylic beetles by using *Osmoderma eremita* Scopoli (Coleoptera: Scarabaeidae) as a model. This beetle is strongly associated with hollow deciduous trees (Luce 1996; Ranius and Nilsson 1997) and it has become a major model for ecological research on insects associated with this habitat in Europe (e.g., Ranius 2001; Ranius and Hedin 2001). Because of its role as an indicator for the species-rich fauna of saproxylic insects, *O. eremita* has high conservation priority according to the European Union's Habitat Directive (Anonymous 1992), and it has recently been intensively surveyed in Europe (Ranius et al. 2005). The most well-known feature of *O. eremita* is its fruity, plum-like or peach-like odor, which is emitted exclusively by males. We identified the odor as (*R*)-(+)-γ-decalactone and demonstrated its function as a sex pheromone used to attract conspecific females (Larsson et al. 2003). We have also shown that the likewise-threatened click beetle *Elater ferrugineus* L. (Coleoptera: Elateridae), whose larvae prey upon the larvae of *O. eremita*, utilizes (*R*)-(+)-γ-decalactone as a kairomone to locate its prey (Svensson et al. 2004). The *Osmoderma* pheromone is produced in exceptionally large amounts and can even be detected inside tree hollows by using traditional headspace sampling techniques (Svensson et al. 2003).

Sexual communication in beetles is often achieved by chiral pheromone compounds (e.g., Birch et al. 1980; Mori et al. 1986; Leal 1991). When one enantiomer of a chiral molecule is used for intraspecific communication, its antipode may function as a behavioral antagonist to avoid cross-species attraction, if the antipode is used by a closely related species. For example, the sympatric scarabs *Popillia japonica* Newman and *Anomala osakana* Sawada utilize the (*R*)- and (*S*)-configuration of japonilure, respectively, as sex pheromones, and strong reciprocal behavioral antagonism to the opposite enantiomer has been observed (Tumlinson et al. 1977; Leal 1996). Similar stereochemical discrimination has also been documented in insect predators that eavesdrop on the pheromone communication systems of their prey (Aldrich 1999), such as the clerid beetle *Thanasimus dubius* F., which only responds to the (*S*)-enantiomer of frontalin, the major enantiomer of the pheromone of its prey *Dendroctonus frontalis* Zimmerman (Payne et al. 1982, 1984).

However, behavioral effects of nonpheromonal enantiomers vary considerably among different insect species (Mori 1998; Leal 1999), and more studies are needed to understand the adaptive significance of these effects, including mechanisms of enantiomeric perception and behavioral data. From an applied perspective, evaluating the enantiomeric specificity in insect pheromone or kairomone systems is important for efficient semiochemical-based monitoring. Because pure enantiomers are usually more expensive than racemic mixtures (in the case of commercially available (*R*)-(+)-γ-decalactone, currently about 15 times as expensive, see the "Methods and Materials" section), use of the latter could significantly reduce the cost for monitoring. We, therefore, conducted electrophysiological analyses and field experiments to evaluate the effects of the (*S*)-enantiomer of γ-decalactone on the behaviors of *O. eremita* and *E. ferrugineus*.

Methods and Materials

Insects *O. eremita* is a large (25–35 mm) scarab beetle living exclusively in hollow deciduous trees, mainly oaks (Luce 1996). It is dependent on hollows with large amounts of wood mold, i.e., a mixture of loose, rotten wood, fragments of insects, fungi, and old bird nests. The development time is normally 3 yrs (Ranius et al. 2005). The species is distributed in most parts of Europe, but a recent survey that included 33 countries indicated that it has decreased in all areas (Ranius et al. 2005). Today, it has a relict distribution and only occurs in isolated populations in, e.g., woodland pastures. In Sweden, it is currently known from only 130 sites (Antonsson et al. 2003), and many populations occupy single isolated trees with few or no suitable trees nearby, and may, therefore, be doomed to extinction. *E. ferrugineus* is a large (15–25 mm) click beetle and larval predator on several saproxylic beetles associated with hollow trees, including *O. eremita* (Hansen 1966; Dajoz 2000). In Sweden, it is currently known from only 25+ sites (Nilsson and Baranowski 1994). Ranius (2002) found a strong correlation of occupancy between *E. ferrugineus* and *O. eremita* with fragments of the predator found almost exclusively in trees with fragments of its prey. The development time is several years (Palm 1959). According to Hansen (1966) and T. Tolasch (personal communication) this species swarms during evenings and nights, and its female-produced sex pheromone was recently identified (Tolasch et al. 2007). The species is rare in Sweden with only 147 adult specimens collected from about 25 localities until 1993 (Nilsson and Baranowski 1994). It is considered endangered in the Swedish Red List of threatened species (Gärdenfors 2005).

Chemicals and Dispensers (*R*)-(+)-γ-Decalactone (97% enantiomeric purity) was purchased from Sigma-Aldrich, Sweden (catalog no.: W236012). Because the (*S*)-enantiomer of the compound is not commercially available, we used a racemic mixture of γ-decalactone (catalog no.:

W236004) to study the electrophysiological and behavioral effects of the (*S*)-enantiomer. Dispensers for traps were made from 2 ml glass vials loaded with 600 µl of neat (*R*)-(+)-γ-decalactone or the neat racemic mixture. Cut strings of cotton dental rolls (Celluron, Paul Hartmann, S.A., France) were inserted as wicks into the glass vials, and dispensers were attached to traps via a metal hook.

Electrophysiology Single-sensillum recordings were performed on both female and male *O. eremita*, but not on *E. ferrugineus*, which is rare in Sweden. To restrain a beetle, it was wrapped with parafilm, placed on a microscope slide, and fixed with dental wax. To get access to olfactory receptor neurons (ORNs), the three lamellae on the antenna were held apart with thin metal pins. A tungsten microelectrode or a thin silver wire was inserted into the abdomen of the insect, serving as a ground electrode, and a second tungsten microelectrode (electrolytically sharpened in KNO_2 solution) was inserted into an olfactory sensillum to establish contact with ORNs. The recording electrode was connected to a ×2 gain probe (Syntech, Hilversum, The Netherlands). A microscope with up to ×500 magnification and a DC-3K micromanipulator with a Piezo translator (PM10) (Märzhauser, Wetzlar-Steindorf, Germany) were used to position the electrode. Charcoal-filtered and humified air passed over the antenna from a glass tube outlet at 10 mm distance from the preparation. Odor stimulation of an ORN was achieved by inserting the tip of a Pasteur pipette, containing defined amounts of a stimulus, into a hole in the glass tube 10 cm before the outlet. The pipette was linked to an air control system (Syntech, Hilversum, The Netherlands), which generated 0.5 sec air puffs through the pipette into the air stream of the glass tube.

Odor Stimuli Neat (*R*)-(+)-γ-decalactone and the racemic mixture were diluted in hexane in decadic steps down to concentrations to be used in electrophysiological experiments. A test stimulus was then applied to a small piece of filter paper inserted into the test pipette, and the solvent was allowed to evaporate. Each pipette was loaded with 10 µl of a stimulus solution, and the amount of compound loaded into a pipette ranged from 100 ng to 100 µg. Pipettes loaded with 10 µl of hexane served as controls. After establishing contact with an ORN, it was stimulated with both test odorants at the highest dose (100 µg) and the control. If responses to the test odorants were not greater than to the control, the neuron was classified as non-responding. If the neuron responded to any of the test odorants, a dose–response trial was often performed by using the test stimuli at increasing concentrations. To enable the receptor neuron to recover after odor stimulation, at least 20 sec passed between each stimulation event. Responses from ORNs were calculated as the total number of spikes during 0.5 sec after the onset of stimulation minus the number of spikes during 0.5 sec before the onset of stimulation. The net response to an odor stimulus was calculated by subtracting the response generated by the control stimulation. In dose–response trials, net responses for females and males were compared for stimulations with the (*R*)-enantiomer and the racemic mixture at each dose using paired *t* tests, and net responses for stimulations with (*R*)-enantiomer were compared between the sexes at each dose using unpaired *t* tests.

Coupled Gas Chromatography and Single-sensillum Recordings To further test for the enantiomeric specificity of ORNs to γ-decalactone in *O. eremita*, gas chromatography linked to single-sensillum recordings (GC–SSR) was performed with a chiral column to resolve the enantiomers in the racemic mixture. A Hewlett-Packard 5890 Series II Plus gas chromatograph was used, equipped with a Cyclosil-B chiral column (30 m×0.25 mm i.d., 0.25 µm film; J&W Scientific, USA). Hydrogen was used as carrier gas (40 cm/sec), injector temperature was 220°C, and the following temperature program was used: 60°C for 2 min, 10°C/min to 170°C, followed by 2°C/min to 200°C, and then 10°C/min increase to 225°C. The transfer line temperature was maintained at 225°C. As stimulus, 100 ng of the racemic mixture were injected into the GC.

Field Trapping Field bioassays were conducted in Bjärka-Säby (58°16′N, 15°46°E) and Brokind (58°12′N, 15°40′E), southeast Sweden, during July–August, 2006 and 2007. The study area included five large stands of old hollow oaks housing some of the largest populations of *O. eremita* and *E. ferrugineus* in Sweden (Nilsson and Baranowski 1994; Ranius 2001; Antonsson et al. 2003). In 2006, two different nondestructive trapping systems were used, allowing beetles to be released alive after examination: Lindgren funnel traps (Phero Tech, Delta, BC, Canada), and custom-built traps consisting of two black plastic sheets (25 cm height×30 cm width, 3 mm thickness) arranged in a cross and attached perpendicular to a black plastic funnel (upper diameter, 27 cm) leading down to a 5-l white plastic container [for a picture of a trap with the same general design, see Ruther et al. (2000)]. In 2006, 20 replicates of 4 traps were used. Each replicate contained two traps of each type, baited with either 600 µl of the (*R*)-enantiomer or 600 µl of the racemic mixture. Custom-built traps were about 1.5 times more efficient than Lindgren traps in catching *O. eremita* (191 vs. 141 catches, $\chi^2=7.53$; $df=1$; $P<0.01$) and *E. ferrugineus* (88 vs. 55 catches, $\chi^2=7.62$; $df=1$; $P<0.01$). Thus, only custom-built traps were used in 2007. Twelve replicates were used, each containing 2 traps baited with either 600 µl of (*R*)-enantiomer or 2×600 µl of racemic

mixture, and a control trap without odorants. Traps were suspended from oak branches at 2–4 m height and at least 10 m apart, and they were checked for the presence of beetles every second day. The relative positions of traps within replicates were changed every eighth day.

In 2006 (but not in 2007), captured *O. eremita* were marked with shallow marks drilled in the elytra (Ranius 2001), and *E. ferrugineus* were marked on the elytra with a permanent marker pen to estimate the proportion of recaptures, which comprised <10% of the total number of captures in *O. eremita* and <4% in *E. ferrugineus*. Statistical analyses on trapping data were based on the total number of captures, including recaptures. For male and female *O. eremita* and *E. ferrugineus*, χ^2 analyses were performed to check for the difference in trap catch among treatments. We found no published information allowing the sex of *E. ferrugineus* individuals to be distinguished easily without genital examination, and to avoid killing them, we did not determine the sex of most captured individuals in 2006, except for a subset of 23 individuals, mostly consisting of individuals found dead in the traps.

Results

Electrophysiology Recordings were performed only from sensilla on the inner lamella of the *O. eremita* antenna. High-quality recordings were obtained from 234 sensilla of 30 females and 101 sensilla of 21 males. Based on their spike amplitude, most sensilla appeared to contain two ORNs, but odor stimulation always affected only one of these neurons, whereas the other was nonresponsive to both stimuli. In 88% of the recordings from both females and males, the ORNs in the sensillum did not respond to the (*R*)-enantiomer or racemic mixture of γ-decalactone. The remaining sensilla (29 in females and 10 in males)

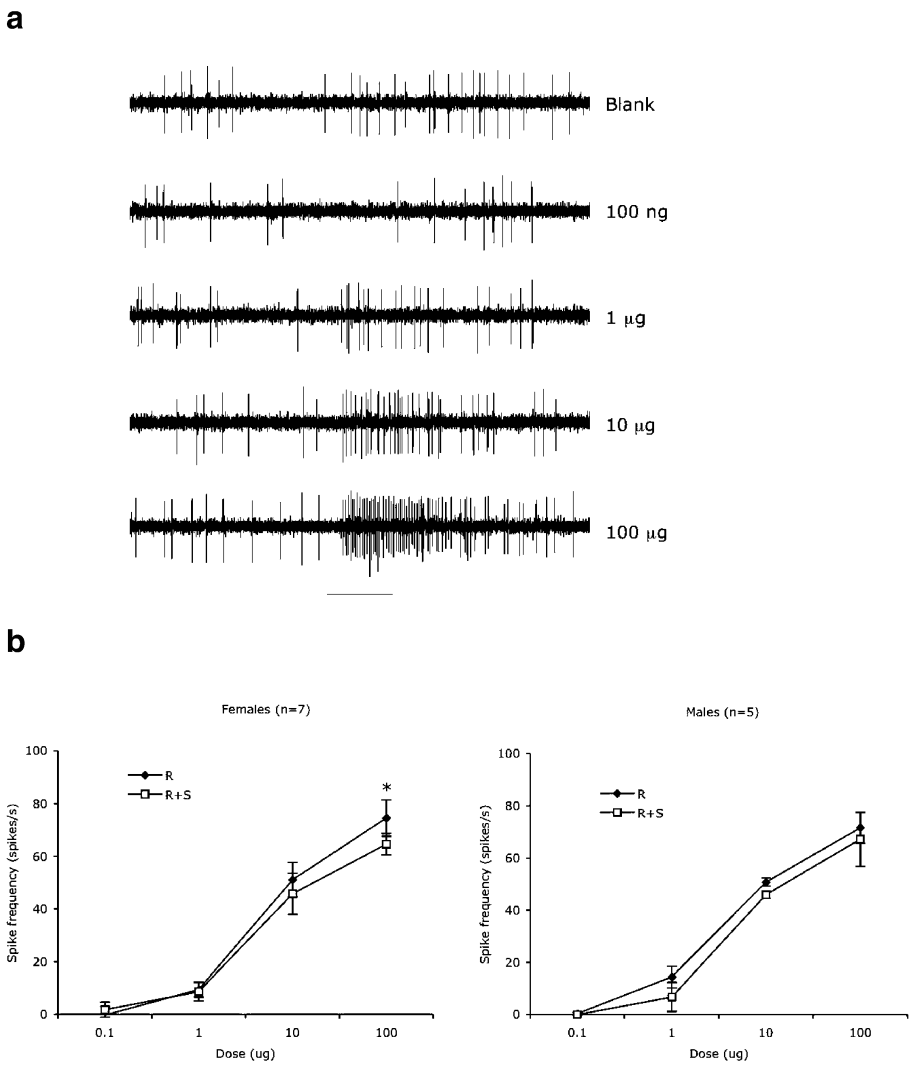

Fig. 1 **a** Single-sensillum recordings from the antenna of a female *O. eremita* showing the response of an ORN to the blank control and to increased doses of (*R*)-(+)-γ-decalactone. The *bar below* the recordings indicates the stimulus duration (0.5 sec). **b** Dose–response relationships of ORNs of female and male *O. eremita* stimulated with the (*R*)-enantiomer (*R*) or a racemic mixture (*R*+*S*) of γ-decalactone. For each dose, the same amount of (*R*)-enantiomer was used in both test stimuli. The response to a stimulus is quantified as the net number of spikes (number of spikes during 0.5 sec after the stimulation minus the number of spikes during 0.5 sec before the stimulation) minus the net blank response. *Error bars* show the standard error of the mean. The *asterisk* indicates a significant difference in spike activity between stimuli within the dose (paired *t* test, $P<0.05$)

contained a receptor neuron that responded to both test odorants (Fig. 1a). No neurons were found that responded primarily to the racemate, which would have indicated specificity to the (*S*)-enantiomer. The electrophysiological recordings revealed that γ-decalactone-sensitive ORNs were sparsely distributed over the whole inner antennal lamella of both sexes, but mainly found in the smooth area close to the ventral edge of the lamella (cf. Larsson et al. 2001, 2003). In dose–response trials, ORNs of females and males showed equal sensitivity to (*R*)-(+)-γ-decalactone (for all doses, $P>0.05$; Fig. 1b). However, a small but significant difference in spike frequency was observed for ORNs of females when stimulated with the (*R*)-enantiomer compared to the racemic mixture at the highest dose ($t=2.96$, $df=6$, $P<0.05$; Fig. 1b). Single-sensillum recordings coupled with gas chromatography confirmed that the γ-decalactone-sensitive ORNs indeed only responded to the (*R*)-enantiomer (Fig. 2). There was a clear separation of the two compounds on the chiral column with the (*R*)-enantiomer eluting 8.5 sec earlier than its antipode, which allowed the γ-decalactone-sensitive receptors to recover between stimulation events.

Trap Catch O. eremita: In 2006, 332 females were caught in odor-baited traps with significantly more caught in traps baited with the (*R*)-enantiomer compared to those baited with the racemic mixture (with half the amount of (*R*)-enantiomer) (200 vs. 132; $\chi^2=13.93$; $df=1$; $P<0.001$; Fig. 3a). Also, 87 captures of males were observed, but no difference was observed in the number of captures between odor treatments (51 vs. 36 for *R* and *R*+*S*, respectively; $\chi^2=2.59$; $df=1$; $P>0.05$; Fig. 3a). In 2007, 122 captures of females in odor-baited traps were observed (Fig. 3b). No significant difference in trap catch was observed between traps baited with the (*R*)-enantiomer and those baited with the racemic mixture (with the same amount of (*R*)-enantiomer) (56 vs. 66; $\chi^2=0.82$; $df=1$; $P>0.05$; Fig. 3b). Seven and three males were captured in attractant-baited traps with (*R*)-enantiomer and the racemic mixture, respectively, and three females were found in control traps.

E. ferrugineus: In 2006, 143 captures of 139 individuals were observed (Fig. 3a). The majority of trapping events (60%) included single individuals, whereas up to six beetles were found in a trap on other occasions. Similar to *O. eremita* females, significantly more insects were caught in traps baited with the (*R*)-enantiomer compared to those baited with the racemic mixture with half the amount of (*R*)-enantiomer (88 vs. 55; $\chi^2=7.62$; $df=1$; $P<0.01$). The subset of 23 beetles used for sex determination contained 22 females and only 1 male. In 2007, only six females were trapped (three for each treatment, data not shown in Fig. 3b).

Discussion

In this study, we show both electrophysiological and behavioral evidence for enantiomeric anosmia in the pheromone communication system of *O. eremita*. Recordings from single sensilla showed that both sexes have

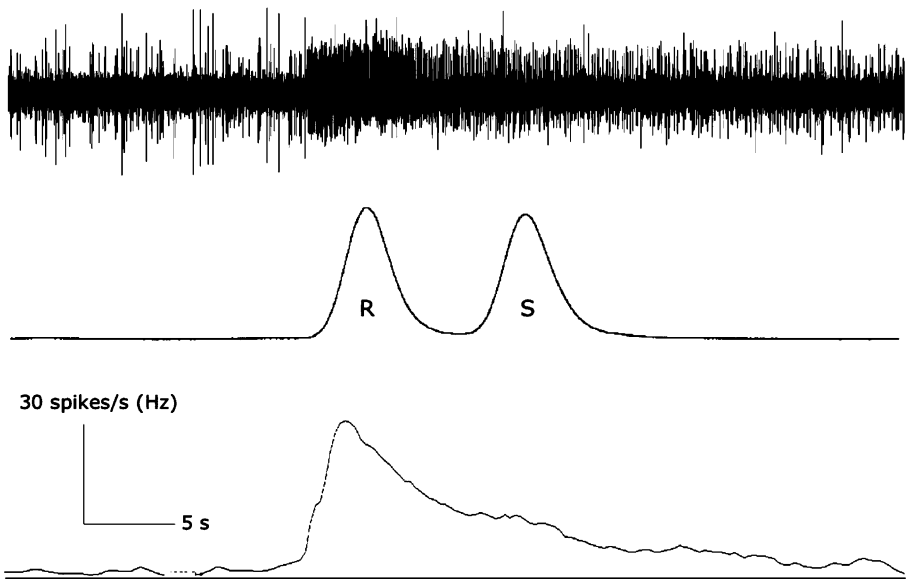

Fig. 2 Example of enantiomer-specific response to γ-decalactone in the ORN of a female *O. eremita* using GC–SSR. The *upper* trace shows the spike activity of the ORN, the *middle* trace shows the FID response to the two enantiomers separated on a chiral column, and the *lower* trace shows the spike activity of the ORN calculated as the number of spikes per second. After responding to the (*R*)-enantiomer, the ORN recovers almost completely and no second activity peak is observed during stimulation with the (*S*)-enantiomer

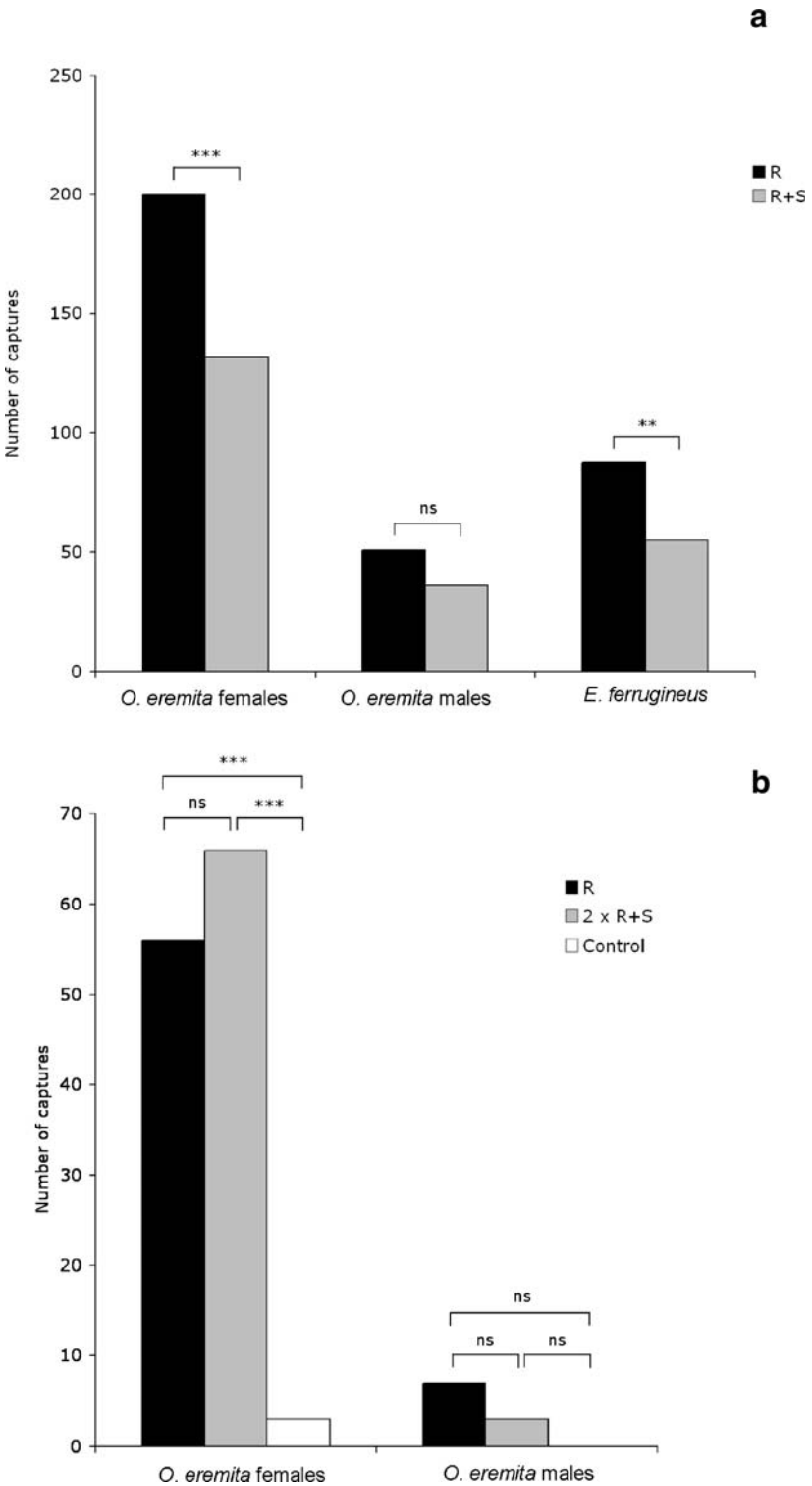

Fig. 3 **a** Trap catch of *O. eremita* and *E. ferrugineus* in 2006 using traps baited with 600 µl of (*R*)-enantiomer (*R*) or 600 µl of the racemic mixture (*R*+*S*) of γ-decalactone (χ^2 test: ***P*<0.01, ****P*<0.001). **b** Trap catch of *O. eremita* in 2007 using traps baited with 600 µl of (*R*)-enantiomer (*R*) or 2×600 µl of the racemic mixture (2×*R*+*S*) of γ-decalactone, and control traps (χ^2 test: ****P*<0.001)

ORNs specific to the (*R*)-enantiomer of γ-decalactone, the compound produced by conspecific males. No neurons tuned to the opposite enantiomer were found in the sensilla investigated. In dose–response trials that used the (*R*)-enantiomer and the racemic mixture as stimuli, the spike frequency of female ORNs was significantly higher for the pure (*R*)-enantiomer than for the mixture (with half the amount of (*R*)-enantiomer) at the highest dose (100 µg), also indicating that those neurons do not respond to the (*S*)-enantiomer (Fig. 1b). These observations were confirmed by using coupled gas chromatography and single-sensillum recordings where the two enantiomers were resolved on a

chiral column: the neurons always responded to the first-eluting (*R*)-enantiomer and never to the later-eluting (*S*)-enantiomer (Fig. 2).

Field trapping data were in concordance with electrophysiological data, and the attraction of both *O. eremita* females and its predator *E. ferrugineus* to attractant-baited traps was correlated with the amount of (*R*)-enantiomer released from lures. In 2006, significantly more captures of *O. eremita* females and *E. ferrugineus* were observed in traps with pure (*R*)-enantiomer compared to the racemic mixture, releasing half the amount of (*R*)-enantiomer. In 2007, no difference in trap catch of *O. eremita* females was observed when both treatments released the same amount of (*R*)-enantiomer. Thus, there is no inhibitory effect of adding the (*S*)-enantiomer to the *O. eremita* pheromone. From a practical point of view, the choice of lure is not critical, and the much cheaper racemic mixture can be used if the lure constitutes a significant part of the trap costs.

Effects of nonpheromonal enantiomers on insect pheromone attraction range from attractive to neutral to strong antagonistic effects (Mori 1998). The lack of inhibitory effects on the attraction of *O. eremita* and *E. ferrugineus* by the antipode of their pheromone/kairomone is in stark contrast to many other cases where the presence of the wrong enantiomer, sometimes at concentrations of only a few percent, results in strong inhibition of response to the pheromone. This phenomenon is common in many insect groups that use chiral pheromones, including scarab (Tumlinson et al. 1977; Leal 1996; Tolasch et al. 2003) and other beetles (Birch et al. 1980; Levinson and Levinson 1999; Lacey et al. 2004), gall midges (Hillbur et al. 2001), and moths (Szöcs et al. 1993; Larsson et al. 2002). The generally accepted explanation for this strong antagonistic effect is that it prevents cross-attraction of sympatric, usually congeneric, species that use the antipode as a pheromone component. Electrophysiological investigations of insect olfactory receptor neurons have demonstrated special sensory adaptations for avoiding enantiomers produced by heterospecifics. Species that use chiral pheromones often possess dedicated olfactory receptor neurons that respond selectively to each enantiomer, also when only one of these constitutes a pheromone component (Okada et al. 1992; Larsson and Hansson 1998; Larsson et al. 2002; Wojtasek et al. 1998).

In several scarab species, racemic mixtures do attract significant numbers of beetles, indicating that these species are indifferent to the presence of nonpheromonal enantiomers (Leal 1999). Absence of biological activity to nonpheromonal enantiomers has been demonstrated in the scarabs *Anomala octiescostata* Burmeister and *A. cuprea* Hope, for which the racemic mixture and pure enantiomer are equally attractive (Leal 1999). Electrophysiological investigations have shown that both species appear to be anosmic to the nonpheromonal enantiomers, entirely lacking receptors for their detection (Larsson et al. 1999, 2001; Leal 1999), which seems to be the case also for *O. eremita*. When the nonpheromonal enantiomer in a racemic mixture cannot be detected, the expected outcome might be a slightly lower attraction to the racemate than to the pure pheromone, considering that the release rate of the attractant is only 50% as high in the racemic mixture.

The high numbers of *E. ferrugineus* observed in attractant-baited traps in 2006 suggest that this click beetle may have higher populations than previously recognized. Scientists used to dealing with pest insects may not consider 139 beetles impressive, but it is close to the total of 147 specimens documented in Swedish collections until 1993 (Nilsson and Baranowski 1994) and is certainly many times more than entomologists have previously encountered during a lifetime. Earlier investigations of only eight *E. ferrugineus* individuals found in attractant-baited traps indicated that both males and females might be attracted to the *O. eremita* pheromone (Svensson et al. 2004). This study, with a larger sample analyzed, showed that the pheromonal signal is exploited almost exclusively by females, presumably to find trees containing *O. eremita*. The much lower number of *E. ferrugineus* captured per trap in 2007 suggests that populations of this predator fluctuate much more than populations of its prey.

Many trees suitable for saproxylic beetles cannot be analyzed with traditional methods, such as wood mold sampling or pitfall trapping, because of the characteristics of their hollows. For example, only about 25% of the oaks in the current study areas that potentially harbor *O. eremita* and *E. ferrugineus* have been investigated because other hollows have been too far up in a tree to be reached by a ladder or the cavity has been too deep to place a trap in the wood mold (Hedin and Mellbrand 2003). With a majority of suitable habitat patches not investigated, estimates of population sizes of focal species may be severely biased, which may in turn affect conservation strategies. With efficient nondestructive trapping systems available, we can now gather detailed ecological data about these species that have not been possible with previously available methods. Attractant-baited traps placed outside tree hollows will only catch those individuals that have left their natal tree and should be used as a complementary method to pitfall traps and other methods to increase the accuracy of population size estimates and analyses of dispersal rates and dispersal distances. Odor traps may also be used for targeted efforts to rediscover species at localities where they have not been observed for sometime. Our data show that attractant-based monitoring could be a powerful tool for conservation purposes, and more research should be focused on the chemical ecology of threatened insects to develop new strategies for their preservation.

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References

ALDRICH, J. R. 1999. Predators, pp. 357–381, in J. Hardie, and A. K. Minks (eds.). Pheromones of Non-Lepidopteran Insects Associated with Agricultural Plants. Wallingford, UK, CAB International.

ANONYMOUS 1992. Directive 92/43 of the Council of the European Community on the conservation of habitats and wild fauna and flora. European Community, Brussels.

ANTONSSON, K., HEDIN, J., JANSSON, N., NILSSON, S. G., and RANIUS, T. 2003. Occurrence of the hermit beetle (*Osmoderma eremita*) in Sweden. *Entomol. Tidskr* 124:225–240 (In Swedish, English summary).

BIRCH, M. C., LIGHT, D. M., WOOD, D. L., BROWNE, L. E., SILVERSTEIN, R. M., BERGOT, B. J., OHLOFF, G., WEST, J. R., and YOUNG, J. C. 1980. Pheromonal attraction and allomonal interruption of *Ips pini* in California by the two enantiomers of ipsdienol. *J. Chem. Ecol* 6:703–717.

BORDEN, J. H. 1997. Disruption of semiochemical-mediated aggregation in bark beetles, pp. 421–438, in R. T. Cardé, and A. K. Minks (eds.). Insect Pheromone Research: New Directions. New York, Chapman and Hall.

BUTENANDT, A., BECKMANN, R., STAMM, D., and HECKER, E. 1959. Über den Sexual-Lockstoff des Seidenspinners *Bombyx mori*. Reindarstellung und Konstitution. *Z. Naturforsch* 14b:283–284.

CARDÉ, R. T., and MINKS, A. K. 1995. Control of moth pests by mating disruption: successes and constraints. *Annu. Rev. Entomol* 40:559–585.

DAJOZ, R. 2000. Insects and Forests. pp. 515–533. Intercept Publishing, UK.

FOSTER, S. P., and HARRIS, M. O. 1997. Behavioral manipulation methods for insect pest-management. *Annu. Rev. Entomol* 42:123–146.

GÄRDENFORS, U. (ed.) 2005. Rödlistade arter i Sverige 2005—The 2005 Red List of Swedish Species. ArtDatabanken, Uppsala, Sweden.

HANNAH, L., CARR, J. L., and LANKERANI, A. 1995. Human disturbance and natural habitat: a biome level analysis of a global data set. *Biodivers. Conserv* 4:128–155.

HANSEN, V. 1966. Danmarks Fauna, Biller XXIII, Smældere of Pragtbiller, Gads Forlag, København, pp. 36–37 (In Danish).

HEDIN, J. and MELLBRAND, K. 2003. Population size of the threatened beetle *Osmoderma eremita* in relation to habitat quality, in J. Hedin (ed.). Metapopulation ecology of *Osmoderma eremita*—dispersal, habitat quality and habitat history. Ph.D. dissertation. Lund University, Lund, Sweden.

HILLBUR, Y., BENGTSSON, M., LÖFQVIST, J., BIDDLE, A., PILON, O., PLASS, E., FRANCKE, W., and HALLBERG, E. 2001. A chiral sex pheromone system in the pea midge, *Contarinia pisi*. *J. Chem. Ecol* 27:1391–1407.

LACEY, E. S., GINZEL, M. D., MILLAR, J. G., and HANKS, L. M. 2004. Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*. *J. Chem. Ecol* 30:1493–1507.

LARSSON, M. C., and HANSSON, B. S. 1998. Receptor neuron responses to potential sex pheromone components in the caddisfly *Rhyacophila nubila* (Trichoptera: Rhyacophilidae). *J. Insect. Physiol* 44:189–196.

LARSSON, M. C., LEAL, W. S., and HANSSON, B. S. 1999. Olfactory receptor neurons specific to chiral sex pheromone components in male and female *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J. Comp. Physiol* 184:353–359.

LARSSON, M. C., LEAL, W. S., and HANSSON, B. S. 2001. Olfactory receptor neurons detecting plant odours and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J. Insect Physiol* 47:1065–1076.

LARSSON, M. C., HALLBERG, E., KOZLOV, M. V., FRANCKE, W., HANSSON, B. S., and LÖFSTEDT, C. 2002. Specialized olfactory receptor neurons mediating intra- and interspecific chemical communication in leafminer moths *Eriocrania* spp. (Lepidoptera: Eriocraniidae) . *J. Exp. Biol* 205:989–998.

LARSSON, M. C., HEDIN, J., SVENSSON, G. P., TOLASCH, T., and FRANCKE, W. 2003. Characteristic odor of *Osmoderma eremita* identified as a male-released pheromone. *J. Chem. Ecol* 29:575–587.

LEAL, W. S. 1991. (*R*,*Z*)-5-(−)-(Oct-1-enyl)oxacyclopentan-2-one, the sex pheromone of the scarab beetle *Anomala cuprea*. *Naturwissenschaften* 78:521–523.

LEAL, W. S. 1996. Chemical communication in scarab beetles: reciprocal behavioral agonist–antagonist activities of chiral pheromones. *Proc. Natl. Acad. Sci. U. S. A* 93:12112–12115.

LEAL, W. S. 1999. Enantiomeric anosmia in scarab beetles. *J. Chem. Ecol* 25:1055–1066.

LEVINSON, A., and LEVINSON, H. 1999. Inhibition of sexual attraction and mating by pheromone enantiomers in male *Lasioderma serricorne*. *Naturwissenschaften* 86:138–140.

LUCE, J. -M. 1996. *Osmoderma eremita* (Scopoli 1763), pp. 64–69, in P. J. van Helsdingen, L. Willemse, and M. C. D. Speight (eds.). Background Information on Invertebrates of the Habitats Directive and the Bern Convention. Part 1: Crustacea, Coleoptera, and Lepidoptera. Council of Europe, Strasbourg.

MORI, K. 1998. Chirality and insect pheromones. *Chirality* 10:578–586.

MORI, M., MOCHIZUKI, K., KOHNO, M., CHUMAN, T., OHNISHI, A., WATANABE, H., and MORI, K. 1986. Inhibitory action of (4*S*, 6*S*, 7*R*)-isomer to pheromonal activity of serricornin, (4*S*, 6*S*, 7*S*)-7-hydroxy-4,6-dimethyl-3-nonanone. *J. Chem. Ecol* 12:83–89.

NILSSON, S. G., and BARANOWSKI, R. 1994. Indicators of megatree continuity—Swedish distribution of click beetles (Coleoptera, Elateridae) dependent on hollow trees. *Entomol. Tidskr* 115:81–97 (In Swedish, English summary).

OKADA, K., WATANABE, A., MORI, M., SHIMAZAKI, K., CHUMAN, T., MOCHIZUKI, F., and SHIBUYA, T. 1992. Olfactory responses to the sex pheromone component and its behavioral inhibitor in the male cigarette beetle, *Lasioderma serricorne*. *J. Insect. Physiol* 38:705–709.

PALM, T. 1959. Die Holz- und Rinden-Käfer der Süd- und Mittelschwedishen Laubbäume. Lund: Opuscula Entomologica supplementum XVI (In German, English summary).

PAYNE, T. L., RICHERSON, J. V., DICKENS, J. C., WEST, J. R., MORI, K., BERISFORD, C. W., HEDDEN, R. L., VITÉ, J. P., and BLUM, M. S. 1982. Southern pine beetle: Olfactory receptor and behavior discrimination of enantiomers of the attractant pheromone frontalin. *J. Chem. Ecol* 8:873–881.

PAYNE, T. L., DICKENS, J. C., and RICHERSON, J. V. 1984. Insect predator–prey coevolution via enantiomeric specificity in a kairomone–pheromone system. *J. Chem. Ecol* 10:487–492.

RANIUS, T. 2001. Constancy and asynchrony of *Osmoderma eremita* populations in tree hollows. *Oecologia* 126:208–215.

RANIUS, T. 2002. *Osmoderma eremita* as an indicator of species richness of beetles in tree hollows. *Biodivers. Conserv* 11:931–941.

RANIUS, T., and HEDIN, J. 2001. The dispersal rate of a beetle, *Osmoderma eremita*, living in tree hollows. *Oecologia* 126:363–370.

RANIUS, T., and NILSSON, S. G. 1997. Habitat of *Osmoderma eremita* Scop. (Coleoptera: Scarabaeidae), a beetle living in hollow trees. *J. Insect Conserv* 1:193–204.

RANIUS, T., AGUADO, L. O., ANTONSSON, K., AUDISIO, P., BALLERIO, A., CARPANETO, G. M., CHOBOT, K., GJURASIN, B., HANSSEN, O., HUIJBREGTS, H., LAKATOS, F., MARTIN, O., NECULISEANU, Z., NIKITSKY, N. B., PAILL, W., PIRNAT, A., RIZUN, V., RUICNESCU, A., STEGNER, J., SÜDA, I., SZWAKO, P., TAMUTIS, V., TELNOV, D., TSINKEVICH, V., VERSTEIRT, V., VIGNON, V., VÖGELI, M., and ZACH, P. 2005. *Osmoderma eremita* (Coleoptera, Scarabaeidae, Cetoniidae) in Europe. *Anim. Biodivers. Conserv* 28:1–44.

RUTHER, J., REINECKE, A., THIEMANN, K., TOLASCH, T., FRANCKE, W., and HILKER, M. 2000. Mate finding in the forest cockchafer, *Melolontha hippocastani*, mediated by volatiles from plants and females. *Physiol. Entomol* 25:172–179.

SVENSSON, G. P., LARSSON, M. C., and HEDIN, J. 2003. Air sampling of its pheromone to monitor the occurrence of *Osmoderma eremita*, a threatened beetle inhabiting hollow trees. *J. Insect Conserv* 7:189–198.

SVENSSON, G. P., LARSSON, M. C., and HEDIN, J. 2004. Attraction of the larval predator *Elater ferrugineus* to the sex pheromone of its prey, *Osmoderma eremita*, and its implication for conservation biology. *J. Chem. Ecol* 30:353–363.

SZÖCS, G., TÓTH, M., FRANCKE, W., SCHMIDT, F., PHILLIP, P., KÖNIG, W. A., MORI, K., HANSSON, B. S., and LÖFSTEDT, C. 1993. Species discrimination in five species of winter-flying Geometrids (Lepidoptera) based on chirality of semiochemicals and flight season. *J. Chem. Ecol* 19:2721–2735.

TOLASCH, T., SÖLTER, S., TÓTH, M., RUTHER, J., and FRANCKE, W. 2003. (*R*)-acetoin—female sex pheromone of the summer chafer *Amphimallon solstitiale* (L.). *J. Chem. Ecol* 29:1045–1050.

TOLASCH, T., von VRAGSTEIN, M., and STEIDLE, J. L. M. 2007. Sex pheromone of *Elater ferrugineus* L. (Coleoptera: Elateridae). *J. Chem. Ecol* 33:2156–2166.

TUMLINSON, J. H., KLEIN, M. G., DOOLITTLE, R. E., LADD, T. L., and PROVEAUX, A. T. 1977. Identification of the female Japanese beetle sex pheromone: inhibition of male response by an enantiomer. *Science* 197:789–792.

WOJTASEK, H., HANSSON, B. S., and LEAL, W. S. 1998. Attracted or repelled?—A matter of two neurons, one pheromone binding protein, and a chiral center. *Biochem. Biophys. Res. Commun* 250:217–222.

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Identification and Characterization of Cuticular Hydrocarbons from a Rapid Species Radiation of Hawaiian Swordtailed Crickets (Gryllidae: Trigonidiinae: *Laupala*)

Sean P. Mullen · Jocelyn G. Millar · Coby Schal · Kerry L. Shaw

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Abstract A previous investigation of cuticular hydrocarbon variation among Hawaiian swordtail crickets (genus *Laupala*) revealed that these species differ dramatically in composition of cuticular lipids. Cuticular lipid extracts of *Laupala* species sampled from the Big Island of Hawaii also possess a greatly reduced number of chemicals (as evidenced by number of gas chromatography peaks) relative to ancestral taxa sampled from the geologically older island of Maui. One possible explanation for this biogeographic pattern is that reduction in chemical diversity observed among the Big Island taxa represents the loss of ancestral hydrocarbons found on Maui. To test this hypothesis, we characterized and identified the structures of cuticular hydrocarbons for seven species of Hawaiian *Laupala*, two from Maui (ancestral) and five from the Big Island of Hawaii (derived) by using gas chromatography-mass spectrometry. Big Island *Laupala* possessed a reduced number of alkenes as well as a reduction in the diversity of methyl-branch positions relative to species sampled from Maui (ancestral), thus supporting our hypothesis of a founder-induced loss of chemical diversity. The reduction in diversity of ancestral hydrocarbons was more severe within one of the two sister lineages on the Big Island, suggesting that post-colonizing processes, such as drift or selection, also have influenced hydrocarbon evolution in this group.

Keywords Chemical communication · Speciation · Mate recognition · Pheromones · *Laupala*

S. P. Mullen
Department of Biological Sciences, Lehigh University, Bethlehem, PA 18015, USA

S. P. Mullen (✉) · K. L. Shaw
Department of Biology, University of Maryland, College Park, MD 02742, USA
e-mail: sem307@lehigh.edu

J. G. Millar
Department of Entomology, University of California, Riverside, CA 92521, USA

C. Schal
Department of Entomology, North Carolina State University, Raleigh, NC 27695, USA

K. L. Shaw
Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14850, USA

Introduction

Endemic Hawaiian swordtail crickets of the genus *Laupala* are a well-characterized species radiation that has been intensively studied as a model system for the evolution of acoustic communication and speciation (Otte 1994; Shaw 1996, 1999, 2000; Shaw and Herlihy 2000; Mendelson and Shaw 2002; Shaw et al. 2007). Phylogenetic evidence suggests that speciation in *Laupala* has proceeded by colonization of newly emerged volcanic islands and subsequent rapid intra-island radiations (Mendelson and Shaw 2005). Unlike examples of ecologically-driven speciation (Funk 1998; Via 1999; Rundle et al. 2000; Nosil et al. 2002), closely related species of *Laupala* have diversified in secondary sexual traits but remain morphologically and ecologically similar.

Species boundaries in *Laupala* have been hypothesized based on differences in acoustic signaling and, to some extent, small metric differences in male genitalia (Otte 1994; Shaw 2000). Molecular evidence supports these delineations (Shaw 1999, 2002; Parsons and Shaw 2001;

Mendelson and Shaw 2002, 2005). Multiple species often occur sympatrically (Otte 1994; Shaw 2000), but males of sympatric species always call with distinct pulse rates (Otte 1994; Shaw 1999, 2000; Parsons and Shaw 2001). Evidence from phonotaxis trials has established that females are preferentially attracted to conspecific pulse rates (Shaw 2000; Shaw and Herlihy 2000; Mendelson and Shaw 2002), thereby contributing to the maintenance of barriers to gene flow in sympatry.

These data suggest that divergence among species of *Laupala* is driven by evolution of male calling songs and that sexual selection on this trait may be responsible for the rapid speciation rates observed among these crickets. Although recent behavioral work suggests that female preference for conspecific pulse rates operates as a long-range signal, females that are not given an opportunity to choose males based on long-range acoustics evidently rely upon additional sexual recognition signals during close-range courtship (Mendelson and Shaw 2006). Reliance upon multiple mate-recognition signals during sexual communication is widespread among animals (Johnstone 1996; Rowe 1999; Candolin 2003), and therefore, it is possible that *Laupala* utilize diverse chemical, tactile, or vibratory cues during courtship and mating (e.g., *Drosophila*, Boake 2005). Specifically, the use of chemical mate-recognition signals has been demonstrated in other cricket species (Balakrishnan and Pollack 1997; Treganza and Wedell 1997), and cuticular compounds may function as close-range sex pheromones in the *Laupala* system as well.

To investigate the possible importance of contact pheromones in *Laupala*, Mullen et al. (2007) examined patterns of chemical variation in cuticular lipids among species of *Laupala* from Maui and the Big Island of Hawaii. Their results demonstrated that (1) significant differences in cuticular lipids exist between males and females, (2) there has been rapid and dramatic evolution of cuticular lipid composition among species in this genus, and (3) there has been a significant reduction in the complexity of cuticular lipid profiles among closely related species from the Big Island of Hawaii, as compared to two outgroup species from Maui. Detailed knowledge of the chemical differences between these two island faunas is important to our understanding of the evolution of cuticular hydrocarbon (CHC) biosynthesis in these and other insects and, furthermore, may provide insights into the evolutionary processes that shape patterns of lipid diversity among species. Specifically, chemical identification will determine whether the reduced number of gas chromatography peaks observed for species on the Big Island, relative to outgroup taxa on Maui (Mullen et al. 2007), reflects a reduction in the chain-length diversity of saturated or unsaturated hydrocarbons, differences in methylation patterns for specific hydrocarbons, or both. This would allow us to test the hypothesis that Big Island *Laupala* species have experienced a founder-induced loss of ancestral cuticular lipid variation.

Methods

Sampling To identify the major cuticular lipid components identified by Mullen et al. (2007) and to test biogeographic hypotheses about patterns of chemical variation, extracts of cuticular lipids of males and females of seven *Laupala* species ($N=14$) were analyzed by coupled gas chromatography-mass spectrometry (GC-MS). Specifically, we sampled two ancestral species of these crickets from the island of Maui (*Laupala makaio* and *Laupala orientalis*) and five derived species from two distinct clades on the Big island of Hawaii (*Laupala pruna* and *Laupala kohalensis* vs *Laupala nigra*, *Laupala paranigra*, and *Laupala kona*; see Mendelson and Shaw 2005). Individual crickets used for GC-MS analysis were collected during 2005 and stored frozen until used for sample preparation.

Extraction and Analysis of Cuticular Lipids Lipids were extracted with high-performance liquid chromatography-grade hexane. All glassware was pre-rinsed three times with hexane to remove possible contaminants and allowed to dry in a fume hood for 5–10 min before beginning extractions. Between each extraction, forceps were rinsed in three separate containers of hexane to avoid cross-contamination of samples. Each cricket carcass was wiped with a wet Kimwipe® to remove any external debris and then submerged in 1 ml hexane for 5 min in a glass vial with a Teflon-lined cap. Extracts were concentrated to ~200 µl under a stream of nitrogen, and 1 µl aliquots were analyzed by GC-MS with an Agilent 6890 GC interfaced to an Agilent 5973 mass selective detector operated in electron impact ionization mode (70 eV). The GC was fitted with a 30 m×0.25 mm I.D. DB-5MS column (J &W Scientific, Folsom, CA, USA), programmed from 100°C for 1 min, 10°C/min to 280°C and held for 20 min. Helium was the carrier gas. The injector and transfer line temperature was 280°C, the ion source temperature was 250°C, and the quadrupole temperature 200°C. Injections were made in splitless mode, with purge on at 0.5 min.

Straight-chain saturated alkanes were identified by their molecular ions and from comparisons of retention times and mass spectra with those of authentic standards. Methyl-branched compounds were identified from their Kovat's retention indices relative to straight chain hydrocarbons, in combination with diagnostic ions from enhanced fragmentations at methyl branch points (Nelson 1993; Nelson and Blomquist 1995; Carlson et al. 1998). Unsaturated alkenes were identified by molecular weight, from retention times

Table 1 Cuticular hydrocarbons present in various *Laupala* species

Retention Time	Compound I.D.	*L. orientalis*	*L. makaio*	*L. pruna*	*L. kohalensis*	*L. paranigra*	*L. nigra*	*L. kona*	Diagnostic Fragments
16.82	2Me-C22	+							281, 309 (324)
17.15	C23	+							324
17.79	3Me-C23	+							309 (338)
18.02	C24	+							338
18.55	C25 monoene						+		350
18.56	2Me-C24	+	+	+	+	+	+	+	309, 337, 352
18.68	C25 monoene	+	+					+	350
18.66–18.68	C25 di- and triene							++	346, 348
18.74	C25 monoene	+	+						350
18.83	C25 diene							+	348
18.86	C25	+	+	+	+			+	352
19.17	9Me-C25	+							140/252 (366)
19.21	7Me-C25	+							112/280, 351 (366)
19.28	5Me-C25	+	+	+					85/309, 351 (366)
19.39	2Me-C25	+		+					323, 351 (366)
19.49	3Me-C25	+	+	+	+				337, 351 (366)
19.49	C26 mono- and diene	+						+	364, 362
19.72	C26			+					366
20.21	C27 triene	+	+			+		+	374
20.31	C27 monoene						+		378
20.33	2Me-C26	++	++	++	++	+	++	+	337, 365, 380
20.36	C27 diene							+	376
20.48–20.54	C27 diene	+++	++		+			++	376
20.53	C27 monoene		++	+	+				378
20.63	C27 monoene		+	+	+				378
20.68	C27		+	+					380
21.09	7Me-C27	+							112/309 (394)
21.20	5Me-C27		+						85, 337 (394)
21.46	3Me-C27		+	+	+				365 (394)
22.36	C29 tetraene		++						400
22.42	C29 diene	+							404
22.47	C29 triene					+			402
22.52	2Me-C28	+	+	+	+	+	+	+	365, 393 (408)
22.59	C29 diene							+	404
22.63	C29 monoene	+							406
22.74	C29 diene	+	++	+		+	+		404
22.86	C29 monoene		+	+					406
23.64	7Me-C29		+	+					112, 337 (422)
23.80	C30 triene					+			416
25.28	C31 monoene				+				434
25.40	C31 monoene				+				434
25.44	C31 tetraene		+	+					428
25.51	C31 trienes					+++	+		430
25.61	C31 diene		+	+			+		432
25.69	C31 monoene				+				434
25.87	C31 triene				+				430
26.01	C31 diene				+				432
27.65	Polyene?			+					
30.02	Polyene?		+						
30.09	C33 triene					++	+		458
30.09	C33 tri-, tetra-, and pentaene						+++		454, 456, 458
30.28	C33 tri- and tetraene			+++	++				456, 458

+ Peaks contributing <10% of total area; ++, 10–50%; and +++, >50%.

slightly shorter than those of the corresponding straight-chain saturated alkanes, and their characteristic patterns of ions with masses 2, 4, or 6 mass units less than the corresponding ions in the spectra of straight-chain alkanes, for monoenes, dienes, and trienes, respectively. The positions and geometries of double bonds were not determined.

Results

Mass spectral analyses indicated that the cuticular lipids of *Laupala* species consisted of a relatively small number of saturated and unsaturated hydrocarbons (c.f., other insects; Howard and Blomquist 2005), varying in chain length from C_{22} to C_{33}. We found considerable variability among species (Table 1, Fig. 1), with the greatest diversity in chain length observed for *L. orientalis* (Maui). In particular, this species possessed a large variety of differentially methylated pentacosanes as well as several different unsaturated C_{27} and C_{29} alkenes. *L. makaio*, the other species sampled from Maui and the sister taxon to *L. orientalis* (Mendelson and Shaw 2005), similarly showed a large variety of hydrocarbons but differed from *L. orientalis* in possessing longer-chain alkenes (C_{29} and C_{31}), and lacking C_{22}–C_{24} alkanes. These two species also showed radically different relative abundances of particular hydrocarbons, with the CHC profile of *L. orientalis* predominantly (~66%) composed of C_{27} dienes.

Patterns of hydrocarbon diversity among the Big Island species were similar to *L. makaio* but typically displayed a marked reduction in hydrocarbon diversity. We found fairly similar hydrocarbon compositions for *L. kohalensis* and *L. pruna*, but these two species varied in the relative compositions of C_{31}–C_{33} tri- and tetraenes. In contrast, the remaining three species, *L. kona*, *L. paranigra*, and *L. nigra*, each showed a drastic reduction in both the number of hydrocarbon-chain lengths and the variety of unsaturated alkenes. The two most closely related species, *L. paranigra* and *L. nigra*, differed mainly in the relative abundance of C_{31} vs C_{33}. C_{33} alkenes made up ~70% of the CHC abundance for *L. nigra*, whereas a single C_{31} triene represented ~65% of the CHC observed for *L. paranigra*. A slightly larger number of *n*-alkenes were observed for *L. kona*, with no single-hydrocarbon type comprising more than 30% of the total.

Differences between Males and Females Our previous GC analyses of CHC variation (Mullen et al. 2007) demonstrated that significant qualitative differences existed among all species sampled. In addition, we showed that males and females of *L. makaio* had different cuticular lipid compositions. One of the goals of the current study was to assay each species for both qualitative and quantitative differences in individual hydrocarbons between males and females. In line with our expectations, we found that *L. makaio* males possessed more C_{31} tetraene than females (Fig. 2; males=48% total abundance vs 5% in females; $P<0.001$). Males and females of this species also appeared

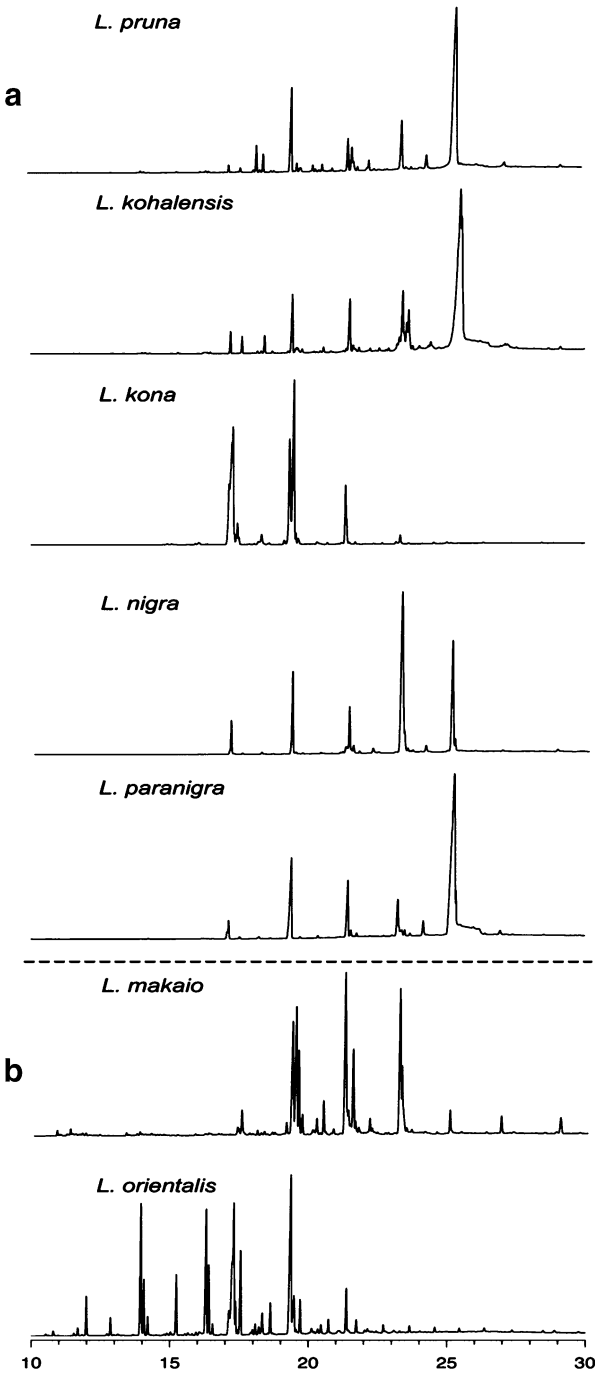

Fig. 1 Gas chromatograms showing differences in epicuticular lipids among Hawaiian *Laupala* sampled from the **a** Big Island of Hawaii (five species) and **b** Maui (two species); adapted with permission from Mullen et al. (2007)

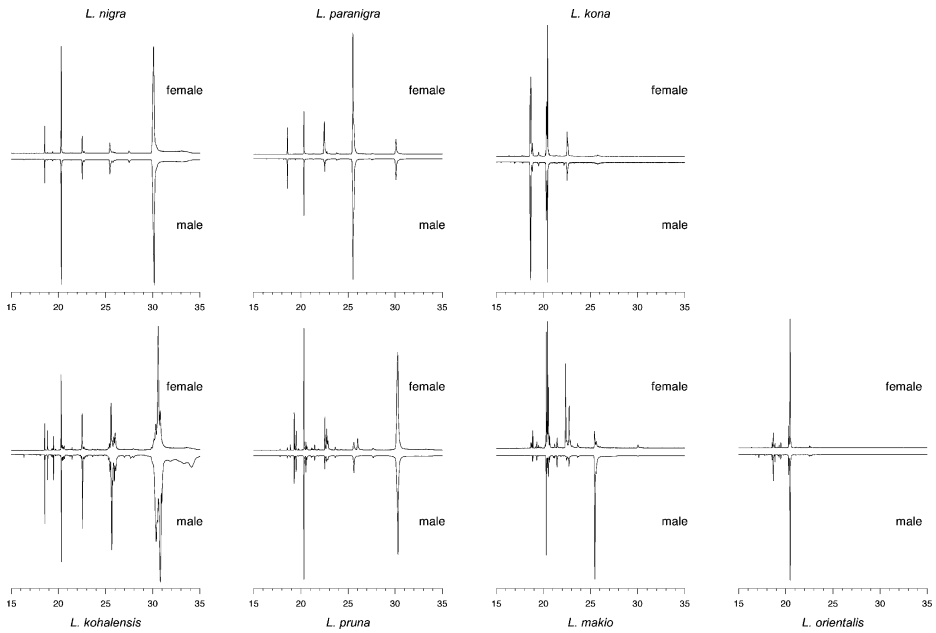

Fig. 2 Total ion chromatograms of extracts of cuticular hydrocarbons for various species of *Laupala*. Chromatograms from females are shown on the *top* and from males on the *bottom*

to differ in the relative ratios of C_{27} alkenes. Surprisingly, males and females of the other species sampled showed little qualitative variation (Fig. 2), even though our previous statistical analysis found significant quantitative variation between males and females when grouped by sex (Mullen et al. 2007).

Discussion

We tested our a priori hypothesis about hydrocarbon evolution in this group, namely, that the reduction in chemical diversity among the Big Island taxa represents the loss of modifications to the ancestral hydrocarbons found on the Maui taxa rather than the de novo origin of chain-length diversity. The chemical identifications supported this hypothesis and indicated that the differences between these two island faunas were due primarily to a decrease in the number of dienic and trienic hydrocarbons on the Big Island taxa compared to the crickets on Maui (Figs. 1 and 2, Table 1). Furthermore, the two ancestral species from Maui also showed a greater diversity of methyl branch positions for a given chain-length hydrocarbon than did species from the Big Island. The differences among species are highlighted by the fact that we identified only two hydrocarbons in common for all the *Laupala* species: 2-Me-C_{26} and 2-Me-C_{28}.

The striking differences between these two lineages in their hydrocarbon composition must reflect modifications in the biosynthesis of hydrocarbon. Straight-chain alkanes and *n*-alkenes are formed via elongation of fatty acyl-CoAs, which are converted to hydrocarbons one carbon shorter in length by decarboxylation (Blomquist 2003; Howard and Blomquist 2005). Chain length is regulated by specific fatty acyl-CoA elongases. Furthermore, methyl-branched hydrocarbons are derived from substitution of methylmalonyl-CoA for malonyl-CoA at specific chain positions during

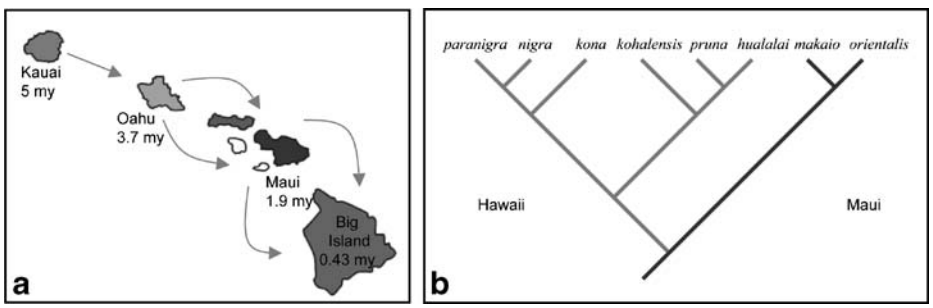

Fig. 3 **a** History of lineage splitting and intra-island radiations within the Hawaiian *Laupala* genus. **b** Simplified cladogram of the evolutionary relationships among the 'pacifica' group species sampled for this study based on Mendelson and Shaw's (2005) amplification fragment length polymorphism phylogeny

elongation (Howard and Blomquist 2005). Although it is unclear how the number and position of methyl-branching units is regulated, work on houseflies (*Musca domestica*, Gu et al. 1997) and the German cockroach (*Blattella germanica*, Gu et al. 1993) suggests that microsomal fatty-acid synthase (FAS) is involved in the production of methyl-branched fatty-acid precursors (Blomquist and Vogt 2003). Thus, the relatively greater diversity of methyl-branched alkanes and alkenes among the Maui species may involve differential regulation of FAS in this lineage.

Differences in Cuticular Composition among Laupala Species on the Big Island of Hawaii A previously unrecognized pattern that emerged as a result of the current analysis was the difference in the composition of cuticular hydrocarbons among the taxa of the Big Island '*pacifica*' species group. This lineage is the most rapidly diverging clade in a rapid species radiation (Mendelson and Shaw 2005). Previous phylogenetic work on these species supports close evolutionary relationships among *L. kohalensis*, *L. pruna*, and *L. hualalai* (Fig. 3). These three species form a recently diverged lineage that is most closely related to the remaining Big Island 'pacifica' group species (*L. kona*, *L. paranigra*, and *L. nigra*). Our results indicate that the *L. kohalensis–L. pruna* clade (*L. hualalai* was not sampled) is more diverse with respect to methyl-branch positions than the *L. kona–L. paranigra–L. nigra* clade. In fact, the former two species possess hydrocarbons that are similar to those found on *L. makaio* on Maui, although the relative amounts of each hydrocarbon vary considerably. The lesser hydrocarbon diversity in the latter species clade suggests that a dramatic reduction in the complexity and diversity of cuticular lipids has occurred in this lineage relative to the remaining Big Island species of the 'pacifica' group.

It is commonly thought that the primary function of insect cuticular hydrocarbons is to regulate water balance (Hadley 1984). Therefore, one possible explanation for the differences in cuticular lipid composition among species on the Big Island is natural selection acting in different environments (e.g., wet vs dry). However, this does not appear to be the case because each of these species occurs in a range of habitats on the Big Island. Furthermore, previous work suggested that closely related, allopatrically distributed species are more similar in their hydrocarbon profiles than are sympatric taxa that share overlapping habitats and ranges (Mullen et al. 2007). An alternative possibility for the variation in cuticular hydrocarbons is that some of these compounds serve as chemical mate-recognition signals and that differences among species reflect lineage-specific adaptations to sexual selection driven by female choice. It is well-recognized that cuticular hydrocarbons serve as recognition signals in arthropods (Howard and Blomquist 2005). While direct behavioral assays have not yet been completed, our preliminary behavioral observations (unpublished data) suggest that courtship between males and females of the species described here involves chemical communication.

Courtship in *Laupala* takes place over the course of several hours and involves a complex series of behavioral elements that are repeated multiple times (Shaw and Lugo 2001). Each courtship unit culminates in the passage of a spermless "microspermatophore" to the female, which is consumed by the female before the next courtship bout. Mating terminates only after the transfer of a much larger "macrospermatophore", that contains all of the sperm for a given mating (Shaw and Khine 2004; de Carvalho and Shaw 2005). Throughout courtship, males emit the same song used for long-range calling. Behaviorally, male singing during courtship is always preceded by antennal contact, and production of each spermatophore occurs while males and females "face" each other and engage in extensive antennal interactions (Shaw and Lugo 2001; Shaw and Khine 2004). Thus, chemical and/or tactile signals are likely exchanged during courtship, and mate choice may be a function of multiple mating signals, rather than simply a byproduct of long-range attraction of females to conspecific male songs.

In summary, the biogeographic differences in CHC diversity among lineages of Hawaiian *Laupala* is broadly consistent with a founder-induced loss of biochemical variation resulting from the colonization history of the Big Island taxa. However, the differences observed between the two major clades of these crickets on the Big Island suggest that post-colonization processes, such as drift or selection, have also played an important role in the evolution of CHC diversity in this system. Further work will focus on complete identification of the hydrocarbons along with behavioral assays to test whether these hydrocarbons function as mate-recognition cues.

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References

BALAKRISHNAN, R., and POLLACK, G. S. 1997. The role of sensory cues in female responses to courting males in the cricket *Teleogryllus oceanus*. *J. Exp. Biol* 200:511–522.

BLOMQUIST, G. J. 2003. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production. *Insect Biochem. Mol. Biol.* 24:231–252.

BLOMQUIST, G. J., and VOGT, R. G. 2003. Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic, London.

BOAKE, C. R. B. 2005. Sexual selection and speciation in Hawaiian *Drosophila*. *Behav. Genet.* 35:297–303.

CANDOLIN, U. 2003. The use of multiple cues in mate choice. *Biol. Rev.* 78:575–595.

CARLSON, D. A., BERNIER, R. U., and SUTTON, B. D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24:1845–1865.

DECARVALHO, T. N., and SHAW, K. L. 2005. Nuptial feeding of spermless spermatophores in the Hawaiian swordtail cricket, *Laupala pacifica* (Gryllidae: Triginodiinae). *Naturwissenschaften* 92:483–487.

FUNK, D. J. 1998. Isolating a role for natural selection in speciation: host adaptation and sexual isolation in *Neochlamisus bebbianae* leaf beetles. *Evolution* 52:1744–1759.

GU, P., WELCH, W. H., and BLOMQUIST, G. J. 1993. Methyl-branched fatty acid biosynthesis in the German cockroach, *Blattella germanica*: Kinetic studies comparing a microsomal and soluble fatty acid synthetase. *Insect Biochem. Mol. Biol.* 23:263–271.

GU, P., WELCH, W. H., GUO, L., SCHEGG, K. M., and BLOMQUIST, G. J. 1997. Characterization of a novel microsomal fatty acid synthetase (FAS) compared to a cytosolic FAS in the housefly, *Musca domestica*. *Comp. Biochem. Physiol* 118B:447–456.

HADLEY, N. F. 1984. Cuticle: Ecological significance, pp. 685–702, in J. Bereiter-Hahn, A. G. Matoltsy, and K. S. Richards (eds.). Biology of the Integument, vol. 1. Springer, Berlin.

HOWARD, R. W., and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol* 50:371–393.

JOHNSTONE, R. A. 1996. Multiple displays in animal communication: 'Backup signals' and 'multiple messages'. *Phil. Trans. Roy. Soc. London B* 351:329–338.

MENDELSON, T. C., and SHAW, K. L. 2002. Genetic and behavioral components of the cryptic species boundary between *Laupala cerasina* and *L. kohalensis* (Orthoptera: Gryllidae). *Genetica* 116:301–310.

MENDELSON, T. C., and SHAW, K. L. 2005. Sexual behavior: rapid speciation in an arthropod. *Nature* 433:375–376.

MENDELSON, T. C., and SHAW, K. L. 2006. Close-range acoustic signaling and mate choice in Hawaiian crickets (Gryllidae: *Laupala*). *Behav. Ecol. Sociobiol* 59:770–776.

MULLEN, S. P., MENDELSON, T. C., SCHAL, C., and SHAW, K. L. 2007. Rapid evolution of cuticular hydrocarbons in a species radiation of acoustically diverse Hawaiian crickets (Gryllidae: Trigonidiinae: *Laupala*). *Evolution* 61:223–231.

NELSON, D. R. 1993. Methyl-branched lipids in insects, pp. 271–315, in D. W. Stanley-Samuelson, and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry, and BiologyUniversity of Nebraska Press, Lincoln, Nebraska.

NELSON, D. R., and BLOMQUIST, G. J. 1995. Insect waxes, pp. 1–90, in R. J. Hamilton (ed.). Waxes: Chemistry, Molecular Biology, and FunctionThe Oily Press, Dundee, Scotland.

NOSIL, P., CRESPI, B. J., and SANDOVAL, C. P. 2002. Host–plant adaptation drives the parallel evolution of reproductive isolation. *Nature* 417:440–448.

OTTE, D. 1994. The Crickets of Hawaii: Origin, Systematics, and Evolution. Orthoptera Soc./Acad. of Nat. Sci. Philadelphia, Philadelphia.

PARSONS, Y. M., and SHAW, K. L. 2001. Species boundaries and genetic diversity among Hawaiian crickets of the genus *Laupala* identified using amplified fragment length polymorphism. *Mol. Ecol.* 10:1765–1772.

ROWE, C. 1999. Receiver psychology and the evolution of multicomponent signals. *Anim. Behav* 58:921–931.

RUNDLE, H. D., NAGEL, L., BOUGHMAN, J. W., and SCHLUTER, D. 2000. Natural selection and parallel speciation in sympatric sticklebacks. *Science* 287:306–308.

SHAW, K. L. 1996. Sequential radiations and patterns of speciation in the Hawaiian cricket genus *Laupala* inferred from DNA sequences. *Evolution* 56:237–255.

SHAW, K. L. 1999. A nested analysis of song groups and species boundaries in the Hawaiian cricket genus *Laupala*. *Mol. Phylogenet. Evol.* 11:332–341.

SHAW, K. L. 2000. Further acoustic diversity in Hawaiian forests: two new species of Hawaiian cricket (Orthoptera: Gryllidae: *Laupala*). *Zool. J. Linn. Soc*. 129:73–91.

SHAW, K. L. 2002. Conflict between mitochondrial and nuclear DNA phylogenies of a recent species radiation: what mtDNA reveals and conceals about modes of speciation in Hawaiian crickets. *Proc. Natl. Acad. Sci. U.S.A.* 99:16122–16127.

SHAW, K. L., and HERLIHY, D. 2000. Acoustic preference functions and song variability in the Hawaiian cricket *Laupala cerasina*. *Proc. Royal Soc. Lond. B* 267:577–584.

SHAW, K. L., and KHINE, A. H. 2004. Courtship behavior in the Hawaiian cricket *Laupala cerasina*: males provide spermless spermatophores as nuptial gifts. *Ethology* 110:81–95.

SHAW, K. L., and LUGO, E. 2001. Mating asymmetry and the direction of evolution in the Hawaiian cricket genus *Laupala*. *Mol. Ecol.* 10:751–759.

SHAW, K. L., PARSONS, Y. M., and LESNICK, S. C. 2007. QTL analysis of a rapidly evolving speciation phenotype in the Hawaiian cricket *Laupala*. *Mol. Ecol.* 16:2879–2892.

TREGANZA, T., and WEDELL, N. 1997. Definitive evidence of cuticular pheromones in a cricket. *Anim. Behav.* 54:979–984.

VIA, S. 1999. Reproductive isolation between sympatric races of pea aphids. I. Gene flow restriction and habitat choice. *Evolution* 53:1446–1457.

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Experimental Evidence for Three Pheromone Races of the Scarab Beetle *Phyllophaga anxia* (LeConte)

Paul S. Robbins · Daniel B. Cash · Charles E. Linn, Jr · Wendell L. Roelofs

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Abstract This study offers experimental evidence for the existence of three pheromone races of the northern genitalic form of *Phyllophaga anxia*: one race in which females produce and males respond mainly to L-valine methyl ester, a second producing and responding to L-isoleucine methyl ester, and a third producing and responding to an intermediate range of blends of the two compounds. At Franklinville, NY, pheromone gland contents of females were analyzed using coupled gas chromatography–electroantennogram detection. Two types of females were found, one that produced greater than 99% L-valine methyl ester and another that produced greater than 99% L-isoleucine methyl ester. Capture–mark–release–recapture field tests with males at Franklinville established that most males were recaptured in traps baited with the same blends with which they were originally captured. The populations characterized at Franklinville, NY, have also been found at numerous locations from eastern Canada and the northeast and north central USA, sometimes in allopatry and sometimes in sympatry. At a site in Carver, MA, *P. anxia* males responded to blends of the methyl esters of L-valine and L-isoleucine, and Carver females produced blends similar to those to which the males responded. Populations responding to blends have been identified only from southeastern Massachusetts and Rhode Island. At a field site near Waterloo, NY, the addition of small proportions of L-isoleucine methyl ester to lures containing L-valine methyl ester did not affect trap captures, but higher proportions of L-isoleucine methyl ester were inhibitory, decreasing trap captures.

Keywords L-Isoleucine methyl ester · L-Valine methyl ester · Sex pheromone · Scarabaeidae · Melolonthinae · Pheromone races

P. S. Robbins (✉) · C. E. Linn, Jr · W. L. Roelofs
Department of Entomology,
New York State Agricultural Experiment Station,
Cornell University,
Geneva, NY 14456, USA
e-mail: psr1@cornell.edu

D. B. Cash
P.O. Box 282, Angelica, NY 14709, USA

Introduction

In a multiyear North American study involving the capture and identification of approximately 57,000 individuals in 61 species of scarab beetles in the genus *Phyllophaga*, Robbins et al. (2006) documented the existence of geographic variation of male response of the northern genitalic form of *Phyllophaga anxia* to various blends of the two sex attractant components of this species. *P. anxia* is described as having two distinctive genitalic types, commonly called northern or southern (see Luginbill and Painter 1953 or Woodruff and Beck 1989 for photographs or scanning electron micrographs of the two forms). *P. anxia* is perhaps the most widely distributed (Luginbill and Painter 1953; Woodruff and Beck 1989) of the approximately 215 species (Evans and Smith 2007) of *Phyllophaga* in the USA and Canada. The larval stages of *P. anxia* are serious root-feeding pests of commercial cranberry crops in Massachusetts and Wisconsin (Franklin 1950; Dittl and Kummer 1997).

In the study of Robbins et al. (2006), traps were baited with various blends of the methyl esters of L-valine and L-isoleucine, the sex pheromone components of this species (Zhang et al. 1997). Three types of *P. anxia* male responders were identified: males that were captured principally in the 100:0 or 90:10 L-valine methyl ester/L-

isoleucine methyl ester baited traps, those that were captured in the 0:100 L-valine methyl ester/L-isoleucine methyl ester baited traps, and those that were captured in traps baited with blends (90:10, 80:20, 60:40, 40:60, 20:80, 10:90) of the two compounds (Fig. 1). No morphological characters were found that delineated these populations (Robbins, personal observation). Males captured with intermediate blends of L-valine methyl ester/L-isoleucine methyl ester were found only in populations from southeastern Massachusetts and Rhode Island, whereas the groups responding to predominantly L-valine methyl ester or L-isoleucine methyl ester alone were captured over a much greater area (Fig. 2). In most locations, either the L-valine methyl ester population or the L-isoleucine methyl ester population dominated the male flight response profile, but at several sites, both populations occurred in large numbers flying on the same dates, producing a bimodal trap response profile (e.g., Fig. 3).

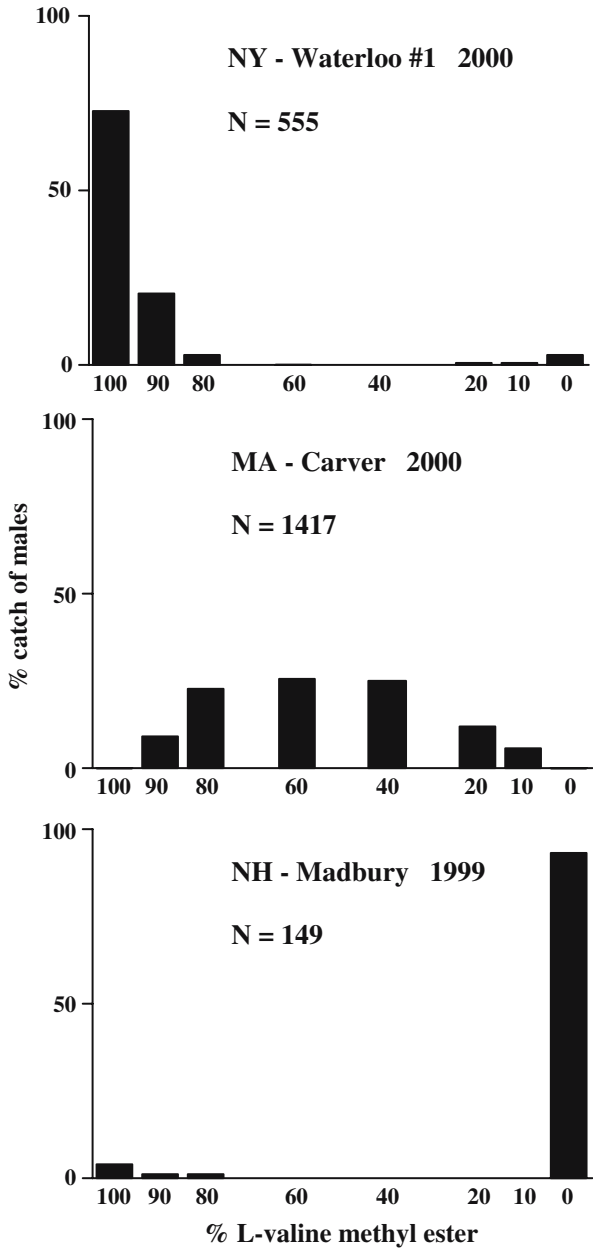

Fig. 1 Captures of male *P. anxia* in traps baited with various blends of the methyl esters of L-valine and L-isoleucine at three different sites. Captures for each lure blend are shown as a percentage of the total catch at that site

To investigate these populations in greater detail, experiments were conducted at three sites. At the first site, near Franklinville, NY, sex pheromone glands from individual females were analyzed to determine whether the female pheromone production curve was also bimodal. Male capture–mark–release–recapture studies were also conducted at Franklinville to determine repeatability of male preference. At the second site, near Carver in southeastern Massachusetts, where males responded to the various blend combinations, the range of ratios of L-valine methyl ester/L-isoleucine methyl ester produced by females was determined, to compare with the male response curves. The third site, Waterloo # 1 (Robbins et al. 2006), near Geneva, NY, was used to test the role of L-isoleucine methyl ester as a minor component in an L-valine methyl ester-responding *P. anxia* population.

Methods and Materials

Franklinville, NY—Single Female Pheromone Gland Analysis Female *P. anxia* were captured with UV light traps at the Franklinville site in 2000 to determine whether the ratios of L-valine methyl ester/L-isoleucine methyl ester produced by females mirrored the bimodal nature of the male response profile recorded in 1999 (Fig. 3). Five light traps were maintained at the site in 2000 from May 1 to June 30. Each day, insects captured in light traps were retrieved, and the sexes were separated. Females were stored individually in ~30-ml plastic cups in a 3:1 mix of greenhouse sand and screened peat moss raised to approximately 12% moisture. Each cup was marked with the date and light trap identification number. Cups containing females were held in a home refrigerator for 2–3 d and then at 10°C for up to 4 d until gland contents were extracted for analysis. Pheromone glands were everted by applying gentle pressure to the abdomen and then excised with iris scissors. Excised glands were soaked individually for 20 min in 200 µl of dichloromethane, removed, and the extract concentrated under a nitrogen stream to approximately 20 µl. Samples were stored at −80°C until analysis.

Single-gland analysis of the Franklinville females was carried out by using a coupled gas chromatograph–electroantennogram detector (GC-EAD). A Hewlett-Packard

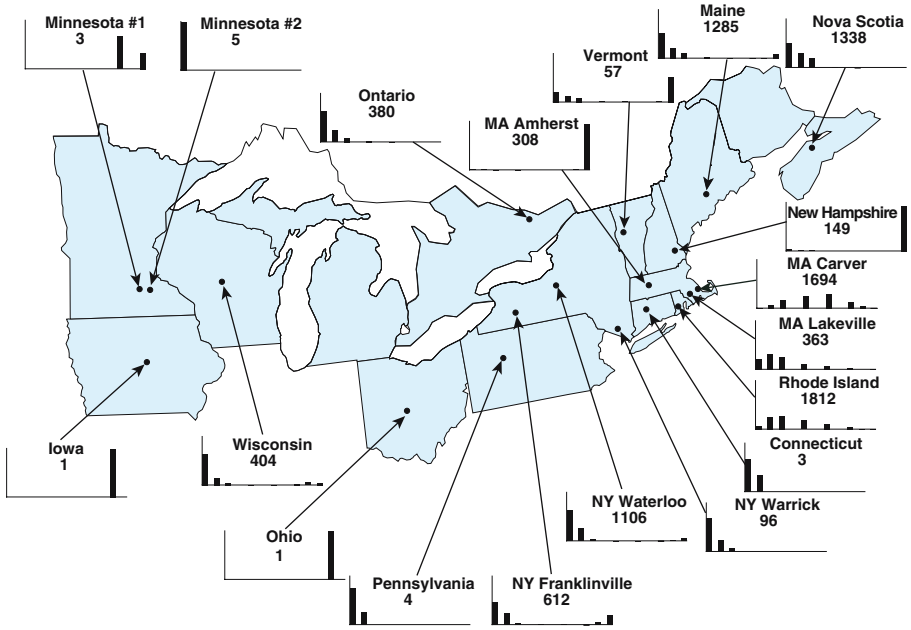

Fig. 2 Captures of male *P. anxia*, northern genitalic form, in traps baited with various blends of the methyl esters of L-valine and L-isoleucine. Total male capture=20,640. Catches from all years are not shown here. *X*- and *Y*-axes are the same as those shown in Fig. 1

5890 series II gas chromatograph equipped with a nonpolar EC-1 Econo-Cap column (30 m×0.25 mm inner diameter [ID], 0.25 μm film thickness; Alltech, Deerfield, IL) was used for the analyses. The carrier gas was N_2 at a head pressure of 138 kPa (flow rate, 2.0 ml/min). Injections were made in splitless mode (split valve opened at 1 min). The temperature program was 40°C for 5 min, then 15°C/min to 250°C, and held for 10 min. Injector, EAD outlet, and flame ionization detector (FID) temperatures were 250°C. The column effluent was combined with N_2 makeup gas (30 ml/min) and then split 1:1 to the FID and EAD.

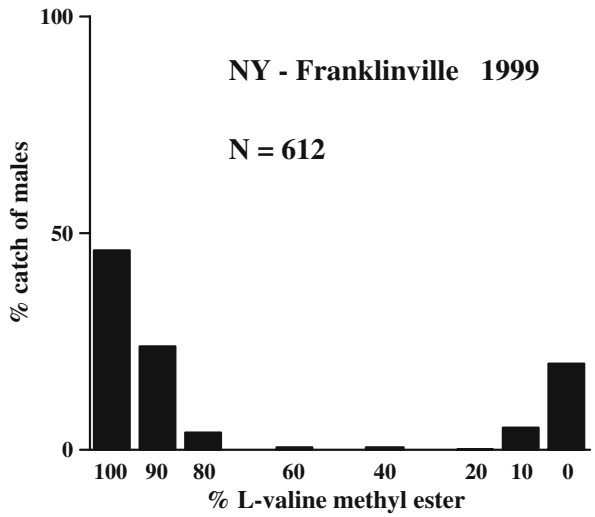

Fig. 3 Captures of male *P. anxia* in traps baited with various blends of the methyl esters of L-valine and L-isoleucine at a bimodal site. Captures for each lure blend are shown as a percentage of the total catch at that site

An extra FID port of the GC was modified and used for the EAD outlet. The EAD transfer line was terminated in a humidified, filtered air stream, which was cooled by a modified condenser flushed with 0°C water. The air stream carried the column effluent over the beetle antennal preparation. See Robbins et al. (2003) for a photograph of the beetle antennal preparation. The output signal from the antenna was amplified by a customized single-step high-input impedance DC amplifier. The resulting signal was recorded on a HP 3390 A integrator synchronized with the GC integrator. These methods were adapted from Nojima et al. (2003a) and Zhang et al. (1997).

Pheromone glands from 67 Franklinville females were extracted and analyzed by GC-EAD in 2000, using antennae from 11 different males. Preliminary work indicated no differences in antennal responses between the races when antennae from males of each of the two races were tested in the GC-EAD apparatus with 1 μl of a hexane solution containing 1 ng/μl of the methyl esters of L-valine and L-isoleucine.

Franklinville, NY—Male Capture–Mark–Release–Recapture

Males of populations in Franklinville, NY, were studied from 2000 to 2002 using capture–mark–release–recapture methods to evaluate blend response specificity. Traps were deployed on May 1, before *P. anxia* flights began, and checked each day until flight activity ceased at the end of June. In 2000 and 2001, the blends of L-valine methyl ester/L-isoleucine methyl ester were tested in the same ratios (100:0, 90:10, 80:20, 60:40, 40:60, 20:80, 10:90, and 0:100) and the same doses (4 mg per septum) as in the 1999 field test. A trap baited with a control septum treated with hexane was deployed in both years. A.C. Oehlschlager

(ChemTica Internacional S.A. [San Jose, Costa Rica]) kindly provided the lures for this study. The traps (see Robbins et al. 2006) were placed at the edge of a mature hardwood forest bordering a large field. Traps were hung on metal stakes ~15 m apart such that the trap bottom was ~30 cm from the ground. Traps were rerandomized each morning after collecting the beetles. In 2000 and 2001, male beetles were collected from each trap/blend combination and marked with a unique paint (Testor Corporation, Rockford, IL) code identifying the date captured and the blend to which the male responded. Marked individuals were distributed randomly among three release sites and allowed to burrow into the soil. The three release sites were 20 m from the trap line and equidistant from each other. Data from recaptured males were recorded, and males were frozen for later identification to species.

In 2002, eight traps were deployed at the same site, consisting of four traps baited with lures containing 4 mg of L-valine methyl ester and four containing 4 mg of L-isoleucine methyl ester. Positions of the L-valine methyl ester and L-isoleucine methyl ester traps were alternated within the trap line at initial placement. Beetles were collected, and trap positions were rotated each day during the flight period. Beetles captured were marked according to the compound to which they responded and released as in 2000 and 2001. Data from recaptured males were recorded, and males were frozen for later identification to species.

Carver, MA—Analysis of Female Pheromone Gland Contents Virgin females were dug from a cranberry bog in Carver, MA, in April of 2001 and 2002 before the beetle flight season. This was the same bog from which the individuals used to identify the *P. anxia* sex pheromone were acquired (Zhang et al. 1997). In the laboratory, females were housed in glass observation cages in a controlled environment room maintained at 25°C during the 16-hr photophase and 20°C during the 8-hr scotophase. Cages contained a 3:1 mix of greenhouse sand and screened peat moss raised to approximately 12% moisture in the bottom (approximately 10 cm deep) in which the females could burrow. A 6-mm mesh wire screening was available for the females to climb on and call from during scotophase. When females were observed calling (Leal et al. 1993; Nojima et al. 2003b) during scotophase, they were removed from the cages, the glands were excised and extracted, and the extracts were stored as described previously. Samples were analyzed on a Shimadzu GC17A in splitless mode, equipped with a nonpolar EC-1 Econo-Cap column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech). Nitrogen was used as the carrier gas. During the 1-min time for splitless injection, the column head pressure was kept at 150 kPa (flow rate, 2.2 ml/min) and then decreased to 78 kPa after the split valve was opened (flow rate, 1 ml/min). The oven temperature was kept at 40°C for 2 min, then programmed at 15°C/min to 150°C (0 min) and then at 30°C/min to 250°C (8 min). Injector and detector temperatures were 200 and 260°C, respectively. A 1:1 mix of L-valine methyl ester and L-isoleucine methyl ester at a concentration of 10 ng/μl each in hexane was used to calibrate the instrument and determine retention times of the two compounds. Ratios of the two compounds were determined by manual integration of the electronically stored peaks. Individuals were then ranked in 5% increments by percent of L-valine methyl ester from 0 to 100%. For example, an individual with 14.8% L-valine methyl ester was placed in the 10% rank, whereas an individual with 16.3% L-valine methyl ester was placed in the 15% rank.

Waterloo, NY—Role of L-Isoleucine Methyl Ester as a Minor Component The male flight response profile at Waterloo # 1 over several years (Robbins et al. 2006) indicated the presence of a large population that responded to lure blends containing a high percentage of L-valine methyl ester. This population provided an excellent site to test the effect on male capture of small amounts of L-isoleucine methyl ester added to the L-valine methyl ester. Traps were placed at the Waterloo no. 1 site, near Geneva, NY, on May 18, 2001 and removed on June 8, 2001. Treatments included L-valine methyl ester/L-isoleucine methyl ester blends of 100:0, 99.75:0.25, 99.5:0.5, 99:1, 97:3, 95:5, 90:10, and a solvent control-baited trap. Red rubber septa (5 mm Thomas Scientific, Swedesboro, NJ) were loaded with 4 mg each of the above blends by dissolving neat compounds in hexane, applying appropriate amounts to the septa, and allowing the hexane to evaporate in a fume hood. Three replications of the eight treatments were placed in three noncontiguous lines at the study site as described above. Traps were rerandomized each time they were checked, three times each week.

Statistics Data were tested for homogeneity of variance using Levene's test and log transformed ($x+1$) if necessary. Data were analyzed with a one-way analysis of variance, $P<0.05$. Fisher's least significant difference test was used for post-hoc comparisons.

Results

Franklinville, NY—Analysis of Pheromone Glands of Females Of the 67 glands analyzed from females caught in light traps at the Franklinville site in 2000, 63 glands contained enough material for GC analysis. The pheromone blend ratios were partitioned into two groups: those with L-valine

methyl ester/L-isoleucine methyl ester blends of approximately greater than 99:1 and those producing blends of approximately less than 1:99, yielding an unequivocal bimodal distribution (Fig. 4). In examining the coupled GC-EAD traces, it was found that in every instance, female glands contained both esters, regardless of the group to which they had been assigned. However, the amount of the lesser compound was often too small to be detected by the GC. Nevertheless, the EAD provided evidence of the presence of trace amounts of these compounds (Fig. 5).

Franklinville, NY—Capture–Mark–Release–Recapture of Males In capture–mark–release–recapture experiments of males, the rate of recapture for 2000 was 13.1% (39 of 297; Fig. 6a) and for 2001 was 10.9% (32 of 293; Fig. 6b). In both 2000 and 2001, males captured in traps baited with blends containing a high proportion of L-valine methyl ester were recaptured in traps baited with those blends. The shapes of the male recapture profiles are nearly identical to those of the capture profiles. In neither year were any males that were captured in the 100% L-isoleucine methyl ester-baited trap recaptured in that trap. In 2001, a single male captured using 100% L-isoleucine methyl ester returned to the 100% L-valine methyl ester-baited trap.

In 2002, the capture–mark–release–recapture field trial was redesigned. Because no males were recaptured in traps baited with the 100% L-isoleucine methyl ester in 2000 or 2001, we hypothesized that the sensitivity of the L-isoleucine methyl ester-responding population to the large amount of L-valine methyl ester emanating from the other blends present at the trapping site hindered their recapture in the single 100% L-isoleucine methyl ester-baited trap. The response curve of males from Franklinville in 1999

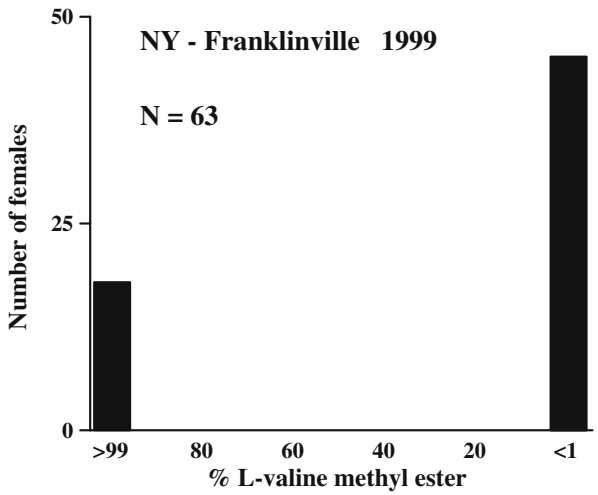

Fig. 4 Percentages of the methyl esters of L-valine and L-isoleucine extracted from pheromone glands of female *P. anxia*, Franklinville, NY, 2000

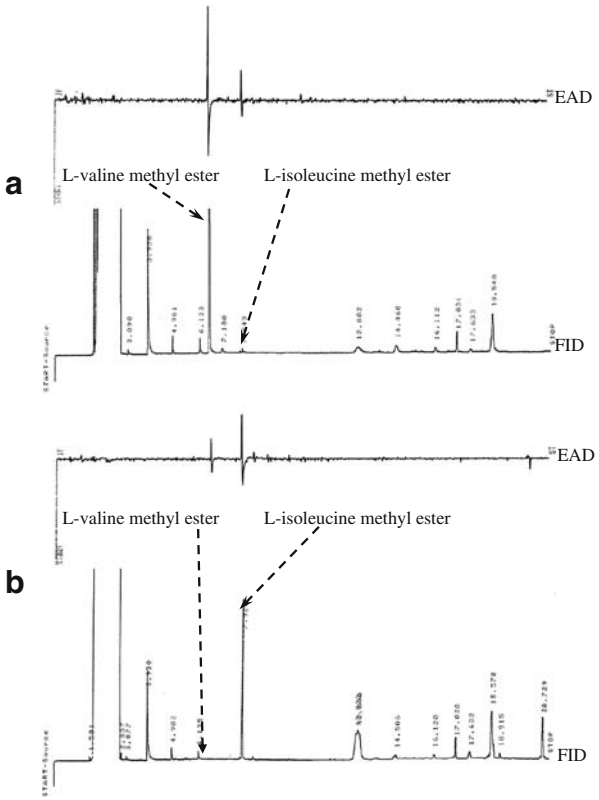

Fig. 5 Simultaneous EAD and FID traces illustrating male antennal responses to the gland contents of the two populations of female *P. anxia* found at Franklinville, NY, 2000. **a** L-Valine methyl ester population; **b** L-isoleucine methyl ester population

(Fig. 3) revealed that the population that responded to the high-percentage L-valine methyl ester blends was more tolerant of the presence of L-isoleucine methyl ester than was the L-isoleucine methyl ester-responding population to the presence of L-valine methyl ester. This phenomenon was also true for other *P. anxia* response profiles, whether or not both populations were present at a given site (Robbins et al. 2006). Bearing these observations in mind, males used for capture–mark–release–recapture in 2002 were presented with only the two pure compounds, 100% L-valine methyl ester and 100% L-isoleucine methyl ester. The capture profile of males in 2002 (Fig. 6c) indicated an increase in the number of beetles captured in the L-isoleucine methyl ester-baited traps relative to 2000 or 2001. The total rate of recapture for 2002 was 16.0% (212 of 1,320). The rate of recapture for the L-valine methyl ester-baited traps was 27.8% (199 of 717) and for the L-isoleucine methyl ester baited traps was 2.1% (13 of 603). A single male captured in a L-valine methyl ester-baited trap was recaptured in a L-isoleucine methyl ester-baited trap.

Carver, MA—Analysis of Pheromone Gland Contents of Females The contents of pheromone glands from 21 females were analyzed during 2001 and 2002. Of these, 17

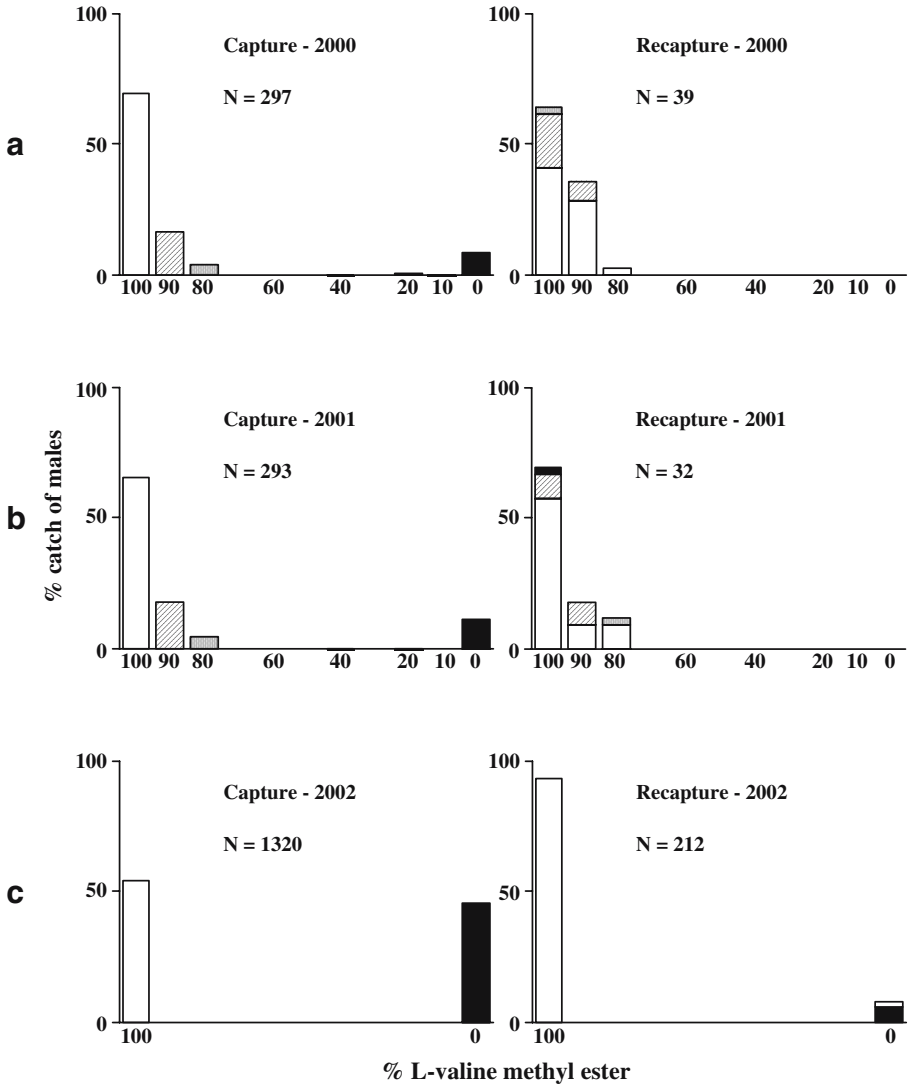

Fig. 6 *P. anxia* male capture–mark–release–recapture in traps baited with various blends of the methyl esters of L-valine and L-isoleucine. Franklinville, NY, **a** 2000, **b** 2001, **c** 2002. *Graph on the left* indicates percent male capture in each blend. *Graph on the right* indicates the percent female recapture in a given blend and the blend from which it was first captured in the graph on the left

extracts had sufficient amounts of the two components to calculate blend ratios. Single-gland analyses of females from the Carver site yielded a histogram of L-valine methyl ester/L-isoleucine methyl ester ratios (Fig. 7) similar to the male response curve found there in 1999 (Fig. 1) as well as other years (Robbins et al. 2006). No females from the Carver site produced blends of greater than 99:1 L-valine methyl ester/L-isoleucine methyl ester or less than 1:99, resembling those from Franklinville, NY. Of the 7,004 male *P. anxia* captured at Carver, MA, over 4 yr of trapping, only 33 were captured in the 100% L-valine methyl ester-baited traps and 22 in the 100% L-isoleucine methyl ester-baited traps. Thus, more than 99% of the males captured at Carver, MA, were captured using blends of the two compounds.

Waterloo, NY—Role of L-isoleucine Methyl Ester as a Minor Component All baited traps captured significantly ($F_{7,\ 16}$= 8.43; P<0.001) more males than the control (hexane only)

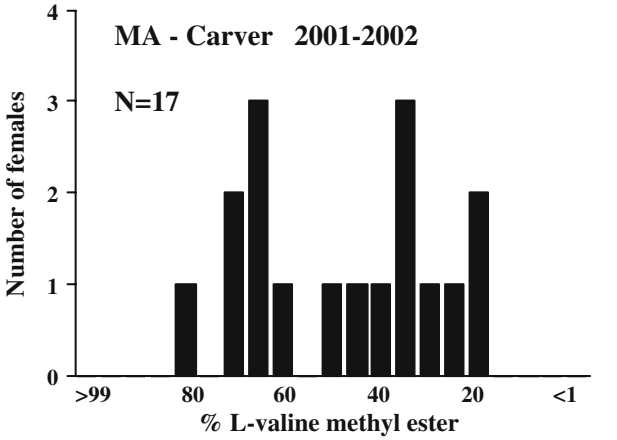

Fig. 7 Percentages of the methyl esters of L-valine and L-isoleucine extracted from pheromone glands of female *P. anxia*, Carver, MA, 2001 and 2002

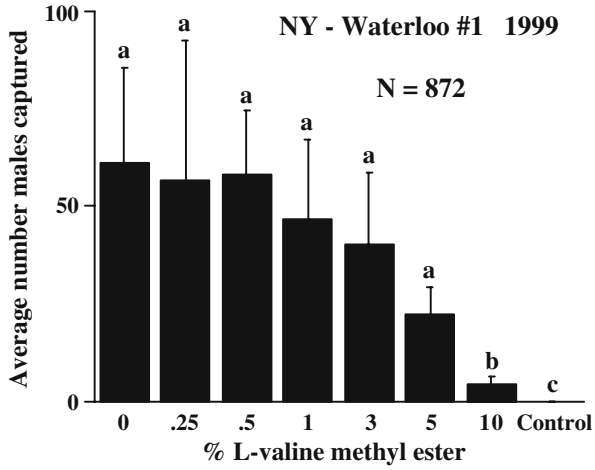

Fig. 8 Average capture/treatment (mean±SE) of male P. anxia in traps baited with various blends of the methyl esters of L-valine and L-isoleucine. Waterloo, NY, 2001. *Bars with the same letter* are not significantly different, $P<0.05$, Fisher's LSD test

traps (Fig. 8). There were no significant differences in numbers of males captured in traps baited with 0, 0.25, 0.5, 1.0, 3.0, and 5.0% L-isoleucine methyl ester (relative to the amount of L-valine methyl ester). The trap baited with 10% L-isoleucine methyl ester captured significantly fewer males than any of the traps baited with the other six blends with lower percentages of L-isoleucine methyl ester or no L-isoleucine methyl ester. Thus, we conclude that isoleucine did not function as a pheromone component at trace levels but rather was antagonistic at increasing doses.

Hand digging at Waterloo no. 1, NY, in April of 1999 yielded three virgin females. GC-EAD analysis of their pheromone gland contents showed a pattern identical to females from the Franklinville L-valine methyl ester group (Robbins, unpublished data), with large amounts of L-valine methyl ester and only trace amounts of L-isoleucine methyl ester.

Discussion

Evidence for Pheromone Races in P. anxia The results from analysis of female pheromone gland contents and capture–mark–release–recapture field tests with males offer experimental evidence for the existence of three pheromone races of the northern genitalic form of *P. anxia*: one in which females produce and males respond mainly to L-valine methyl ester, a second that employs L-isoleucine methyl ester, and a third that produces and responds to an intermediate range of blends of the two compounds. The variation in the sex pheromone system of *P. anxia* stands in direct contrast to the intraspecific uniformity of male pheromone response profiles found for 23 other *Phyllophaga* species captured at more than one geographical location (Robbins et al. 2006).

At Franklinville, NY, pheromone gland contents of females were analyzed by using GC coupled with EAD. One fraction of the population produced greater than 99% L-valine methyl ester, whereas the other fraction produced greater than 99% L-isoleucine methyl ester. Capture–mark–release–recapture field tests of males at Franklinville established that most but not all males were recaptured in traps baited with the same ratios of compounds in which they were originally captured. In 2000 and 2001, the trap lures were 100:0, 90:10, 80:20, 60:40, 40:60, 20:80, 10:90, and 0:100 L-valine methyl ester/L-isoleucine methyl ester. The results from those trapping tests revealed that 94% of the males captured in the 100:0 and 90:10 combinations were recaptured in traps baited with those same mixtures. In 2002, traps were baited with L-valine methyl ester or L-isoleucine methyl ester alone. In that test, of the males recaptured, 99.5% captured initially in traps baited with 100% L-valine methyl ester were recaptured in traps baited with the same source, whereas 100% captured in traps baited with 100% L-isoleucine methyl ester were recaptured in traps baited with 100% L-isoleucine methyl ester. Thus, the two *P. anxia* populations found at Franklinville, NY, are sympatric and synchronic, although it is unknown if they have different diel activity cycles.

Pheromone response curves of males resembling those of the two *P. anxia* pheromone races found at Franklinville, NY, are distributed from eastern Canada and the northeast USA to the north central USA (Fig. 2), sometimes in allopatry and sometimes in sympatry. Populations responding to blends have been found only in southeastern Massachusetts and Rhode Island. The blend responders appear as two groups. The first group, represented by a male capture profile generated from the Carver site (Fig. 9), was found at three locations (see the Carver, MA, and Plympton, MA, sites in Robbins et al. 2006) at which the overwhelming majority of males were captured in traps baited with blends of L-valine methyl ester/L-isoleucine methyl ester. Female *P. anxia* at Carver, MA, were found to produce blends of L-valine methyl ester/L-isoleucine methyl ester that ranged from 16:84 to 79:21 (Fig. 7). The male response profiles generated from capture data at the Carver site (Fig. 9) overlapped female production profiles by about 10% at each end, suggesting that the male response window was wider than the range of blends produced by the Carver females. In 4 yr of field testing at Carver, MA, and 1 yr each at Plympton no. 1, MA, and no. 2, MA, a total of 7,717 male *P. anxia* were captured. Of this total, only 53 (0.7%) were captured in traps baited with L-valine methyl ester alone, and 24 (0.3%) were captured in traps baited with L-isoleucine methyl ester alone.

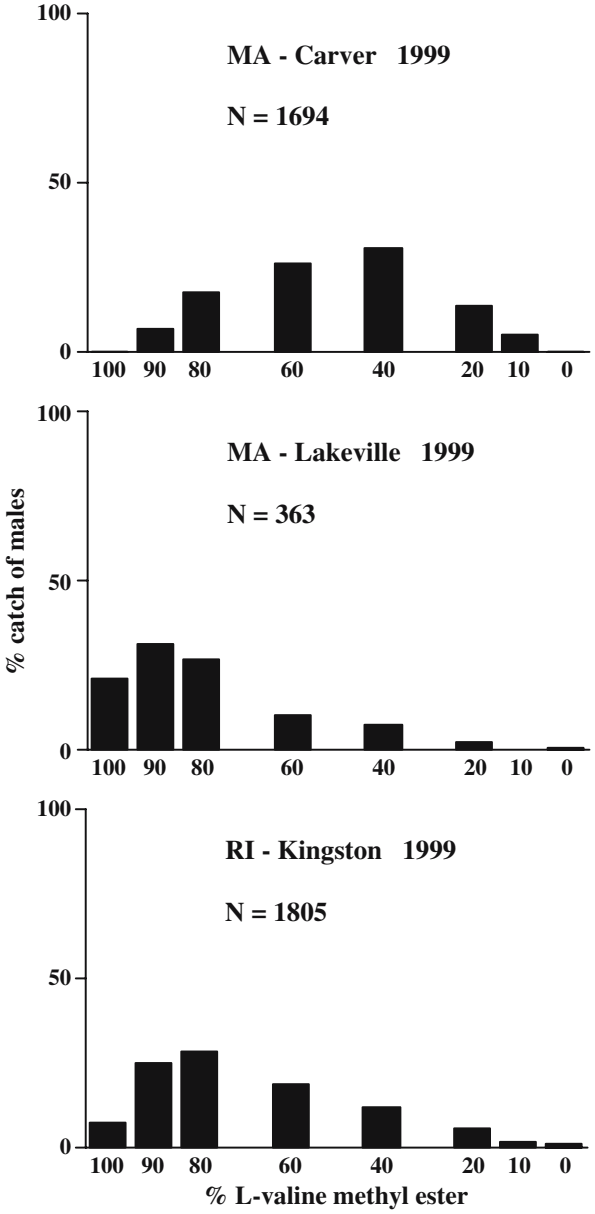

Fig. 9 Captures of male *P. anxia* in traps baited with various blends of the methyl esters of L-valine and L-isoleucine at test sites in southeast Massachusetts and Rhode Island. Captures for each lure blend are shown as a percentage of the total catch at that site

The second group responding to blends is represented by male capture profiles generated from 2 yr of field testing at Lakeville, MA, and Kingston, RI (Fig. 9). Although the majority of males at these sites were captured in traps baited with blends of L-valine methyl ester/L-isoleucine methyl ester, male capture curves were shifted to the L-valine methyl ester end of the blends presented, relative to those noted at Carver, MA. At the Carver and Plympton sites, an average of 0.7% of the males were captured in traps baited with 100% L-valine methyl ester, but in Lakeville, MA, and Kingston, RI, an average of 15.5% of the males were captured in traps baited with 100% L-valine methyl ester.

Intraspecific Variation in Insect Sex Pheromone Systems Sex pheromone variation is well documented in numerous insect species (Löfstedt 1990; Phelan 1997), particularly those in the order Lepidoptera. The turnip moth, *Agrotis segetum*, is widely distributed throughout Europe, the near East, and Africa. Various ratios of (*Z*)-5-decenyl acetate, (*Z*)-7-dodecenyl acetate, and (*Z*)-9-tetradecenyl acetate were required to attract males throughout Eurasia and North Africa, whereas in sub-Saharan Africa, (*Z*)-5-decenyl acetate alone attracted males (Arn et al. 1983; Löfstedt et al. 1986; Tóth et al. 1992; Wu et al. 1999). The larch budmoth, *Zeiraphera diniana*, consists of two host races that feed on larch and Cembran pine in the European Alps. The host races exist in both allopatry and sympatry, with each race producing and responding to its own pheromone. Cembran pine populations of *Z. diniana* utilize (*E*)-9-dodecenyl acetate as their pheromone, whereas the larch populations utilize (*E*)-11-tetradecenyl acetate (Baltensweiler et al. 1978; Baltensweiler and Priesner 1988). In contrast, analyses of populations of the pink bollworm moth, *Pectinophora gossypiella*, sampled in several areas of the world (Haynes and Baker 1988), and the cabbage looper moth, *Trichoplusia ni*, sampled across the USA (Haynes and Hunt 1990), demonstrated little variation in female produced pheromone blends.

The first case of intraspecific geographical variation in sex pheromone response was demonstrated in the Coleoptera. Lanier et al. (1972) utilized pine bolts infested with male pine engraver (*Ips pini*) from California, Idaho, and New York to perform reciprocal field tests at each of the three locations. In New York, beetles responded much more strongly to the infested bolts from New York than to either the infested bolts from California or Idaho. In both California and Idaho, the local populations preferred infested bolts from Idaho over bolts from California. In California, the local population showed only a small response to the New York bolts. Lanier et al. (1980) quantified ratios of (+)- and (−)-ipsdienol from *I. pini* males from Idaho and New York and field tested the two compounds in both locations. They concluded that the differences in production of and response to the enantiomers of ipsdienol in the populations at each of the locations explained their earlier work with infested bolts. Later, Miller et al. (1989) demonstrated that both chirality and quantity of the enantiomers of ipsdienol varied both between and within populations, even populations as close in proximity as southwestern and southeastern British Columbia.

The sex pheromone variation observed in *P. anxia* resembles none of the examples described above but rather

is reminiscent of the patterns seen in the European corn borer, *Ostrinia nubilalis*. In *O. nubilalis*, two pheromone races use blends of (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate. The *E* race produces a 99:1 blend of *E*/*Z* isomers, whereas the *Z* race produces a 3:97 blend of *E*/*Z* isomers. In the field, these races occasionally mate, and hybrids are produced. Hybrid females produce intermediate blend ratios of approximately 35:65 *Z*/*E* (Roelofs et al. 1985; Glover et al. 1991), and hybrid male moths preferentially fly to blends of the *E*/*Z* isomers in the flight tunnel, although more hybrid males of either *E*×*Z* or *Z*×*E* crosses fly to the *Z* blend alone than to the *E* blend alone. Despite of the fact that natural hybridization between the *E* and *Z* pheromone races is well documented in the field, an in-depth genetic analysis of sex pheromone production and perception in this species predicts that no self-sustaining population of hybrids can exist (Roelofs et al. 1987). Therefore, the origin of the stable blend-responding populations of *P. anxia* is intriguing.

In *O. nubilalis*, the differences in the blend ratios of (*E*) and (*Z*)-11-tetradecenyl acetate are due to a single change in the activity of the reductase enzyme that determines the final blend ratio in pheromone production (Linn and Roelofs 1995). Roelofs et al. (1987) speculated that a few simple genetic substitutions could result in the *E* strain evolving from the *Z* strain. However, changes in the sex pheromone production system would have to evolve by a different mechanism in *P. anxia* because the raw materials are not synthesized *de novo*. L-Valine and L-isoleucine are essential amino acids (Chapman 1982) and must either be sequestered during larval feeding or produced by bacterial endosymbionts. Our results from Franklinville showed that both amino acid methyl esters were present in the female pheromone glands of females, one in large amounts and the other in very small amounts, whereas at Carver, the compounds were present in the pheromone glands of females in intermediate blends. Therefore, the titers of the amino acids before esterification or the formation of the methyl esters themselves must be regulated in some manner in all the pheromone races. If bacteria are involved in production of sex pheromones in *P. anxia*, as they are in *Costelytra zealandica*, another melolonthine scarab beetle (Henzell and Lowe 1970; Hoyt and Osborne 1971), their identification and coevolution with the many *Phyllophaga* species will be of interest.

Genitalic Morphology and Pheromone Races in P. anxia Although this study was undertaken to examine the sex pheromone variation encountered in the northern genitalic form of *P. anxia*, this discussion would not be complete without including mention of the southern genitalic form. Luginbill and Painter (1953) note that *P. anxia*, the most widely distributed *Phyllophaga* species in North America, is a variable species in both size and genitalic characters. In an examination of *P. anxia* from collections at Rutgers University, the University of Kentucky, and the University of Alabama, Robbins found both distinctly northern and southern forms of genitalia from each of the three states, as well as genitalic forms that can be regarded as intermediates (Robbins, unpublished data). Luginbill and Painter (1953) also record the presence of intermediate forms. The southern form has been captured only in traps baited with 100% L-isoleucine methyl ester (Robbins et al. 2006). Although little is known about close range courtship and mating behaviors in *Phyllophaga*, there is nothing that precludes a male of the southern genitalic form flying upwind to a female of the northern genitalic form producing L-isoleucine methyl ester or vice versa. Successful matings between northern and southern forms may explain the existence of intermediate genitalic phenotypes.

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References

ARN, H., ESBJERG, P., BUES, R., TÓTH, M., SZÖCS, G., GUERIN, P., and AUSCHER, S. 1983. Field attraction of *Agrotis segetum* males in four European countries to mixtures containing three homologous acetates. *J. Chem. Ecol.* 9:267–276.

BALTENSWEILER, V. W., and PRIESNER, E. 1988. A study of pheromone polymorphism in *Zeiraphera diniana* Gn. (Lep., Tortricidae). 3. Specificity of attraction to synthetic pheromone sources by different male response types from two host races. *J. Appl. Entomol.* 106:217–231.

BALTENSWEILER, V. W., PRIESNER, E., ARN, H., and DELUCCHI, V. 1978. Evidence for different sexual attractants used by the larch form and cembran pine form of the gray larch bud moth *Zeiraphera diniana*. *Mitt. Schweiz. Entomol. Ges.* 51:133–142.

CHAPMAN, R. F. 1982. The Insects: Structure and Function. Harvard University Press, Cambridge, MA.

DITTL, T. G., and KUMMER, L. D. 1997. Major Cranberry Insect Pests of Wisconsin. www.wiscran.org/cranbugs.pdf.

EVANS, A. V., and SMITH, A. B. T. 2007. An Electronic Checklist of the New World Chafers (Coleoptera: Scarabaeidae: Melolonthinae), Version 2. Ottawa, Canada. 349 pp. http://www.museum.unl.edu/research/entomology/nwmelos.htm.

FRANKLIN, H. J. 1950. Cranberry Insects of Massachusetts. Bulletin 445. Parts II–VII. Massachusetts Agricultural Experimental Station, Amherst, MA.

GLOVER, T. J., KNODEL, J. J., ROBBINS, P. S., ECKENRODE, C. J., and ROELOFS, W. L. 1991. Gene flow among three races of European corn borers (Lepidoptera: Pyralidae) in New York state. *Env. Entomol.* 20:1356–1362.

HAYNES, K. F., and BAKER, T. C. 1988. Potential for evolution of resistance to pheromones. Worldwide and local variation in chemical communication system of pink bollworm moth, *Pectinophora gossypiella*. *J. Chem. Ecol.* 14:1547–1560.

HAYNES, K. F., and HUNT, R. E. 1990. Interpopulational variation in emitted pheromone blend of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:509–519.

HENZELL, R. F., and LOWE, M. D. 1970. Sex attractant of the grass grub beetle. *Science* 168:1005–1006.

HOYT, C. P., and OSBORNE, G. O. 1971. Production of an insect sex attractant by symbiotic bacteria. *Nature* 230:472–473.

LANIER, G. N., BIRCH, M. C., SCHMITZ, R. F., and FURNISS, M. M. 1972. Pheromones of *Ips pini* (Coleoptera: Scolytidae): variation in response among three populations. *Can. Entomol.* 104:1917–1923.

LANIER, G. N., CLASSON, A., STEWART, T., PISTON, J. J., and SILVERSTEIN, R. M. 1980. *Ips pini*: The basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

LEAL, W. S., SAWADA, M., MATSUYAMA, S., KUWAHARA, Y., and HASEGAWA, M. 1993. Unusual periodicity of sex pheromone production in the large black chafer *Holotrichia parallela*. *J. Chem. Ecol.* 19:1381–1391.

LINN, C. E., and ROELOFS, W. L. 1995. Pheromone communication in moths and its role in the speciation process, pp. 263–300, in D. M. Lambert, and H. G. Spencer (eds.). Speciation and the Recognition Concept The Johns Hopkins University Press, Baltimore, MD.

LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C., LÖFQVIST, J., LANNE, B. S., VAN DER PERS, J. N. C., and HANSSON, B. S. 1986. Pheromone dialects in European turnip moths *Agrotis segetum*. *Oikos* 46:250–257.

LUGINBILL, P., and PAINTER, H. R. 1953. May Beetles of the United States and Canada. US Department of Agriculture Technical Bulletin no. 1040.

MILLER, D. R., BORDEN, J. H., and SLESSOR, K. N. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 15:233–248.

NOJIMA, S., LINN, C. E. , MORRIS, B. D., ZHANG, A., and ROELOFS, W. L. 2003a. Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol.* 29:319–334.

NOJIMA, S., ROBBINS, P. S., SALSBURY, G. A., MORRIS, B. D., ROELOFS, W. L., and VILLANI, M. G. 2003b. L-Leucine methyl ester: The female-produced sex pheromone of the scarab beetle *Phyllophaga lanceolata*. *J. Chem. Ecol.* 29:2439–2446.

PHELAN, P. L. 1997. Evolution of mate-signaling in moths: phylogenetic considerations and predictions from the asymmetric tracking hypothesis, pp. 240–256, in J. C. Choe, and B. J. Crespi (eds.). Mating Systems in Insects and ArachnidsCambridge University Press, Cambridge, MA.

ROBBINS, P. S., CROCKER, R. L., NOJIMA, S., MORRIS, B. D., ROELOFS, W. L., and VILLANI, M. G. 2003. Methyl 2-(methylthio)benzoate: the unique sulfur-containing sex pheromone of *Phyllophaga crinita*. *Naturwissenschaften* 90:517–520.

ROBBINS, P. S., ALM, S. R., ARMSTRONG, C. D., AVERILL, A. L., BAKER, T. C., BAUERNFIEND, R. J., BAXENDALE, F. P., BRAMAN, S. K., BRANDENBURG, R. L., CASH, D. B., COUCH, G. J., COWLES, R. S., CROCKER, R. L., DELAMAR, Z. D., DITTL, T. G., FITZPATRICK, S. M., FLANDERS, K. L., FORGATSCH, T., GIBB, T. J., GILL, B. D., GILREIN, D. O., GORSUCH, C. S., HAMMOND, A. M., HASTINGS, P. D., HELD, D. W., HELLER, P. R., HISKES, R. T., HOLLIMAN, J. L., HUDSON, W. G., KLEIN, M. G., KRISCHIK, V. L., LEE, D. J., LINN, C. E., LUCE, N. J., MACKENZIE, K. E., MANNION, C. M., POLAVARAPU, S., POTTER, D. A., ROELOFS, W. L., OYALS, B. M., SALSBURY, G. A., SCHIFF, N. M., SHETLAR, D. J., SKINNER, M., SPARKS, B. L., SUTSCHEK, J. A., SUTSCHEK, T. P., SWIER, S. R., SYLVIA, M. M., VICKERS, N. J., VITTUM, P. J., WEIDMAN, R. B., WEBER, D. C., WILLIAMSON, R. C., and VILLANI, M. G. 2006. Trapping *Phyllophaga* spp. (Coleoptera: Scarabaeidae: Melolonthinae) with sex attractants in the United States and Canada. *J. Insect Sci.* 6:124.

ROELOFS, W. L., DU, J. W., TANG, X. H., ROBBINS, P. S., and ECKENRODE, C. J. 1985. Three European corn borer populations in New York based on sex pheromones and voltinism. *J. Chem. Ecol.* 11:829–836.

ROELOFS, W., GLOVER, T., TANG, X.-H., SRENG, I., ROBBINS, P., ECKENRODE, C., LÖFSTEDT, C., HANSSON, B., and BENGTSSON, B. O. 1987. Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proc. Natl. Acad. Sci. USA* 84:7585–7589.

TÓTH, M., LÖFSTEDT, C., BLAIR, B. W., CABELLO, T., FARAG, A. I., HANSSON, B. S., KOVALEV, B. B., MAINI, S., NESTEROV, E. A., PAJOR, I., SAZONOV, A. P., SHAMSHEV, I. V., SUBCHEV, M., and SZÖCS, G. 1992. Attraction of male turnip moths *Agrotis segetum* (Lepidoptera: Noctuidae) to sex pheromone components and their mixtures at 11 sites in Europe, Asia, and Africa. *J. Chem. Ecol.* 18:1337–1347.

WOODRUFF, R. E., and BECK, B. E. 1989. Arthropods of Florida and Neighboring Land Area: the Scarab Beetles of Florida (Coleoptera: Scarabaeidae). Part II. The May or June Beetles (genus *Phyllophaga*), vol. 13. Florida Department of Agriculture and Consumer Services, Tallahassee, FL(226 pp).

WU, W., COTTRELL, C. B., HANSSON, B. S., and LÖFSTEDT, C. 1999. Comparative study of pheromone production and response in Swedish and Zimbabwean populations of turnip moth, *Agrotis segetum*. *J. Chem. Ecol.* 25:177–196.

ZHANG, A., ROBBINS, P. S., LEAL, W. S., LINN, C. E. , VILLANI, M. G., and ROELOFS, W. L. 1997. Essential amino acid methyl esters: major sex pheromone components of the cranberry white grub, *Phyllophaga anxia* (Coleoptera: Scarabaeidae). *J. Chem. Ecol.* 23:231–245.

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E-2-Ethylhexenal, *E*-2-Ethyl-2-Hexenol, Mellein, and 4-Hydroxymellein in *Camponotus* Species from Brunei

Heather L. Voegtle · Tappey H. Jones · Diane W. Davidson · Roy R. Snelling

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Abstract *E*-2-ethyl-2-hexen-1-ol (**1**), mellein (**4**), and 4-hydroxymellein (**5**) were identified as the major volatile compounds in the head and/or thorax of *Camponotus quadrisectus*. Neither **1** nor **5** have been previously detected in insects. Also identified were small amounts of *m*-cresol (**2**) and 6-methyl salicylic acid (**3**). *E*-2-ethylhexenal (**6**) and small amounts of **3** were identified in heads of *Camponotus irritibilis* from Kuala Belalong, Brunei. Compounds **2**–**4** occur in other Bornean camponotines with hypertrophied mandibular glands, and **4** is widespread in the tribe. The possibility of semiochemical parsimony (multiple functions) for these mandibular gland compounds is reviewed in the context of existing data on mandibular gland products of other camponotines, reported biological activities of the compounds, and secondary loss of metapleural glands in this ant group.

Keywords Ants · Borneo · *Camponotus* · Mandibular glands · *E*-2-ethyl-2-hexenal · *E*-2-ethyl-2-hexenol · Mellein · 4-hydroxymellein

H. L. Voegtle · T. H. Jones (✉)
Department of Chemistry, Virginia Military Institute,
Lexington, VA 24450, USA
e-mail: jonesth@vmi.edu

D. W. Davidson
Department of Biology, University of Utah,
257 South, 1400 East,
Salt Lake City, UT 84112–0840, USA

R. R. Snelling
Los Angeles County Museum of Natural History,
900 Exposition Blvd.,
Los Angeles, CA 90007, USA

Introduction

Mandibular gland products of hyperdiverse and ubiquitous carpenter ants (genus *Camponotus*) have been studied in relation to several different functions (Hölldobler and Wilson 1990). The economic importance of these ants drove early studies of compounds proposed as male sex pheromones (Brand et al. 1973a; Blum et al. 1987; Seidel et al. 1990; Torres et al. 2001). However, the few studies that included sterile workers and reproductive females identified similar products in those castes (Duffield and Blum 1975; Seidel 1988; Blum et al. 1988; but see Bellas and Hölldobler (1985) for a *Polyrhachis* species, also Camponotini). In workers of certain species, mandibular gland volatiles also function in nestmate recognition (Jaffe and Sánchez 1984) and alarm/defense. For example, in *Camponotus gigas*, mandibular gland secretions appear to be used in fighting; they flow prodigiously from mandibular gland orifices when majors are engaged in combat (D. W. Davidson, personal observations). Additionally, some members of the *Camponotus* (*Colobopsis*) *cylindricus* complex deploy secretions of hypertrophied mandibular gland reservoirs in suicidal fights over territory (Jones et al. 2004; D.W. Davidson, unpublished data).

This report describes the identification of three compounds not previously reported from insects, *E*-2-Ethyl-2-hexen-1-ol (**1**) and 4-hydroxymellein (**5**) are two of the major components of the mandibular glands of workers of *Camponotus quadrisectus* (F. Smith), and *E*-2-ethyl-2-hexenal (**6**) is a major component from workers of a coexisting species, *Camponotus irritibilis* (F. smith). In addition, the mandibular glands of *C. quadrisectus* contain mellein (**4**) as a major component.

Methods and Materials

Field Work Ants were collected and observed at various times of the year at the Kuala Belalong Field Studies Center (KBFSC), run by the Universiti Brunei Darussalam, and located in the Batu Apoi Forest Reserve, Temburong District, Brunei (4°32′N, 115°10′E). Workers from each of two *C. quadrisectus* nests, and one nest of *C. irritibilis*, were collected separately in vials containing small amounts of methanol. In one of two collections from a *C. quadrisectus* nest, several workers were trisected into head, thorax, and gaster, with replicates of each body segment stored independently in vials containing methanol. Both *C. quadrisectus* colonies nested in live trees, one at ∼1.5 m height and the second at an undetermined height. After feeding, workers of *C. irritibilis* climbed high into the trees, likely to a high canopy nest that we could not locate. Activities of one colony of each species were monitored sporadically for 5–6 weeks on each of three trips to KBFSC. Voucher specimens of both species were deposited in the entomological collections of both the Brunei Museum and the Los Angeles County Natural History Museum (DWD-KB series 05A-30 and 05A-42, respectively).

Chemical Analyses Gas chromatography-mass spectrometry (GC-MS) was carried out in the EI mode with a Shimadzu QP-5000 equipped with a 30 m×0.25 mm i.d. RTX-5 column. The instrument was programmed from 60 to 250°C at 10°C min^{-1}. Nuclear magnetic resonance spectrometry (NMR) was carried out in $CDCl_3$ solutions by using a Varian Mercury 400 MHz NMR spectrometer. For samples of less than 1 mg, a Shigemi tube was used (5 mm symmetrical microtube matched with $CDCl_3$, insert length 190 mm, bottom length 8.0 mm). Vapor phase Fourier Tranform-Infrared Spectrophotometry (FT-IR) spectra were obtained with a Hewlett-Packard model 5965B detector interfaced with a Hewlett-Packard 5890 gas chromatograph fitted with a 30 m×0.25 mm RTX-5 Amine column. Preparative HPLC was performed on a Waters machine equipped with a 1525EF Binary HPLC pump, a 2987 Dual λ Absorbance Detector set at 254 nm, and Empower software. A prepacked C_{18} reversed-phase (RP) column (Nova-Pak, 3.9×150 mm, 4 μm, 60 Å) was used for the analyses with a solvent system of 60% methanol/40% H_2O (both solvents HPLC grade) and a flow rate of 1.0 ml min^{-1}.

4-Hydroxymellein **(5)**: Methanol extracts from *C. quadrisectus* were purified by reversed-phase HPLC. The fraction containing the unknown with a molecular ion of *m/z*=194 was collected, and the solvent was removed *in vacuo* to provide an isomeric mixture of **5** as a white solid; GC-FTIR: ν_{max} 3636, 3159, 3074, 2994, 2916, 1709, 1615, 1587, 1465, 1368, 1336, 1300, 1224, 1163, 1107, 1047, 986, 954, 825, 884 cm^{-1}; ^{1}H NMR ($CDCl_3$, 400 MHz) δ *cis*: 10.98 (1H, s, 8-OH), 7.47 (1H, t, *J*=7.2 Hz, H-6), 6.96 (1H,d, *J*=8.4 Hz, H-7), 6.86 (1H, d, *J*=6.8 Hz, H-5), 4.64 (1H, dq, H-3), 4.52 (1H, bs, H-4), 1.53 (3H, d, *J*=6.8 Hz, 3-Me); *trans*: 10.94 (1H, s, 8-OH), 7.47 (1H, t, *J*=7.2 Hz, H-6), 6.96 (1H, d, *J*=8.4 Hz, H-7), 6.86 (1H, d, *J*=6.8 Hz, H-5), 4.64 (1-H, dq, H-3), 4.56 (1H, bs, H-4), 1.46 (3H, d, *J*=5.6 Hz, 3-Me); ^{13}C NMR data, see Table 2; EI-MS: *m/z* 194 [M^+] (33), 165 (2), 150 (66), 137 (2), 122 (50), 121 (100), 104 (5), 93 (20), 77 (5), 65 (25), 43 (20).

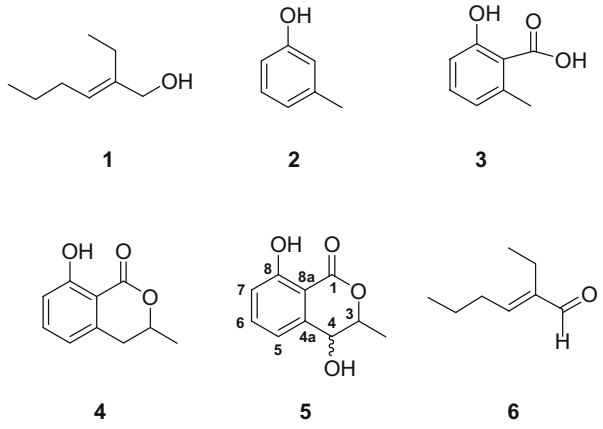
Results

Chemical Analyses GC-MS analysis of the methanol extracts from whole *C. quadrisectus* revealed three major components, *E*-2-ethyl-2-hexenol (**1**), mellein (**4**), and 4-hydroxymellein (**5**), along with small amounts of *m*-cresol (**2**) and 6-methylsalicylic acid (**3**) as its methyl ester (Fig. 1, Table 1). Straight chain alkanes, alkenes, and fatty acid methyl esters were frequently detected in smaller amounts. Extracts of heads and thoraxes had components **1**, **4**, and **5** as major compounds (92% in heads and 78% in thoraxes), while the gasters contained these compounds as minor components (ca. 16% of the total composition, with the remainder being the hydrocarbons listed in Table 1). *E*-2-Ethyl-2-hexenol (**1**) was suggested by its mass spectrum and confirmed by direct comparison with a synthetic sample (Iseki et al. 1992), while *m*-cresol (**2**) and 6-methylsalicylic acid (**3**) and its methyl ester, likely a consequence of storage in methanol, were suggested by

Table 1 Relative amounts of compounds identified from extracts of *Camponotus quadrisectus* and *C. irritibilis*

Compounds[a]	*C. quadrisectus*	*C. irritibilis*
E-2-Ethyl-2-hexen-1-ol (**1**)	40.5	–
E-2-Ethylhexenal (**6**)	–	54.8
m-Cresol (**2**)	0.5	–
Undecane	0.74	4.65
Ethyl octanoate	0.15	
Octanoic acid	0.48	
Tridecene	0.14	1.86
Tridecane	0.24	2.26
6-Methyl salicylic acid (**3**)	1.65	2.35
Tetradecane	0.1	
Pentadecene	0.6	
Pentadecane	0.7	1.31
Mellein (**4**)	30.8	–
Hexadecane	0.24	–
Heptadecane	0.30	–
4-Hydroxymellein (**5**)	20.6	–

[a] Gas chromatographic percentages including fatty acid methyl esters.



1 = *E*-2-ethyl-2-hexen-1-ol

2 = *m*-cresol

3 = 6-methyl salicylic acid

4 = mellein

5 = 4-hydroxymellein

6 = *E*-2-ethylhexenal

Fig. 1 Structures of important compounds identified in *Camponotus quadrisectus* and *C. irritibilis*

their mass spectra and confirmed by comparison with authentic samples. Mellein (**4**) was identified from its mass spectrum and direct comparison with published data (Dimitriadis et al. 1997).

The EI mass spectrum of **5** showed a parent ion at *m/z*= 194, 16 larger than the mass of mellein, indicating an additional oxygen, along with an ion at *m/z*=150 (M- CO_2) and analogous to the same fragmentation observed in the mass spectrum of mellein. The base peak at *m/z*=121/122 suggested that the second hydroxyl group must be somewhere other than the aromatic ring. The mass spectrum of **5** was similar to the published data for 4-hydroxymellein (Findlay et al. 1995). The C=O absorption at 1,709 cm^{-1} and the C–O absorptions at 1,224 and 1,105 cm^{-1} in the GC-FTIR spectrum of **5** corresponded to those (at 1,707, 1,220, and 1,101 cm^{-1}) observed in the spectrum of mellein obtained from the same sample. This strongly suggested a similar dihydroisocoumarin. Additionally, absorptions at 3,636 and 3,159 cm^{-1} in the spectrum of **5** indicated the presence of two hydroxyl groups. The latter matched the one at 3,157 cm^{-1} in the spectrum of mellein resulting from the hydrogen bonded phenolic hydroxyl group at C-8, while the absorption at 3,636 cm^{-1} indicated a non-hydrogen bonded OH.

To confirm the location of the second hydroxyl group on the non-aromatic ring, the ant extract was purified by RP HPLC to yield compound **5** as a white solid. The ^{1}H NMR spectrum of **5** resembled published spectra of 4-hydroxymellein (Findlay et al. 1995; Asha et al. 2004) and indicated the presence of both isomers of this compound. The presence of two distinct phenolic OH singlets at 10.98 and 10.94 ppm corresponded to the values reported for the *cis* and *trans* isomers, respectively, with the former being the major component (2.5:1 *cis:trans*). Doublets at 1.46 and 1.53 ppm for the methyl group on C-3 and broad singlets at 4.52 and 4.56 ppm for the hydrogen on C-4 also confirmed these assignments. Since the isomeric mixture broadened many signals throughout the ^{1}H NMR spectrum, the ^{13}C spectrum of **5** was obtained by using a low volume Shegimi tube. The intensities of the clearly observable pairs of signals for C-3, C-4, C-4a, and the methyl group, confirmed the 2.5:1 *cis/trans* isomeric ratio of **5**. With the exception of the signal for C-1, the ^{13}C NMR spectrum of the mixture of *cis* and *trans* **5** from *C. quadrisectus* corresponded to those previously reported for the separated isomers of 4-hydroxymellein (Table 2; Asha et al. 2004). On the other hand, the C-1 ^{13}C carbonyl signals of other dihydroisocoumarins, along with that of ethyl salicylate, are reported to appear from 170.2, to 173.0 ppm (Avantaggiato et al. 1999; Kongsaeree et al. 2003; Saito et al. 2007), more closely matching our observation of 169.4 ppm for C-1 than that reported by Asha et al. (2004). Some signals (C-1, C-8, and C-8a,) from the minor *trans* isomer of **5** were not apparent, possibly because of their long relaxation times and the predominance of the *cis* isomer.

The GC-MS analysis of the methanol extracts from *Camponotus irritibilis* revealed the presence of one major component, *E*-2-ethylhexenal (**6**), along with small amounts of 6-methylsalicylic acid (**3**) and its methyl ester, and smaller amounts of normal alkanes, alkenes, and fatty acid methyl esters frequently detected in the methanol extracts of ants (Table 1). The structure of **6**, was suggested by its mass spectrum (NIST/EPA/NIH 1999) and confirmed by direct comparison with an authentic sample (Häusermann 1951).

Discussion

This is the first report of *E*-2-ethyl-2-hexene-1-ol (**1**) and the analogous aldehyde (**6**) from insects. *C. quadrisectus*

Table 2 ^{13}C NMR DATA (δ) FOR *cis*- and *trans*-4-Hydroxymellein (**5**) Compared with literature values[a]

Carbon	*Cis* 5	Literature Values	*Trans* 5	Literature Values
1	169.4	162.0	–	161.96
3	78.4	77.9	80.1	79.8
4	67.5	67.1	69.4	69.1
4a	140.7	140.8	141.3	141.0
5	118.1	118.1	116.4	116.1
6	137.1	137.0	137.0	136.7
7	118.8	118.4	118.5	117.8
8	162.4	162	–	161.96
8a	107.1	106.9	–	106.7
9	16.3	15.9	18.2	17.8

[a] From Asha et al. 2004

and *C. irritibilis* are distinctive morphologically, but the presence of **1** and **6** as major components in the respective taxa, together with the unusual nature of these compounds, suggest a close phylogenetic chemical relatedness. Similar hydrocarbon profiles in the two species (Table 1) also support this.

In *C. quadrisectus*, the hydrocarbons are localized to the gaster, and presumably, this is also the case for *C. irritibilis*, although only whole ants were analyzed.

In contrast to **1** and **6**, phenolic acetogenins **2**, **3**, and **4,** are widespread in camponotines. However, 4-hydroxymellein (**5**), a major component in *C. quadrisectus*, has not been reported previously from insect sources. The monocyclic aromatics **2** and **3** have been identified, respectively, from worker mandibular glands in eight taxa from the *Colobopsis cylindricus* complex in Borneo (Jones et al. 2004; T.H. Jones, unpublished data) and three such taxa, plus some phylogenetically distant *Camponotus* (Brand et al. 1973a, b; Duffield and Blum 1975; Torres et al. 2001; Jones et al. 2004 and unpublished data).

The more volatile of the major mandibular gland components in *C. quadrisectus* and *C. irritibilis*, *E*-2-ethyl-2-hexene-1-ol (**1**) and the analogous aldehyde (**6**), may function in alarm/defensive roles. Aldehyde **6** is the aldol condensation product of butanal, with **1** being its subsequent carbonyl reduction product. In the formicine ant *Oecophylla longinoda*, the four carbon homolog of **6**, *E*-2-butyl-2-octenal, the aldol condensation product of hexanal, has been shown, along with hexanal, to be an alarm pheromone (Bradshaw et al. 1979). Similar, but more highly branched enals, the aldol condensation products of branched chain aldehydes, have been detected in the heads of an Australian *Calomyrmex* species, although their function was not determined (Brown and Moore 1979). Additionally, unbranched, unsaturated aldehydes, functionally similar to **6,** along with their corresponding alcohols, are common defensive compounds in Hemiptera (Blum 1981). It should be noted that unsaturated alcohols homologous to **1,** found in *C. quadrisectus*, were not detected in either *Oecophylla longinoda* or in the *Calomyrmex* species, suggesting that **1** and **6** in the Brunei *Camponotus* are not merely minor artifacts from aldehyde self condensation.

With somewhat lower volatilities, dihydroisocoumarins, such as mellein (**4)** and its analogs, occur in many species of carpenter ants, in which functions likely differ by source organ or gland. Historically, mellein has been reported from male mandibular glands of various *Camponotus* species, and is thought to serve as a sex pheromone (Brand et al. 1973a; Blum et al. 1987; Seidel et al. 1990; Torres et al. 2001). However, in at least three *Camponotus* species, mellein in the worker rectal bladder functions as a trail pheromone (Bestmann et al. 1992, 1999; Übler et al. 1995; Kohl et al. 2003), and appears to be synthesized *de novo*, although microbial contributions have not been explicitly ruled out (Bestmann et al. 1997).

Despite these previously reported functions, the four major mandibular gland components described here also possess documented antiseptic activity, and could play an additional role in nest hygiene. Antifungal activity has been demonstrated for *E*-2-ethyl-2-hexene-1-ol (**1**) and the analogous aldehyde (**6**) (Lyr and Banasiak 1983; Casperson et al. 1986), while the dihydroisocoumarins **4** and **5** are well-known metabolites of diverse fungi (e.g., Sasaki et al. 1970) and display both antiseptic and phytotoxic activities (e.g., Berestetskii et al. 1979; Kansoh and El-Gindi 2004). Consistent with possible use of these compounds as antiseptics are observations of *C. quadrisectus* workers applying them to edges of nest entrances and swollen (possibly diseased) lenticels on the host trunk (D. W. Davidson, unpublished data).

Blum (1996) has suggested that ants and other arthropods may benefit from "semiochemical parsimony", in which exocrine products serve multiple roles simultaneously. As one of perhaps several functions of camponotine mandibular gland compounds, antisepsis would not be surprising, because other (metapleural) glands with antiseptic function have been lost secondarily in this group (Hölldobler and Engel-Siegel 1984). Whatever the combined functions of these compounds, the presence of substantial quantities of mandibular gland compounds in the thorax (above) could indicate that these functions have selected for production of larger amounts of the compounds and enlargement of glandular reservoirs into the anterior thorax, which is distinctly light colored (like the head) in contrast to the remainder of the mesosoma.

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References

ASHA, K. N., CHOWDHURY, R., HASAN, C. M., and ASHID, M. A. 2004. Steroids and polyketides from *Uvaria hamiltonii* stem bark. *Acta Pharm.* 54:57–69.

AVANTAGGIATO, G., SOLFRIZZO, M., TOSI, L., ZAZZERINI, A., FANIZZI, F. P., and VISCONTI, A. 1999. Isolation and characterization of phytotoxic compounds produced by *Phomopsis helianthi*. *Nat. Toxins* 7:119–127.

BELLAS, T., and HÖLLDOBLER, B. 1985. Constituents of mandibular and Dufour''s glands of an Australian *Polyrhachis* weaver ant. *J. Chem. Ecol.* 11:525–538.

BERESTETSKII, O. A., MAMEDOV, T. A., and BOROVKOV, A. V. 1979. Phytotoxic metabolites of the soil fungus *Aspergillus ochraceus* Wilgelm. *Doklady Vsesoyuznoi Akademi Sel'skokhozyaistvennykh Nauk imeni V. I. Lenina* 12:15–17.

BESTMANN, H. J., KERN, F., SCHAFER, D., and WITSCHEL, M. C. 1992. **3,4-**Dihydroisocumarine, eine neue Klasse von Spurpheromonen bei Ameisen. *Angew. Chem.* 104:757–758.

BESTMANN, H. J., ÜBLER, E., and HÖLLDOBLER, B. 1997. First biosynthetic studies on trail pheromones in ants. *Angew. Chem. Int. Ed. Engl.* 36:395–397.

BESTMANN, H. J., LIEPOLD, B., KRESS, A., and HOFMANN, A. 1999. (2S, 4R, 5S)-2,4-Dimethyl-5-hexanolide: ants of different species *Camponotus* can distinguish the absolute configuration of their trail pheromone. *Chem. Eur. J.* 5:2984–2989.

Blum, M. S. 1981. Chemical Defenses of Arthropods. Academic Press, New York.

BLUM, M. S., MOREL, L., and FALES, H. M. 1987. Chemistry of the mandibular gland secretion of the ant *Camponotus vagus*. *Comp. Biochem. Physiol.* 86B:251–252.

BLUM, M. S., SNELLING, R. R., DUFFIELD, R. M., HERMANN, H. R. Jr., and LLOYD, A. 1988. Mandibular gland chemistry of *Camponotus (Myrmothrix) abdominalis*: Chemistry and chemosystematic implications (Hymenoptera: Formicidae), pp. 481–489, in J. C. Trager (ed.). Advances in MyrmecologyLeiden, New York City, New York.

BLUM, M. S. 1996. Semiochemical parsimony in the arthropoda. *Annu. Rev. Entomol.* 41:353–374.

BRADSHAW, J. W. S., BAKER, R., and HOWSE, P. E. 1979. Multicomponent alarm pheromones in the mandibular glands of major workers of the African weaver ant, *Oecophylla longinoda*. *Physiol. Entomol.* 4:15–25.

BRAND, J. M., DUFFIELD, R. M., MACCONNELL, J. G., BLUM, M. S., and FALES, H. M. 1973a. Caste-specific compounds in male carpenter ants. *Science* 179:388–389.

BRAND, J. M., FALES, H. M., SOKOLOSKI, F. A., MACCONNELL, J. G., BLUM, M. S., and DUFFIELD, R. M. 1973b. Identification of mellein in the mandibular gland secretions of carpenter ants. *Life Sci.* 13:201–211.

BROWN, W. V., and MOORE, B. P. 1979. Volatile secretory products of an Australian formicine ant of the genus *Calomyrmex* (Hymenoptera: formicidae). *Insect Biochem.* 9:451–460.

CASPERSON, G., BANASIAK, L., LYR, H., and SUNKEL, M. 1986. Wirkung von 2-Ethyl-2-en-1-al und verwandter Verbindungen auf Wachstum und Ultrastruktur von Pilzen. *J. Basic Microbiol.* 26:259–269.

DIMITRIADIS, C., GILL, M., and HARTE, M. F. 1997. The first stereospecific approach to both enantiomers of mellein. *Tetrahedron* 8:2153–2158.

DUFFIELD, R. M., and BLUM, M. S. 1975. Identification, role, and systematic significance of 3-octanone in the carpenter ant, *Camponotus shaefferi*. *Whr. Comp. Biochem. Physiol.* 51B:281–282.

FINDLAY, J. A., BUTHELEZI, S., LAVOIE, R., and PEÑA-ODRIQUEZ, L. 1995. Bioactive isocoumarins and related metabolites from conifer endophytes. *J. Nat. Prod.* 58:1759–1766.

HÄUSERMANN, M. 1951. Zur disproportionierung aliphatischer aldehyde. *Helv. Chim. Acta.* 34:1482–1491.

HÖLLDOBLER, B., and ENGEL-SIEGEL, H. 1984. On the metapleural glands of ants. *Psyche* 91:201–224.

HÖLLDOBLER, B., and WILSON, E. O. 1990. The Ants. Belknap Press, Cambridge, MA.

ISEKI, Y., KUDO, M., MORI, A., and INOUE, S. 1992. Asymmetric epoxidation of allylic alcohols catalyzed by titanium alkoxide-peptide and -.alpha.-amino acid complexes anchored by phenolic Schiff base. *J. Org. Chem.* 57:6329–6331.

JAFFE, K., and SÁNCHEZ, C. 1984. Nestmate recognition and territorial behavior in the ants *Camponotus rufipes*. *Insect. Soc.* 31:302–315.

JONES, T. H., CLARK, D. A., EDWARDS, A. A., DAVIDSON, D. W., SPANDE, T. F., and SNELLING, R. R. 2004. The chemistry of exploding ants, *Camponotus* spp. (*cylindricus* complex). *J. Chem. Ecol.* 30:1479–1492.

KANSOH, A. L., and EL-GINDI, O. D. 2004. Studies on the bioactive compounds of *Streptomyces* sp. *Egypt. J. Biomed. Sci.* 15:386–401.

KOHL, E., HÖLLDOBLER, B., and BESTMANN, H. J. 2003. Trail pheromones and Dufour gland contents in three *Camponotus* species (*C. castaneus, C. balzani, C. sericeiventris*: Formicidae, Hymenoptera). *Chemoecology* 13:113–122.

KONGSAEREE, P., PRABPAI, S., SRIUBOLMAS, N., VONGVEIN, C., and WIYAKRUTTA, S. 2003. Antimalarial dihydroisocoumarins produced by *Geotrichum* sp., an endophytic fungus of *Crassocephalum crepidoides*. *J. Nat. Prod.* 66:709–711.

LYR, H., and BANASIAK, L. 1983. Alkenals, volatile defense substances in plants, their properties and activities. *Acta Phytopath. Acad. Sci. Hungaricae.* 18:3–12.

NIST/EPA/NIH 1999. Mass Spectral Library on CD-ROM, Version 1.7. U.S. Secretary of Commerce, Gaithersburg, MD.

SAITO, T., HAYAMIZU, K., YANAGISAWA, M., and YAMAMOTO, O. 2007. Spectral data base for organic compounds. <*http://www.aist.go.jp/RIODB/SDBS*>.

SASAKI, M., KANEKO, Y., OSHITA, K., TAKAMATSU, H., ASAO, Y., and TOKOTSUKA, T. 1970. Studies on the compounds produced by molds. Part VII. Isolation of isocoumarin compounds. *Agr. Biol. Chem.* 34:1296–1300.

SEIDEL, J. L. 1988. The monoterpenes of *Gutierrezia sarothrae*; chemical interactions between ants and plants in neotropical ant gardens. Ph.D. dissertation. University of Utah, Salt Lake City.

SEIDEL, J. L., EPSTEIN, W. W., and DAVIDSON, D. W. 1990. Neotropical ant gardens I. Chemical consituents. *J. Chem. Ecol.* 16:1791–1816.

TORRES, J. A., SNELLING, R. R., BLUM, M. S., FLOURNEY, R. C., JONES, T. H., and DUFFIELD, R. M. 2001. Mandibular gland chemistry of the four caribbean species of *Camponotus* (Hymenoptera: Formicidae). *Biochem. Sys. Ecol.* 29:673–680.

ÜBLER, E., KERN, F., BESTMANN, H. J., HOLLDOBLER, B., and ATTYGALLE, A. B. 1995. Trail pheromone of two Formicine ants, *Camponotus silvicola* and *C. rufipes* (Hymenoptera: Formicidae). *Naturwissenschaften* 82:523–525.

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Identification of a Sex Pheromone Produced by Sternal Glands in Females of the Caddisfly *Molanna angustata* Curtis

Christer Löfstedt · Jan Bergmann · Wittko Francke · Erling Jirle · Bill S. Hansson · Vladimir D. Ivanov

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Abstract In the caddisfly *Molanna angustata*, females produce a sex pheromone in glands with openings on the fifth sternite. Gas chromatographic analyses of pheromone gland extracts with electroantennographic detection revealed four major compounds that stimulated male antennae. These compounds were identified by means of gas chromatography–mass spectrometry and enantioselective gas chromatography as heptan-2-one, (*S*)-heptan-2-ol, nonan-2-one, and (*S*)-nonan-2-ol in the approximate ratio of 1:1:4:10, respectively. Field tests showed that the mixture of the two alcohols was attractive to males whereas addition of the corresponding ketones reduced trap catches. The sex pheromone of *M. angustata*, a species in the family Molannidae within the suborder Integripalpia, is similar to the pheromones or pheromone-like compounds previously reported from six other trichopteran families, including members of the basal suborder Annulipalpia. This suggests that minimal evolutionary change of the pheromone chemistry has taken place within the leptoceroid branch of integripalpian Trichoptera compared to the ancestral character state.

Keywords *Molanna angustata* · Molannidae · Trichoptera · Sex pheromone · Nonan-2-ol · Heptan-2-ol · Nonan-2-one · Heptan-2-one

C. Löfstedt (✉) · E. Jirle · B. S. Hansson
Department of Ecology, Lund University,
SE-223 62 Lund, Sweden
e-mail: christer.lofstedt@ekol.lu.se

J. Bergmann · W. Francke
Institute of Organic Chemistry, University of Hamburg,
Martin-Luther-King-Platz 6,
20146 Hamburg, Germany

V. D. Ivanov
Department of Entomology, St. Petersburg State University,
199034 St. Petersburg, Russia

Present address:
J. Bergmann
Institute of Chemistry, Catholic University of Valparaíso,
Avda. Brasil 2950,
Valparaíso, Chile

Present address:
B. S. Hansson
Department of Neuroethology,
Max Planck Institute for Chemical Ecology,
Hans-Knöll-Straße 8,
07745 Jena, Germany

Introduction

The insect order Trichoptera (caddisflies) comprises more than 12,000 described extant species distributed among 46 families (Morse 1997, 1999, 2003). Whereas sex pheromone communication has been thoroughly investigated in the sister order Lepidoptera (moths and butterflies) and hundreds of moth sex pheromones from all of the major lepidopteran families have been identified, the corresponding information on caddisfly pheromone is limited. So far, pheromones or compounds with putative pheromonal activity have been described from only a dozen caddisfly species. Improved knowledge about sex pheromone communication in caddisflies would contribute not only to a better understanding of the reproductive biology in Trichoptera, but would also help to reconstruct the evolution of pheromone communication systems within the superorder Amphiesmenoptera that comprise the two orders already mentioned.

Pheromones occur in various evolutionary lineages of Trichoptera (Kelner-Pillault 1975; Wood and Resh 1984;

Resh and Wood 1985; Solem 1985; Resh et al. 1987; Löfstedt et al. 1994; Bjostad et al. 1996). Attraction of males to sticky traps baited with insect extracts has established that the pheromones are produced in sternal glands situated ventrally on the fourth to fifth female abdominal segment (Resh and Wood 1985). Chemically, the trichopteran pheromones identified so far consist of short-chain secondary alcohols and the corresponding methyl ketones (Löfstedt et al. 1994; Bjostad et al. 1996). Other compounds identified from the sternum V glands have no known pheromonal function and may be defensive secretions rather than pheromones (Duffield et al. 1977; Ansteeg and Dettner 1991). Nevertheless, in some species, strong antennal responses to the compounds suggest that they could be pheromone components (Bergmann et al. 2001, 2002, 2004). The short-chain alcohols and corresponding methyl ketones identified from caddisflies are similar to the sex pheromone components identified from the archaic moth family Eriocraniidae (Zhu et al. 1995) and Nepticulidae (Tóth et al. 1995).

Caddisflies of the family Molannidae are advanced representatives of the leptoceroid branch within the suborder Integripalpia (Ross 1964; Weaver 1984). Field studies suggested the presence of both a swarming behavior (Solem 1984; Ivanov 1985, 1992; Solem and Petersson 1987) and female pheromone signaling (Solem and Petersson 1987) in the Molannidae, whereas no evidence for pheromone communication has been found in the family Leptoceridae, another family from the same evolutionary lineage (Petersson and Solem 1987).

Adults of the genus *Molanna* J. Curtis, 1834, show crepuscular activity at lakeshores. The experiments by Solem and Petersson (1987) on *Molanna angustata* J. Curtis, 1834, showed that whole body extracts of females attract conspecific males. The present study was performed to identify the structures of the sex pheromone components produced by female *M. angustata* and to confirm that they elicit electrophysiological responses and mediate reproductive behaviors in these insects.

Methods and Materials

Insects and Preparation of Extracts Males and females of *M. angustata* were collected manually or by sweep netting at lakeshores in south Sweden. The most abundant populations were found at the lakes Krageholmssjön and Snogeholmssjön situated about 60 km southeast of Lund. These populations were used as the source of material for chemical analyses. Only one species of *Molanna* occurred at these sites. Caddisflies of the genus *Molanna* are readily distinguishable from other local trichoptera by the shape of their wings and their pale coloration.

Adults were taken to the laboratory where females were separated from males based on their genitalia. Both sexes were placed in plastic containers with water droplets on the walls, and the containers were placed in a room with controlled climate (16–20°C, 60–70% relative humidity) and a reversed light/dark cycle (16L:8D) to invert the diurnal rhythm of the insects. Females spent about 40 h under these conditions before extraction of the pheromone glands. Early in the scotophase, insects to be dissected were freeze killed (−20°C, approximately 1 h) before dissection.

The fourth and fifth abdominal sternites from females were dissected and cleared from fat body components so that the glands situated in the anterodorsal part of the fifth and the posterodorsal part of the fourth sternite were exposed. Sternites with attached epithelial tissue and secretory glands were put into a micro-extraction vial that contained 100 µl of dichloromethane to produce composite extracts of several females. This vial was placed in a larger screw cap vial with solvent on the bottom to saturate the atmosphere and thereby reduce the loss of solvent from the actual extract. The sample was extracted for 12 h at room temperature. Then, the extract was transferred to a clean micro-vial that was sealed and stored in the freezer at −20°C. A similar protocol was used to obtain extracts from the female abdominal tips (seventh to ninth abdominal segments), female heads, and male fourth to fifth sternites. In total, approximately 70 µl of extract of fourth and fifth sternites were collected from 24 females; similar amounts of extracts were obtained from 24 female heads, 24 abdominal tips, and from 14 male fourth to fifth sternites. These extracts of females and males were analyzed by gas chromatography with electroantennographic detection (GC-EAD) and coupled gas chromatography–mass spectrometry (GC–MS). The female sternal extract was used also for field behavioral bioassays.

Electroantennography and Gas Chromatography with Electroantennographic Detection A freshly cut male antenna was placed with its basal end in a reference glass electrode that contained Beadle–Ephrussi Ringer solution (155 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$). This electrode was grounded via an Ag/AgCl wire. The antennal tip was removed, and the apical end of the antenna was inserted into a recording electrode and connected through a Ag/AgCl wire to a high-impedance DC amplifier (Syntech, Hilversum, The Netherlands) with automatic baseline drift compensation. The DC potential was recorded on a computer using an IDAC A/D converter and software (Auto Spike v. 3.0; Syntech). Filtered and humidified air continuously flowed over the mounted antenna (approximately 0.5 m/s). Extracts of different parts of the insect bodies were tested for electroantennography (EAG) activity by depositing one insect equivalent inside the tip of a

Pasteur pipette and blowing 1 ml of air through the pipette and onto the antenna.

A Hewlett-Packard 5890 Series II Plus gas chromatograph equipped with an HP Innowax column (30 m× 0.25 mm i.d., Hewlett-Packard, Palo Alto, CA, USA) was used for chromatographic analyses. An effluent split allowed simultaneous flame ionization detection (FID) and EAD of the separated compounds. Hydrogen was used as carrier gas, and the effluent split ratio was approximately 1:1. Samples were injected splitless with injector temperature 225°C, and the split valve was opened 1 min after injection. The column temperature was generally maintained at 40°C for 5 min after injection and then linearly increased to 230°C at a rate of 10°C/min. The outlet for the EAD was placed in a purified humidified air stream flowing over the antennal preparation at a speed of 0.5 m/s. Two to five microliters of an extract containing two to three female equivalents were injected onto the column for analysis.

Structure Elucidation Preliminary GC–MS analysis of active extracts was performed with a Hewlett-Packard 5890 gas chromatograph linked to a 5972 mass spectrometer and equipped with a DB-Wax 30 m×0.25 mm column (J&W Scientific, Folsom, CA, USA), similar to the column used for GC-EAD analyses. Further analyses of the extracts were carried out by using a VG 70/70 mass spectrometer (Vacuum Generators, Manchester, UK) linked to an HP 5890GC (Hewlett-Packard) equipped with a 30 m× 0.25 mm i.d., 0.25 μm film thickness Optima 5MS column (Macherey-Nagel, Düren, Germany) or a 50 m×0.25 mm i.d., 0.25 μm film FFAP column (CS Chromatographic Service, Langenwehe, Germany). Identification of volatile compounds was carried out by means of GC–MS on the basis of comparison with known mass spectra (McLafferty and Stauffer 1989) and by gas chromatographic retention times upon coinjection of natural products and synthetic reference samples. Separation of enantiomers of the chiral secondary alcohols was achieved with a 25 m×0.25 mm i.d. fused silica column coated with a 60:40 mixture of octakis-(6-*O*-methyl-2,3-di-*O*-pentyl)-γ-cyclodextrin and OV1701 (custom-made by Prof. W. A. König, Univ. Hamburg, Germany) with hydrogen or helium as carrier gas and a mass spectrometer as detector. Experimental conditions were 50°C for 12 min, then to 120°C at 3°C/min.

Synthetic Compounds Heptan-2-one and nonan-2-one and the enantiomers of heptan-2-ol and nonan-2-ol were purchased from Aldrich. All compounds were ≥98% chemically pure. The optically active compounds had ee's of at least 98%.

Field Trapping Attraction to extracts and synthetic compounds was examined in the field at the lake Krageholmssjön in 1996 and at the lakes Snogeholmssjön and Sövdesjön in 2004. Lund type II traps with sticky bottoms (Anderbrant et al. 1989) were distributed along the water line. Traps within a replicate (four to seven traps depending on the experiment) were placed in a group, with individual traps separated by 2–3 m distance (1996) or approximately 10 m distance (2004). Distances between replicates were not less than 50 m. Traps were positioned with the bottom approximately 0.3 m above the water surface. Rubber septum dispensers (Arthur H. Thomas Co., 5×9 mm red rubber septa, Catalog No. 1780-J07) were loaded with different mixtures and doses of the test compounds dissolved in dichloromethane (500 μl in 1996 and 100 μl in 2004). The solvent was allowed to evaporate, and the baits were used the same day or stored in the freezer (−20°C) in aluminum bags until used. The dispensers were suspended 1–2 cm from the trap ceiling on an insect pin.

The dose of the synthetic pheromone blends refers to the amount of the major component nonan-2-ol. Thus, a 100 μg dose indicates 100 μg of nonan-2-ol, and other compounds were added as required in the relative amounts in which they occurred in extracts. The blank (control) dispensers were loaded with pure dichloromethane.

Five field-trapping experiments were carried out. The first took place July 18–29, 1996 and was designed to compare the activity of different doses of two alcohols to pheromone gland extracts of females and to a synthetic four-compound blend. The alcohol lures contained a mixture of (*S*)-nonan-2-ol and (*S*)-heptan-2-ol in the proportion found in female extracts wish doses of nonan-2-ol of 10, 100, or 1,000 μg per trap. The synthetic four-compound blend contained 100 μg (*S*)-nonan-2-ol and the corresponding amounts of (*S*)-heptan-2-ol, nonan-2-one, and heptan-2-one. The pheromone extracts originated from the same sample as was used for the GC-EAD experiments, and a septum was loaded with 1.7 female equivalents.

A second experiment, similar to the first, was carried out July 29–August 10, 1996, but in this experiment, pheromone extracts were exchanged for a bait that contained ketones only. A third trapping experiment was designed to examine the effects of the individual alcohols and their corresponding (*R*)-enantiomers. The experiment was performed August 14–22, 1996. The baits tested contained the individual compounds in amounts corresponding to 100 μg of nonan-2-ol. Baits were loaded with both (*S*)-alcohols, both corresponding (*R*)-alcohols, both corresponding ketones, only (*S*)-nonan-2-ol, or only (*S*)-heptan-2-ol.

A fourth experiment was carried out July 28–August 18, 2004 to confirm the attractiveness of (*S*)-nonan-2-ol and (*S*)-heptan-2-ol. Attraction to traps baited with the individual alcohols (100 and 10 μg, respectively, based on their relative abundance in gland extracts), and the mixture of the two compounds was examined. The final experiment was carried out August 23–September 17, 2004 to investigate

the influence of the ratio between (*S*)-nonan-2-ol and (*S*)-heptan-2-ol. Traps were baited with 100 μg of (*S*)-nonan-2-ol plus different amounts of (*S*)-heptan-2-ol (1, 3, 10, and 30 μg).

Traps were checked and re-randomized every 2–4 days during each experiment. Each reading of a trap catch was then treated as a replicate in the statistical analysis. The statistical significance of the trapping results was analyzed by generalized linear models assuming Poisson distribution and corrected for over-dispersion (SAS 9.1, proc Glimmix).

Results

EAG Recordings In preliminary tests that used insect extracts, the only distinct EAG responses recorded were those of antennae of males to extracts of females. Antennae of females were not receptive to any of the stimuli tested. Antennae of males responded strongly to extracts of the female fourth and fifth sternite, whereas head or abdominal tip extracts of females did not elicit significant EAG responses. Extracts obtained from fourth and fifth sternites of males also did not elicit any response from the antennae of males.

Gas Chromatography with Electroantennographic Detection Four EAD responses with matching peaks in the FID chromatogram (peaks 1–4) were observed consistently in the earlier part of the chromatogram during three separate analyses. In addition, in all of these analyses, three EAD responses at longer retention times (peaks 5–7) but without any visible matching FID peaks were also observed (Fig. 1). GC-EAD analyses with extracts of males and extracts from other parts of the female did not show any EAD-active peaks.

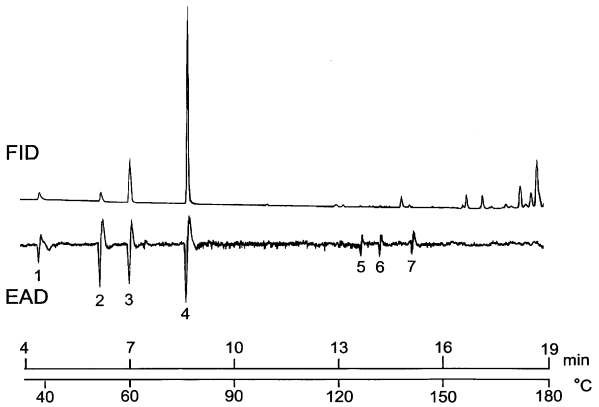

Fig. 1 Gas chromatographic analysis of an extract of female *M. angustata* fourth and fifth abdominal sternites. Simultaneous FID and EAD using a male antenna as detector. EAD-active compounds 1–4 were identified as heptan-2-one (1), (*S*)-heptan-2-ol (2), nonan-2-one (3), and (*S*)-nonan-2-ol (4)

Structure Elucidation Compounds 1–4 in the extracts of sternites IV–V of females were identified as simple methylketones and the corresponding secondary alcohols. Compound 1 was identified as heptan-2-one, characterized by a weak signal for the molecular ion at *m*/*z* 114, an intense signal at *m*/*z* 58 (as a result of a McLafferty rearrangement), and a base peak at *m*/*z* 43 caused by α-cleavage at the carbonyl group. Compound 2 was identified as heptan-2-ol, characterized by small signals at *m*/*z* 101 (M^+-methyl) and *m*/*z* 98 (M^+-water) and a dominant base peak at *m*/*z* 45 caused by α-cleavage at the alcohol group; the molecular ion at *m*/*z* 116 was not observed. The mass spectrometric fragmentations of the two compounds were identical with those reported in the literature (McLafferty and Stauffer 1989). The same was true for the homologs, nonan-2-one (3) and nonan-2-ol (4); the mass spectra of which showed fragmentation patterns similar to those of the C7 compounds. Diagnostic fragments were M^+142 and *m*/*z* 58 as well as *m*/*z* 43 for the ketone, and *m*/*z* 129 (M^+-15), *m*/*z* 126 (M^+-18), and *m*/*z* 45 for the alcohol (M^+=144, was not observed).

Enantioselective gas chromatography was carried out with a modified cyclodextrin as the stationary phase and pure stereoisomers of heptan-2-ol and nonan-2-ol as references revealed the natural products to be the (*S*)-enantiomers of high stereoisomeric purity. The enantiomers of heptan-2-ol and nonan-2-ol were almost baseline separated with α=1.010–1.013 [retention time of the later eluting (*R*)-enantiomer divided by retention time of the earlier eluting (*S*)-enantiomer] showing a valley down to 3% of the peak height. No traces of the (*R*)-enantiomers were detected in the natural products.

We were not able to identify the compounds that elicited the three EAG responses in the later part of the chromatogram (Fig. 1). None of the identified compounds were detected in extracts of males.

The amount of the major compound nonan-2-ol was estimated to be 100 ng or more per female. Based on total ion current counts, the approximate relative amounts (within parentheses) of the four identified compounds were heptan-2-one (1), heptan-2-ol (1), nonan-2-one (4), and nonan-2-ol (10).

Field Trapping In the first of five trapping experiments, the attractiveness of a female extract was compared to three different doses of a mixture of (*S*)-heptan-2-ol and (*S*)-nonan-2-ol, and a mixture of the alcohols plus heptan-2-one and nonan-2-one (Fig. 2). In total, 142 males were captured in this experiment. There was no overall, statistically significant treatment effect. Numerically, the traps baited

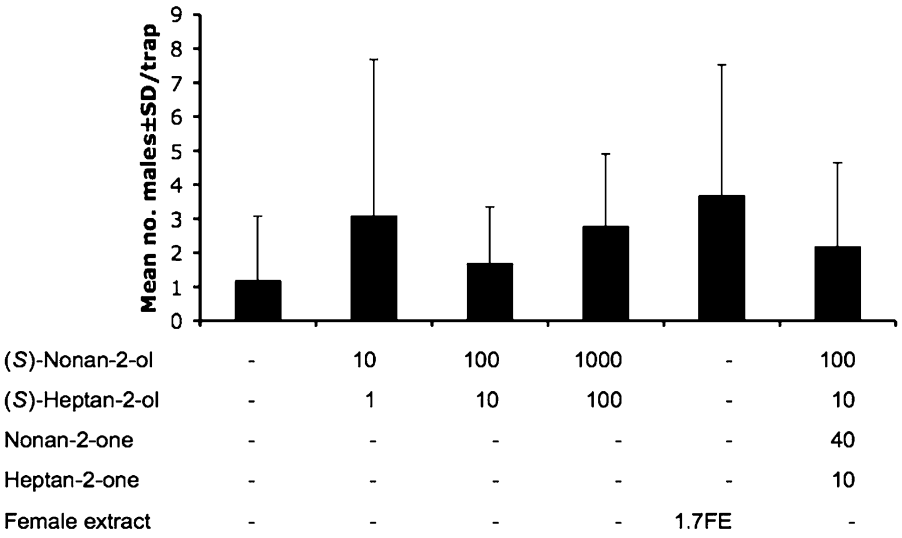

Fig. 2 Attraction of male *M. angustata* to female extracts, alcohols and alcohols, mixed with ketones (Experiment 1). Amounts of synthetic compounds in micrograms. *FE* Female equivalent. Statistical significance of treatment effects (least squares means) analyzed by generalized linear model assuming Poisson error: $F_{5,52}$= 1.04, P=0.403

with female extracts trapped the largest number of males followed by the alcohols in the 10 and 1,000 μg doses. In the second experiment (Fig. 3), the same synthetic baits and an additional bait that contained only the two ketones were tested. In this experiment, we observed a significant overall treatment effect. The three alcohol baits followed by the mixture of alcohols and ketones were the most attractive ones, significantly different from the control and the mixture of the ketones.

In experiment 3 (Fig. 4), the activities of (*S*)-heptan-2-ol and (*S*)-nonan-2-ol as single components were compared with the activity of the mixture of these two compounds, the activity of the mixture of the corresponding (*R*) enantiomers, and the activity of the mixture of heptan-2-one and nonan-2-one. The overall effect was statistically significant, and the mixture of the (*S*) alcohols and (*S*)-nonan-2-ol on its own were more attractive than (*S*)-heptan-2-ol, the mixture of the ketones, or the mixture of the (*R*) alcohols. Numerically, the alcohols showed the highest trap catches, but when tested for statistical significance, all treatments were not different from the blank.

Thus, high variation in trap catches confounded the results in experiments 1–3, carried out during the first field season. Only a few significant differences were found by using pairwise comparisons, and no pronounced differences were found between different baits or between baited traps and the control. Thus, we decided to perform two additional experiments several years later. Experiment 4 (Fig. 5) conclusively demonstrated that the mixture of (*S*)-heptan-2-ol and (*S*)-nonan-2-ol in their natural ratio was significantly attractive to male *M. angustata*. Traps baited with (*S*)-nonan-2-ol as a single component gave the second highest trap catch, but this treatment was not significantly different from the results obtained with (*S*)-heptan-2-ol (P=0.09) or the control. The final experiment (Fig. 6) demonstrated a significant effect of the relative amount of (*S*)-heptan-2-ol

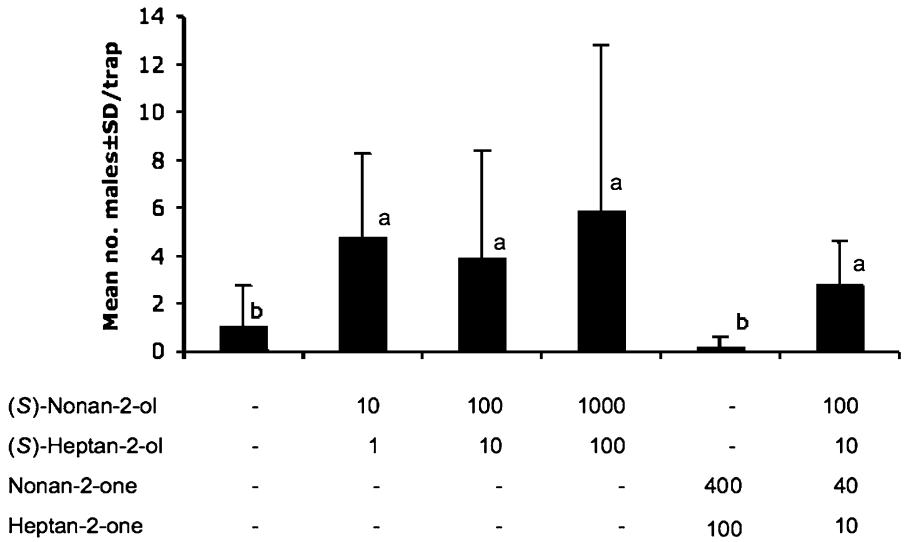

Fig. 3 Field trapping of *M. angustata*, comparing attractiveness of alcohols, ketones, and alcohols mixed with ketones (Experiment 2). Statistical significance of treatment effects (least squares means) analyzed by generalized linear model assuming Poisson error: $F_{5,54}$= 2.98, P=0.019. Values followed by the same letter are not significantly different (P> 0.05)

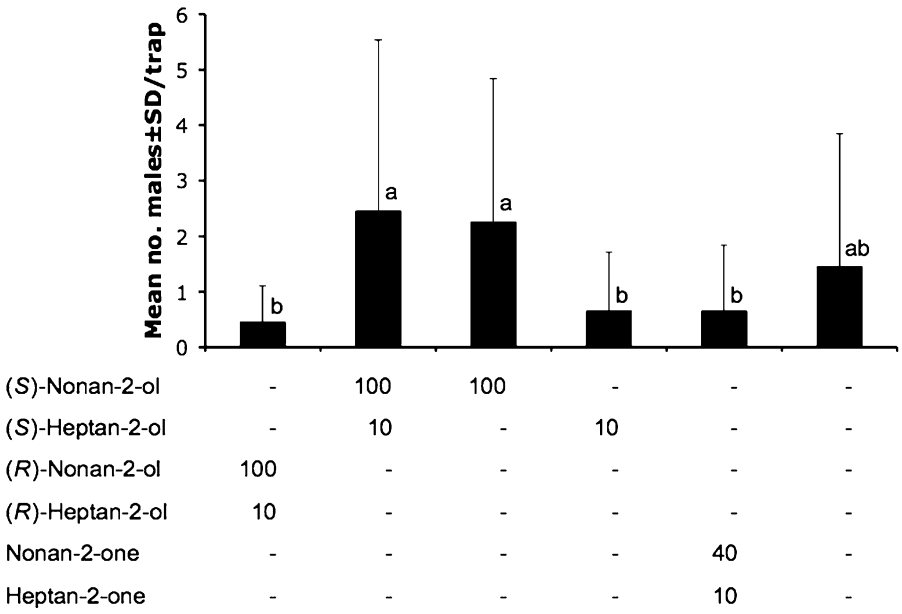

Fig. 4 Field trapping of *M. angustata*, comparing attractiveness of different alcohol baits and a mixture of ketones (Experiment 3). Statistical significance of treatment effects (least squares means) analyzed by generalized linear model assuming Poisson error: $F_{5,84}$= 2.93, P=0.017. Values followed by the same letter are not significantly different (P> 0.05)

added to a fixed amount of the major component (*S*)-nonan-2-ol. The highest trap catches were obtained with the 100:10 mixture, but this treatment was the only one that was significantly different from the 100:1 mixture that appeared to be least attractive.

Discussion

Our study showed that female *M. angustata* produce a mixture of (*S*)-nonan-2-ol and (*S*)-heptan-2-ol that attracts conspecific males. (*S*)-Nonan-2-ol is the major component both quantitatively and with respect to behavioral activity. The electroantennographic responses of male antennae to female extracts were strong and reproducible, and males were attracted to the synthetic pheromone in field bioassays.

We experienced problems in our studies of pheromone communication in *M. angustata*, similar to those reported in studies of other species of caddisflies. For example, (Löfstedt et al. 1994) obtained unexpectedly high catches in unbaited control traps, whereas catches in traps baited with synthetic pheromone or insect extracts were relatively low, albeit still significantly higher than in the controls. In many other species of caddisflies, we have identified pheromone component candidates with high electrophysiological activity but have failed to demonstrate any attraction to these compounds in field trapping experiments (J. Bergmann, W. Francke, V. D. Ivanov, and C. Löfstedt, unpublished). In contrast, in trapping experiments with similar compounds that target primitive moths, large numbers of moths were attracted, and unbaited traps never trapped more than a few insects, if any (Zhu et al. 1995; Kozlov et al. 1996). This suggests that the problem is not a general technical one, related to solvent

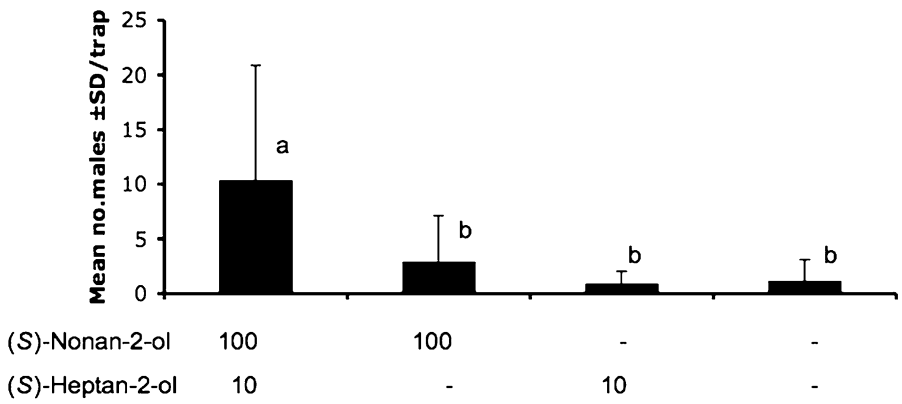

Fig. 5 Field trapping of *M. angustata*, comparing attractiveness of (*S*)-heptan-2-ol, (*S*)-nonan-2-ol, and their mixture (Experiment 4). Statistical significance of treatment effects (least squares means) analyzed by generalized linear model assuming Poisson error: $F_{3,54}$=10.06, P<0.001. Values followed by the same letter are not significantly different (P> 0.05)

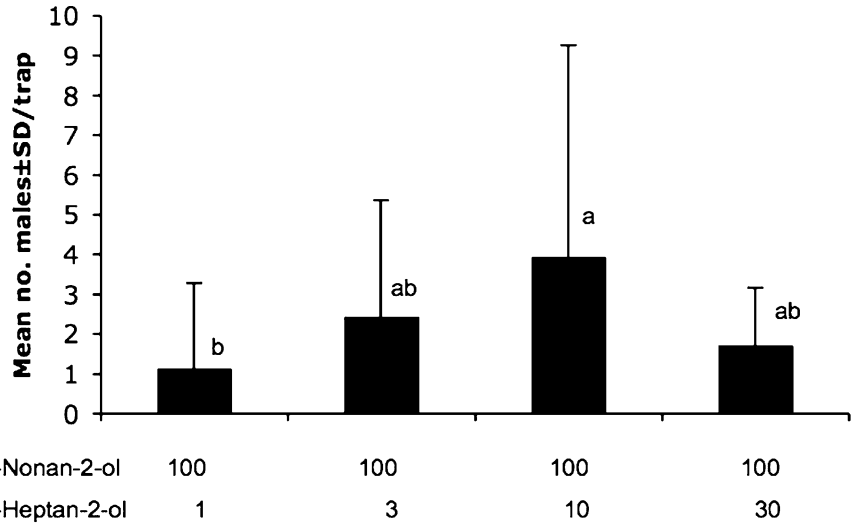

Fig. 6 Field trapping of *M. angustata*, comparing different amounts of (*S*)-heptan-2-ol added to (*S*)-nonan-2-ol (Experiment 5). Statistical significance of treatment effects (least squares means) analyzed by generalized linear model assuming Poisson error: $F_{3,73}$= 2.83, P=0.044. Values followed by the same letter are not significantly different (P> 0.05)

impurities or the release of the alcohols and ketones from the dispensers, but rather a problem specific to trapping of caddisflies and related to the behavior, biology, or pheromone blend of these insects.

In southern Sweden, species of the genus *Molanna* are nocturnal or crepuscular, so their natural behavior is difficult to observe. Observations of adults of *Molanna* sp. at more northern latitudes during the polar day (when they perform mating behavior under conditions of full illumination) revealed the formation of aggregations near the shoreline, where part of the population exhibits characteristic flight patterns, whereas another part of the population crawls over the vegetation. These aggregations are loose, and their borders are not distinct. Thus, this is not a typical lekking (or swarming) formation, as has been observed in other Trichoptera from the families Hydropsychidae and Leptoceridae. On the Kola Peninsula (polar day) and in the Leningrad district (in the fading twilight), the females preferred to stay on the vegetation, and males were most abundant among the flying insects (V. D. Ivanov, personal observations). Possible reasons for the weak and uneven results in our trapping studies, thus, include both the short range of pheromone-mediated attraction in caddisflies ("weak pheromones") and the highly aggregated distribution of insects along the shoreline.

The mixture of (*S*)-nonan-2-ol and (*S*)-heptan-2-ol in the female-produced ratio was the most attractive bait in the field. Adding the ketones to the blend did not increase the attractiveness but rather the opposite. The alcohols and the ketones are biosynthetically related, and we do not know if the females release only the alcohols or the ketones as well. It is noteworthy that, in our GC-EAD analyses, three responses were observed at longer retention times, but no corresponding FID peaks could be detected, and no compounds responsible for this activity could be identified. These EAD peaks may represent biologically active trace components of the pheromone blend.

The four compounds that we identified from *M. angustata* females have been identified previously from several other caddisflies (Duffield 1981; Löfstedt et al. 1994; Bergmann et al. 2002). In *Rhyacophila fasciata*, a mixture of the racemates of heptan-2-ol and nonan-2-ol was attractive to males (Löfstedt et al. 1994). The enantiomeric composition of female extracts was not investigated, and the enantiomers were not tested in trapping experiments. In *R. nubila*, males have antennal receptors specific for the enantiomers of heptan-2-ol (Larsson and Hansson 1998), and male eriocraniid moths likewise have specific receptors for (*R*)- and (*S*)-enantiomers of methyl carbinols that have been identified as pheromone components in this moth genus (Zhu et al. 1995; Kozlov et al. 1996; Larsson et al. 2002). Females of *R. nubila* produce (*R*)-heptan-2-ol and (*R*)-nonan-2-ol of 99.5% ee and 90% ee, respectively (J. Bergmann, W. Francke, C. Löfstedt, and P. Valeur, unpublished). It is interesting to note that unbranched methylketones and the corresponding alcohols are widespread among Hymenoptera and that those methyl carbinols are components of the sex pheromone of the stingless eusocial bee *Scaptotrigona postica* (Engels et al. 1990, 1997). In Trichoptera, different combinations of alcohols and ketones or, similar to Lepidoptera and Coleoptera (Francke and Schulz 1999), the enantiomeric composition of chiral pheromone components may account for the species specificity of caddisfly pheromones. Caddisflies also use vibrational and acoustic signals for communication (Ivanov 1993, 1994), which may contribute to the species specificity of the communication systems. These signals have not been recorded for *M. angustata*, and the specific sternal structures that usually produce these signals are not present in this species. Males have been shown to perform

the zigzagging flight similar to that of searching moths and resembling the swarming flight of Leptoceridae (Ivanov 1985), with the exception that the *Molanna* males never form swarms, even at high population densities.

Three suborders are recognized within the Trichoptera. Annulipalpia is concluded to be the most basal suborder, and Spicipalpia and Integripalpia form a clade (Kjer et al. 2002). Both Annulipalpia and Integripalpia are monophyletic, but whether or not Spicipalpia is monophyletic remains equivocal. Methyl carbinols and methylketones, similar or identical to those found in *M. angustata*, have been reported in the families Philopotamidae, Polycentropodidae, and Hydropsychidae that belong to the Annulipalpia, as well as in Rhyacophilidae and Glossosomatidae that belong to the Spicipalpia. In addition to their occurrence in the Molannidae, such compounds also have been found in Phrygaenidae belonging to a different branch within the Integripalpia (Löfstedt et al. 1994, Bergmann et al. 2002). Thus, this type of pheromone component produced in the sternum V gland may be a basal character in both Trichoptera and Lepidoptera and, thus, of the superorder Amphiesmenoptera (Löfstedt and Kozlov 1996), a conclusion further corroborated by the present study. The alcohols and ketones produced by females were not found in extracts of male *M. angustata*, which resembles the typical situation in Lepidoptera. In contrast, most other species of caddisflies studied previously have male-produced pheromone-like compounds similar to those found in conspecific females (Löfstedt et al. 1994; Bergmann et al. 2002, 2004) that elicit electrophysiological responses from antennae of both females and males.

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References

ANDERBRANT, O., LÖFQVIST, J., JÖNSSON, J., and MARLING, E. 1989. Effects of pheromone trap type, position and colour on the catch of the pine sawfly *Neodiprion sertifer* (Geoff.) Hym., Diprionidae. *J. Appl. Entomol*. 107:365–369.

ANSTEEG, O., and DETTNER, K. 1991. Chemistry and possible biological significance of secretions from a gland discharging at the 5th abdominal sternite of adult caddisflies (Trichoptera). *Entomol. Gen*. 15:303–312.

BERGMANN, J., LÖFSTEDT, C., IVANOV, V. D., and FRANCKE, W. 2001. Identification and assignment of absolute configuration of methyl-branched ketones from limnephilid caddisflies. *Eur. J. Org. Chem*. 16:3175–3179.

BERGMANN, J., LÖFSTEDT, C., IVANOV, V. D., and FRANCKE, W. 2002. Electrophysiologically active volatile compounds from six species of caddisflies. *Nova Suppl. Ent*. 15:37–46.

BERGMANN, J., LÖFSTEDT, C., IVANOV, V. D., and FRANCKE, W. 2004. Identification and synthesis of new bicyclic acetals from caddisflies (Trichoptera). *Tetrahedron Lett*. 45:3669–3672.

BJOSTAD, L. B., JEWETT, D. K., and BRIGHAM, D. L. 1996. Sex pheromone of caddisfly *Hesperophylax occidentalis* (Banks) (Trichoptera: Limnephilidae). *J. Chem. Ecol*. 22:103–121.

DUFFIELD, R. M. 1981. Nonan-2-ol in the exocrine secretion of the nearctic caddisfly, *Rhyacophila fuscula* (Walker) (Rhyacophilidae: Trichoptera). *Proc. Entomol. Soc. Wash*. 83:60–63.

DUFFIELD, R. M., BLUM, M. S., WALLACE, J. B., LLOYD, H. A., and REGNIER, F. E. 1977. Chemistry of the defense secretion of the caddisfly *Pycnopsyche scabripennis* (Trichoptera: Limnephilidae). *J. Chem. Ecol*. 3:649–656.

ENGELS, W., ENGELS, E., LÜBKE, G., SCHRÖDER, W., and FRANCKE, W. 1990. Volatile cephalic secretions of drones, queens and workers in relation to reproduction in the stingless bee, *Scaptotrigona postica* (Hymenoptera: Apidae: Trigonini). *Entomol. Gener*. 15:91–101.

ENGELS, W., ENGELS, E., and FRANCKE, W. 1997. Ontogeny of cephalic volatile patterns in queens and mating biology of the neotropical stingless bee, *Scaptotrigona postica*. *Inverteb. Reprod. Develop*. 30:251–256.

FRANCKE, W., and SCHULZ, S. 1999. Pheromones, pp. 197–261, in S. D. Barton, and K. Nakanishi (eds.). Comprehensive Natural Products Chemistry, Vol. 8. Elsevier, Amsterdam.

IVANOV, V. D. 1985. Behavior of caddisflies in flight. *Latvijas Entomologs*. 28:85–94, (In Russian).

IVANOV, V. D. 1992. Evolution of flight of caddisflies, pp. 351–357, in C. Tomaszewski (ed.). Proc. of 6th Int. Symp. on Trichoptera, Adam Mickiewicz University Press, Lodz.

IVANOV, V. D. 1993. Principles of the sexual communication in caddisflies (Insecta, Trichoptera), Sensory Systems of Arthropods. pp. 609–626, in K. Wiese et al. (eds.). Birkhäuser Verlag, Basel.

IVANOV, V. D. 1994. Vibratory signalling in caddisflies (Insecta, Trichoptera). *Zool. Zhurnal*. 73:55–70, In Russian.

KELNER-PILLAULT, S. 1975. Attirance sexuelle chez un Trichoptère: *Enoicyla pusilla* Burmeister. *Bull. Soc. Entomol. Fr* 80:252–257.

KJER, K. M., BLAHNIK, R. J., and HOLZENTHAL, R. W. 2002. Phylogeny of caddisflies (Insecta, Trichoptera). *Zool. Scr*. 31:83–91.

KOZLOV, M. V., ZHU, J., PHILIPP, P., FRANCKE, W., ZVEREVA, E. L., HANSSON, B. S., and LÖFSTEDT, C. 1996. Pheromone specificity in *Eriocrania semipurpurella* (Stephens) and *E. sangii* (Wood) (Lepidoptera: Eriocraniidae) based on chirality of semiochemicals. *J. Chem. Ecol*. 22:431–454.

LARSSON, M. C., and HANSSON, B. S. 1998. Receptor neuron responses to potential sex pheromone components in the caddisfly *Rhyacophila nubila* (Trichoptera: Rhyacophilidae). *J. Insect Physiol*. 44:189–196.

LARSSON, M. C., HALLBERG, E., KOZLOV, M. V., FRANCKE, W., HANSSON, B. S., and LÖFSTEDT, C. 2002. Specialized olfactory receptor neurons mediating intra- and interspecific chemical communication in leafminer moths *Eriocrania* spp. (Lepidoptera: Eriocraniidae). *J. Exp. Biol*. 205:989–998.

LÖFSTEDT, C., HANSSON, B. S., PETERSSON, E., VALEUR, P., and RICHARDS, A. 1994. Pheromonal secretions from glands on the 5th abdominal sternite of hydropsychid and rhyacophilid caddisflies (Trichoptera). *J. Chem. Ecol*. 20:153–170.

LÖFSTEDT, C., and KOZLOV, M. 1996. A phylogenetic analysis of pheromone communication in primitive moths, pp. 473–489, in R.T. Cardé, and A.K. Minks (eds.). Insect Pheromone Research: New Directions, Chapman & Hall, New York.

MCLAFFERTY, F. W., and STAUFFER, D. B. 1989. The Wiley/NBS Registry of Mass Spectral Data. Wiley, New York.

MORSE, J. C. 1997. Checklist of world Trichoptera, pp. 339–342 in R.W. Holzenthal, and O.S. Flint, Jr. (eds.). Proc. 8th Int. Symp. Trichoptera. Columbus, OH: Ohio Biological Survey.

MORSE, J. C., editor. 1999. Trichoptera World Checklist. http://entweb.clemson.edu/database/trichopt/index.htm, effective 22 May 1999, updated 28 July 2000, 8 January 2001, December 6, 2006.

MORSE, J. C. 2003. Trichoptera (Caddisflies), pp. 1145–1151, in V. H. Resh, and R. T. Carde (eds.). Encyclopedia of Insects. Elsevier.

PETERSSON, E., and SOLEM, J. O. 1987. Male mate recognition in Leptoceridae, pp. 157–160, in M. Bournaud, and H. Tachet (eds.). Proc. 5th Int. Symp. Trichoptera. Dr. W. Junk Publishers, Dordrecht.

RESH, V. H., JACKSON, J. K., and WOOD, J. R. 1987. Techniques for demonstrating sex pheromones in Trichoptera, pp. 161–164, in M. Bournaud, and H. Tachet (eds.). Proc. 5th Int. Symp. on Trichoptera. Dr. W. Junk Publishers, Dordrecht.

RESH, V. H., and WOOD, J. R. 1985. Site of sex pheromone production in three species of Trichoptera. *Aquat. Insects* 7:65–71.

ROSS, H. H. 1964. Evolution of caddis worm cases and nets. *Am. Zool* 4:209–220.

SOLEM, J. O. 1984. Adult behavior of north European caddisflies, pp. 375–382, in J. C. Morse (ed.). Proc. 4th Int. Symp. Trichoptera. Series Entomologica, 30. Dr. W. Junk Publishers, The Hague.

SOLEM, J. O. 1985. Female sex pheromones in *Rhyacophila nubila* (Zetterstedt) (Trichoptera, Rhyacophilidae) and arrival pattern to sticky traps. *Fauna Norv. Ser. B.* 32:80–82.

SOLEM, J. O., and PETERSSON, E. 1987. Demonstration of female sex pheromones and adult behavior in *Molanna angustata* (Trichoptera: Molannidae). *Entomol. Gen*. 12:115–118.

TÓTH, M., SZÖCS, G., NIEUKERKEN, E. J., VAN, PHILIPP, P., SCHMIDT, F., and FRANCKE, W. 1995. Novel type of sex pheromone structure identified from *Stigmella malella* (Stainton) (Lepidoptera: Nepticulidae). *J. Chem. Ecol*. 21:13–27.

WEAVER, J. S. 1984. Evolution and classification of Trichoptera, part I: Groundplan of Trichoptera, pp. 413–419, in J.C. Morse (ed.). Proc. 4th Int. Symp. Trichoptera. Series Entomologica, 30. Dr. W. Junk Publishers, The Hague.

WOOD, J. R., and RESH, V. H. 1984. Demonstration of sex pheromones in caddisflies (Trichoptera). *J. Chem. Ecol*. 10:171–175.

ZHU, J., KOZLOV, M. V., PHILIPP, P., FRANCKE, W., and LÖFSTEDT, C. 1995. Identification of a novel moth sex pheromone in *Eriocrania cicatricella* (Zett.) (Lepidoptera: Eriocraniidae) and its phylogenetic implications. *J. Chem. Ecol*. 21:29–43.

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New Contact Sex Pheromone Components of the German Cockroach, *Blattella germanica*, Predicted from the Proposed Biosynthetic Pathway

Dorit Eliyahu · Satoshi Nojima · Kenji Mori · Coby Schal

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Abstract Upon contacting the cuticle of a sexually mature female, a male German cockroach exhibits a characteristic courtship behavior: he turns away from the female and raises his wings, thereby exposing tergal glands. The glandular secretion stimulates the female to mount the male and feed, thus positioning her appropriately for copulation. A multicomponent contact sex pheromone produced by females is responsible for eliciting courtship behavior. The most abundant pheromone components are 3,11-dimethylnonacosan-2-one and 3,11-dimethylheptacosan-2-one, oxidation products of the abundant hydrocarbon analogs 3,11-dimethylnonacosane and 3,11-dimethylheptacosane, respectively. The C_{29}-dimethyl ketone is thought to be further metabolized to two less abundant pheromone components, 29-hydroxy-3,11-dimethylnonacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one. Based on this proposed biosynthetic pathway of pheromone production, we hypothesized that 3,11-dimethylheptacosan-2-one also would be oxidized to give two candidate pheromone components, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one. By using bioassay-guided fractionation and chemical analyses of cuticular extracts of virgin females and synthesis of the (3*S*,11*S*)-isomer of each of the two predicted pheromone components, we showed that the epicuticle of the German cockroach does indeed contain these two compounds. The contact sex pheromone of the female German cockroach, thus may consist of at least six biosynthetically related components.

Keywords 27-Oxo-3,11-dimethylheptacosan-2-one · 27-Hydroxy-3,11-dimethylheptacosan-2-one · Sex pheromone · *Blattella germanica* · German cockroach · Methyl ketone · Alcohol · Aldehyde

Introduction

Sex pheromones of insects are most often produced and emitted as blends of related chemicals rather than single components. Furthermore, in recent years, it has become apparent that many pheromone molecules are produced from the action of tissue-specific enzymes on intermediates of fatty acid metabolic pathways, giving rise, among other compounds, to related hydrocarbons, alcohols, ketones, epoxides, acetates, and aldehydes (reviewed by Howard and Blomquist 2005). In many cases, each of the pheromone components alone is relatively ineffective, but a blend of two or more components may act as a "minimal blend" that stimulates attraction and/or courtship behavior (McNeil 1991). Unlike the vast majority of volatile sex pheromones, however, each of the four known contact sex pheromone components of the German cockroach, *Blattella germanica*, can independently elicit courtship, and some "minor" less abundant components are far more active than the major most abundant components (reviewed by Gemeno and Schal 2004). Therefore, chemical communication in *B. germanica* is of interest not only because this species is a pest of medical and veterinary importance but also because the chemistry, biochemistry, and behavioral

D. Eliyahu · S. Nojima · C. Schal (✉)
Department of Entomology and W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC 27695-7613, USA
e-mail: coby_schal@ncsu.edu

K. Mori
Photosensitive Materials Research Center, Toyo Gosei Co., Ltd, Wakahagi 4-2-1, Inba-mura, Inba-gun, Chiba 270-1609, Japan

ecology of its sexual communication system are relatively well understood.

The German cockroach lives in aggregations. Nevertheless, sexually receptive females release a volatile pheromone, blattellaquinone (Nojima et al. 2005), that attracts males from a distance. Upon contact with the antennae and mouth parts, the male perceives the female's contact sex pheromone on her cuticle and performs a characteristic courtship display. This behavior includes a rotation of the male's body, turning away from the female so as to orient his abdominal tip toward the female's head. Thus positioned, he raises the wings to almost 90°, exposing specialized glands on the seventh and eighth tergites. These glands secrete a mixture of lipids, proteins, and sugars that synergistically serve as feeding stimulants (Nojima et al. 1999a, b, 2002; Kugimiya et al. 2002). The female then mounts the male to feed on the tergal secretion, and this places her in the correct alignment for copulation (Roth and Willis 1952). Hence, the contact sex pheromone blend encodes sex- and species-specific information, and it triggers and mediates close-range sexual behavior. It is, therefore, imperative for mating.

The most abundant component of the female contact sex pheromone is (3*S*,11*S*)-dimethylnonacosan-2-one (**1**, Fig. 1; Nishida et al. 1974), which is derived through hydroxylation and subsequent oxidation of the abundant cuticular hydrocarbon 3,11-dimethylnonacosane (Chase et al. 1992). Based on the well-established biochemical scheme of conversion of hydrocarbons to methyl ketone pheromones in the housefly (reviewed by Blomquist 2003) and the presence of the hydrocarbon 3,11-dimethylheptacosane on the cuticular surface of females, we predicted and later confirmed through synthesis that 3,11-dimethylheptacosan-2-one (**4**) also served as a sex pheromone component in this cockroach (Jurenka et al. 1989; Schal et al. 1990; Eliyahu et al. 2004). This component is less abundant on the female's cuticular surface, and its biological activity is significantly lower in dose-response studies than that of its C_{29} homolog.

Two less abundant sex pheromone components have also been identified: the alcohol 29-hydroxy-3,11-dimethylnonacosan-2-one (**2**) and the aldehyde 29-oxo-3,11-dimethylnonacosan-2-one (**3**). Interestingly, the alcohol has been shown to be about tenfold more effective at eliciting behavioral responses than the C_{29}-dimethyl ketone (reviewed by Nishida and Fukami 1983). The biochemical pathway that gives rise to the alcohol and aldehyde components has not been elucidated, but Chase et al. (1992) proposed that female-specific hydroxylation and subsequent oxidation reactions that result in the dimethyl ketones might act at the C-29-position of the dimethyl ketone to produce the 29-hydroxy- and 29-oxo-dimethyl ketone pheromone components.

Although this hypothesis has yet to be tested with labeled precursors, it predicts that if the same mechanism converts 3,11-dimethylheptacosane to the corresponding dimethyl ketone pheromone, then its two oxidation analogs, 27-hydroxy- and 27-oxo-3,11-dimethylheptacosan-2-one, might also be present as pheromone components in the female German cockroach. We now report behavioral and analytical results that show that 27-hydroxy-3,11-dimethylheptacosan-2-one and 27-oxo-3,11-dimethylheptacosan-2-one (**5** and **6**, respectively, Fig. 1) are found on the cuticular surface of adult females, and we confirm their pheromonal activity by synthesis and bioassay of the synthetic compounds.

Methods and Materials

Insects Blattella germanica cockroaches, representing an insecticide-susceptible strain originally obtained from American Cyanamid in 1989, were kept in groups at 27°C under a 12:12 L/D photoperiod with continuous access to dry LabDiet rat chow (#5001; PMI Nutrition International, Brentwood, MO, USA) and water. Nymphs that hatched within 2–3 d were reared in synchronous cohorts, and newly emerged adult males and females were separated daily from cages containing late instar nymphs.

Behavioral Assay Male sexual response was tested by using a modification of the assay developed by Roth and Willis (1952). An antenna of a 14- to 21-d-old adult male *B. germanica* was excised, inserted into a small mass of

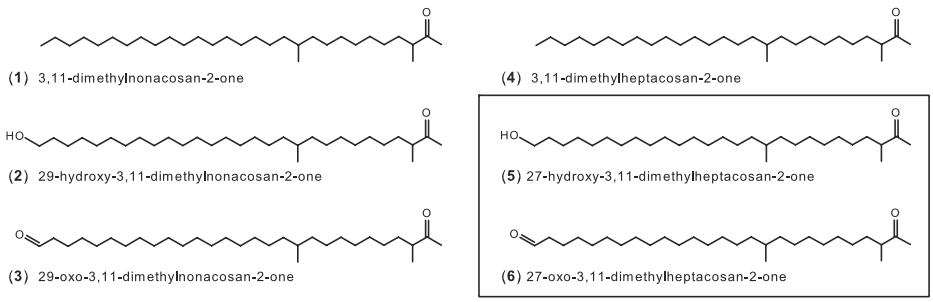

Fig. 1 Components of the contact sex pheromone of female *B. germanica*. Compounds **5** and **6**, *within the box*, are identified as new pheromone components in this paper

modeling clay at the end of a glass Pasteur pipette, and extracted for 1–2 sec in hexane to remove cuticular lipids. A test fraction or standard solution was applied with a 10-µl syringe (Hamilton, Reno, NV, USA) in 3 µl hexane to the distal 1 cm of the test antenna. The hexane was allowed to evaporate, and the antenna was used immediately to test the responses of at least 3 groups of 10 males, 14- to 21-d-old, that were housed individually in 9×9×7.5-cm plastic cages supplied with rat chow and water. All assays were conducted during mid-scotophase, avoiding the first and last 2 hr of the scotophase. The antennae of each male were gently stroked with the test antenna, and a positive response was recorded when the male executed a courtship response, rotating his body relative to the stimulus and raising his wings within 30 sec. This is an unmistakable response that occurs only in a sexual context and is never elicited by male test antennae either unfortified with female pheromone or treated with hexane alone.

This behavioral assay was used to identify chromatographic fractions that elicited behavioral responses, to conduct dose-response studies with synthetic compounds, and to test for synergistic interactions among the three C_{27} pheromone components.

Extraction and Fractionation Sexually mature virgin females, 6- to 7-d-old, were freeze-killed and extracted in groups of 50 females in a 20-ml vial with ~6 ml hexane (Optima; Fisher Scientific, Waltham, MA, USA) for 1 min. The extract was transferred to a clean vial, and the hexane was slowly reduced to 100 µl under a gentle stream of N_2. The extract was fractionated by flash column chromatography: 14.5-cm glass Pasteur pipettes loaded with 200 mg of silica gel (100–200 mesh; Fisher Scientific) were activated at 110°C for 30 min and washed with ~1 ml hexane. The extract was loaded and eluted successively with 4 ml hexane, 2 ml each of 1, 2, 5, 10, 20, and 40% diethyl ether (Optima; Fisher Scientific) in hexane, 2 ml diethyl ether, 2 ml ethyl acetate (Optima; Fisher Scientific), and 2 ml methanol (HPLC grade; Fisher Scientific). Each fraction was tested in the courtship behavioral assay, and active fractions were further fractionated on a normal phase

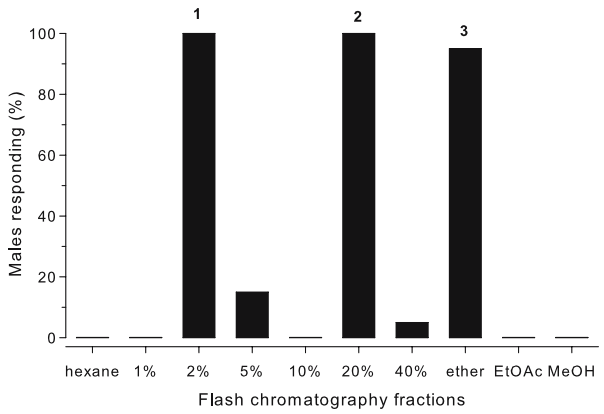

Fig. 2 Percentage of males (N>90 per fraction) responding to flash column chromatography fractions of female cuticular extract (0.05 female equivalents). The fractions in which synthetic compounds **1** (3,11-dimethylnonacosan-2-one), **2** (29-hydroxy-3,11-dimethylnonacosan-2-one), and **3** (29-oxo-3,11-dimethylnonacosan-2-one) would elute are indicated. Numbers (fractions) between hexane and ether represent percent ether in hexane. *EtOAc* Ethyl acetate, *MeOH* methanol

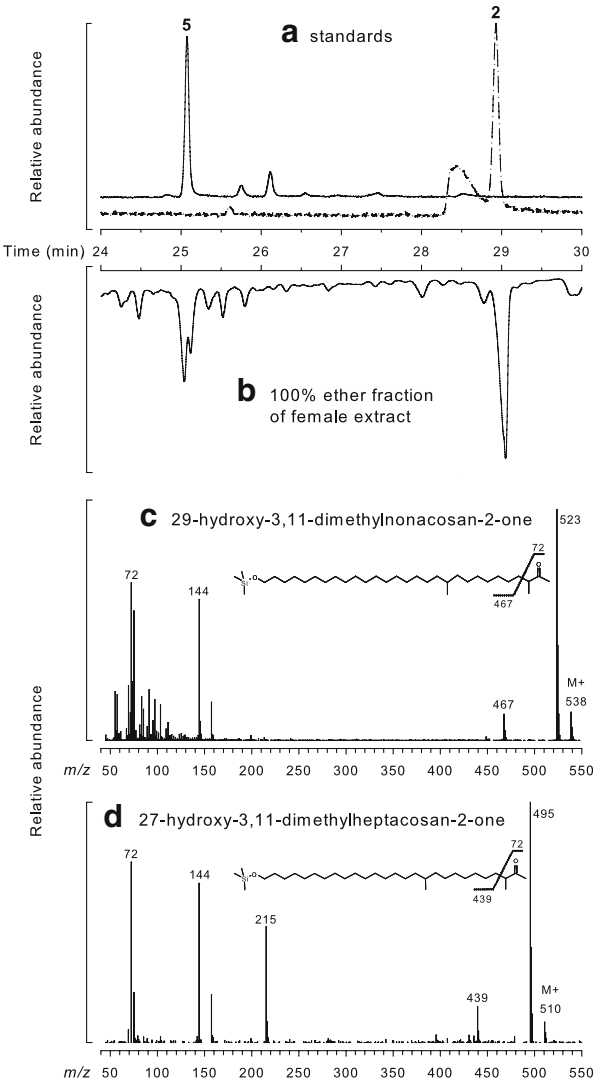

Fig. 3 Gas chromatograms and mass spectra of synthetic and natural alcohols. **a** Two separate total ion chromatograms of 160 ng of the silylated synthetic compound **2**, 29-hydroxy-3,11-dimethylnonacosan-2-one (*dotted line*) and 100 ng of the silylated synthetic compound **5**, 27-hydroxy-3,11-dimethylheptacosan-2-one (*solid line*). **b** The silylated 100% ethyl ether fraction of a composite extract of 120 females fractionated by flash column chromatography. **c** and **d** The 70-eV electron impact mass spectra of peaks in the silylated extract of females with retention times corresponding with those of compounds **2** and **5**, respectively. The fragment at *m*/*z* 72 is the result of McLafferty rearrangement of the 3-methyl-2-one-moiety

high performance liquid chromatography (HPLC) column (Econosphere, 5 μm silica, 250×4.6 mm; Alltech, Deerfield, IL, USA) on an HP1050 HPLC (Hewlett-Packard, Palo Alto, CA, USA). Supellapyrone (Charlton et al. 1993) was added as internal standard and monitored at 296 nm with an HP1050 diode array detector; the *B. germanica* sex pheromone components have no UV absorption. The sample was eluted isocratically at 1 ml min^{-1} with 99% hexane and 1% 2-propanol (HPLC grade; Fisher Scientific). One-minute fractions were collected and tested in behavioral bioassays on at least 30 males.

An Agilent 5975 mass selective detector, operated in electron impact ionization mode and coupled to an Agilent 6890 GC (Agilent, Santa Clara, CA, USA) was used for chemical structure determinations of compounds in active fractions. The GC was operated in splitless injection mode and fitted with a 30-m×0.25-mm ID DB-5MS column (Agilent). The oven was programmed from 60°C to 300°C at 15°C min^{-1} after an initial delay of 2 min and held at 300°C for 20 min. Injector temperature was 280°C, MS quadrupole 150°C, MS source 230°C, and transfer line 250°C.

Microchemical Reactions 1,1-Dimethylhydrazine (DMH, 98% pure; Sigma-Aldrich, St. Louis, MO, USA) derivatization was used to stabilize the thermally unstable oxo-dimethyl ketones (Nishida and Fukami 1983). The biologically active HPLC fraction was concentrated under N_2 to ~50 μl in a conical reaction vial, DMH (5 μl) was added, the vial was capped and incubated in a 60°C glass bead bath for 30 min, and the resulting solution immediately subjected to gas chromatography–mass spectrometry (GC-MS) analysis.

Silylation of the hydroxyl group was conducted before GC-MS analysis of the hydroxy-dimethyl ketones. The biologically active 100% ether fraction from flash chromatography of 120 females was concentrated under N_2 in a conical reaction vial, 80 μl *N*-methyl-*N*-trimethylsilyl trifluoroacetamide (MSTFA, Alltech) were added, the reaction mixture was incubated in an 80°C bead bath for 30 min, and the mixture was analyzed immediately by GC-MS.

Synthesis of 27-Hydroxy- and 27-Oxo-3,11-dimethylheptacosan-2-one (3*S*,11*S*)-27-Hydroxy-3,11-dimethylheptacosan-2-one and its oxidation product (3*S*,11*S*)-27-oxo-3,11-dimethylheptacosan-2-one were synthesized by K.M. The synthesis was executed based on an improved version of the previous synthesis of (3*S*,11*S*)-29-hydroxy-3,11-dimethylnonacosan-2-one (Mori et al. 1981). Oxidation of the alcohol to the aldehyde was carried out with Dess–Martin periodinane to avoid racemization at C-3. Details of the synthesis will be published separately by K.M. We used an enantioselective synthesis because the natural isomer of compound **1** is (3*S*,11*S*). We surmise that the natural isomers of compounds **4**, **5,** and **6** also have the (3*S*,11*S*) configuration, although this has not been shown analytically.

Statistical Analysis For dose-response studies, the probit and logistic procedures were used to estimate the dose to which 50% of the males would respond (RD_{50}) and to compare the RD_{50} values for the 3 components. The analysis was performed with SAS (SAS Institute 2003).

Results

Identification of New Components Three flash chromatography fractions (2, 20, and 100% ether fractions) elicited sexual responses from male cockroaches in behavioral bioassays (Fig. 2). These fractions corresponded to the synthetic pheromone components 3,11-dimethylnonacosan-2-one (**1**, Fig. 1), 29-oxo-3,11-dimethylnonacosan-2-one (**3**, Fig. 1), and 29-hydroxy-3,11-dimethylnonacosan-2-one (**2**, Fig. 1), respectively. The elution pattern of the components corresponded to that reported by Nishida and Fukami (1983).

Table 1 Amounts and ratios of 6 sex pheromone components on the cuticular surface of adult female *B. germanica*

Pheromone Component	C_{27} Components[d]		C_{29} Components[d]	
	Amount (ng)	Ratio	Amount (ng)	Ratio
Dimethylketone[a]	97	100	470	100
Hydroxy-dimethylketone[b]	5.3	5.5	25.6	5.4
Oxo-dimethylketone[c]	0.15	0.15	0.5	0.11

[a] 3,11-Dimethylheptacosan-2-one and 3,11-dimethylnonacosan-2-one, respectively
[b] 27-Hydroxy-3,11-dimethylheptacosan-2-one and 29-hydroxy-3,11-dimethylnonacosan-2-one, respectively
[c] 27-Oxo-3,11-dimethylheptacosan-2-one and 29-oxo-3,11-dimethylnonacosan-2-one, respectively
[d] Amounts and ratios of sex pheromone components determined from GC analyses of a composite extract of the cuticular surface of 100 virgin adult females, adjusted to nanogram per female. Ratios relative to 100% of the respective dimethyl ketone

To determine if the 100% ether fraction contained the predicted 27-hydroxy-3,11-dimethylheptacosan-2-one, hydroxylic components in this fraction were converted to silyl ethers and subjected to GC-MS analysis. The silyl ether of compound **2**, 29-hydroxy-(3*S*,11*S*)-dimethylnonacosan-2-one (Fig. 3a) was found at retention time 29.04 min, and its identity was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound (Fig. 3c). The silyl ether of the predicted candidate 27-hydroxy-3,11-dimethylheptacosan-2-one was found at retention time 25.14 min (Fig. 3b) by careful examination of the fragmentation patterns of all components. The mass spectrum of compound **5** was somewhat noisy because of its low abundance in the extract and other components eluting very close to it. However, it showed diagnostic fragments at *m*/*z* 510 (expected molecular weight, 0.08 intensity), *m*/*z* 495 (base peak, loss of a methyl group), an intense *m*/*z* 72 from McLafferty rearrangement of the 3-methyl-2-one-moiety, supporting the structure of a methylketone with a methyl group in the alpha position (Fig. 3d). Silylation also produced a fragment at *m*/*z* 144 in compounds **2** and **5**. The intense fragment at *m*/*z* 215 in the MS of the silylated heptacosanone derivative appears to be an artifact resulting from compounds that co-elute with **5**. The identity of compound **5** was confirmed by comparing both its GC retention time and MS fragmentation pattern with those of the authentic compound.

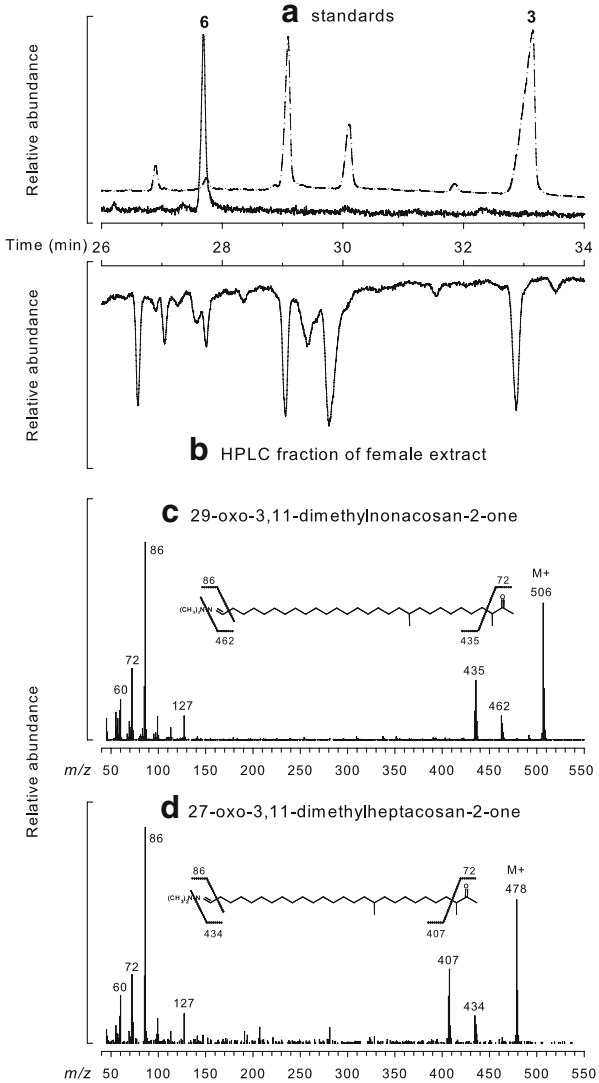

Fig. 4 Gas chromatograms and mass spectra of synthetic and natural aldehydes. **a** Two separate total ion chromatograms of 300 ng of the derivatized synthetic compound **3**, 29-oxo-3,11-dimethylnonacosan-2-one (*dotted line*), and 100 ng of the derivatized synthetic compound **6**, 27-oxo-3,11-dimethylheptacosan-2-one (*solid line*). **b** The similarly derivatized active HPLC fraction of the combined 20% ethyl ether fractions from a composite extract of 120 females fractionated by flash chromatography. **c** and **d** The electron impact mass spectra of peaks in the extract of females with retention times that corresponded with those of compounds **3** and **6**, respectively. The fragment at *m*/*z* 72 is the result of McLafferty rearrangement of the 3-methyl-2-one-moiety, and the intense fragment at *m*/*z* 86 represents McLafferty rearrangement of the 1,1-dimethylhydrazone moiety

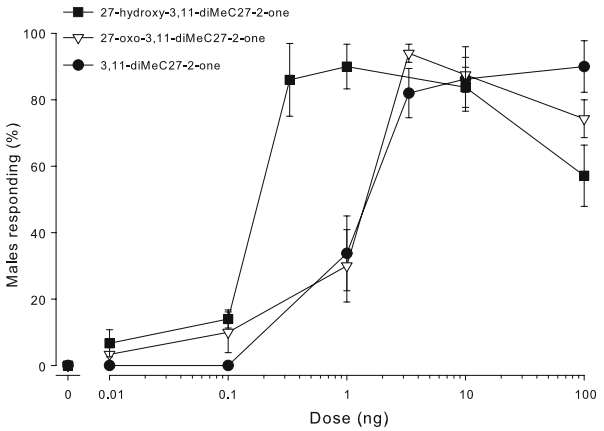

Fig. 5 Dose–response curves of 3 C_{27} sex pheromone components showing mean (±SE) percentage of males responding to 3,11-dimethylheptacosan-2-one (**4**), 27-hydroxy-3,11-dimethylheptacosan-2-one (**5**), and 27-oxo-3,11-dimethylheptacosan-2-one (**6**). (*N*=30–80 per compound/dose combination)

The amount of 27-hydroxy-3,11-dimethylheptacosan-2-one was estimated by comparison to authentic compound **2** to be ~5.3 ng per female, which is about fivefold less than the amount of 29-hydroxy-3,11-dimethylnonacosan-2-one found on the cuticular surface of sexually receptive females. Each of these alcohols is about 18-fold less abundant than the equivalent chain length dimethyl ketone from which the alcohol presumably arises (Table 1).

The 20% ether fraction was subjected to further bioassay-guided fractionation by HPLC. Two discrete fractions that elicited behavioral responses were obtained: the first 1-min fraction co-eluted with synthetic compound **3**, 29-oxo-3,11-dimethylnonacosan-2-one, whereas the second fraction

Table 2 Probit analysis of dose-response studies of 3 sex pheromone components

Sex Pheromone Component	Slope	Intercept	RD_{50} (ng)[a]	95% Fiducial Limits
3,11-Dimethylheptacosan-2-one (**4**)	0.590	−0.376	1.67 a	0.814, 2.818
27-Hydroxy-3,11-dimethylheptacosan-2-one (**5**)	0.763	0.664	0.18 b	0.042, 0.456
27-Oxo-3,11-dimethylheptacosan-2-one (**6**)	1.105	−0.093	1.18 a	0.567, 2.199

[a] Estimated dose to which 50% of males respond. Values followed by different letters are significantly different ($P<0.05$, Probit and logistic procedures, SAS Institute 2003).

eluted several minutes later (data not shown). To determine if the first fraction also contained the predicted 27-oxo-3,11-dimethylheptacosan-2-one, aldehyde components in this fraction were reacted with 1,1-dimethylhydrazine and subjected to GC-MS analysis. The hydrazone derivative of compound **3** (Fig. 4a) was found at retention time 32.87 min, and the identity was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound (Fig. 4b). The hydrazone derivative of the predicted candidate 27-oxo-3,11-dimethylheptacosan-2-one was found at retention time 27.74 min (Fig. 4a) by screening of the fragmentation patterns of all components. Compound **6** showed diagnostic fragments at *m/z* 72 (0.32 intensity relative to base peak) and 86, indicating McLafferty rearrangements of alpha-methyl ketone and 1,1-dimethylhydrazone moieties, $–CH_2–CH(CH_3)–CO–CH_3$ and $–CH=N–N(CH_3)_2$, respectively. In addition, it showed diagnostic fragments of the 1,1-dimethylhydrazone derivative at *m/z* 407, 434, and 478, from M^+-44 and M^+-86, and the expected molecular ion of the derivative, respectively (Fig. 4c). The identity of compound **6** was confirmed by comparing the GC retention time and MS fragmentation pattern with those of the authentic compound. Although DMH reacts with carbonyl groups in general, the proximity of the ketone group to the methyl branch in compounds **3** and **6** appears to hinder its reaction with the carbonyl group at position 2. Accordingly, derivatizations of compound **1** did not result in a hydrazone (data not shown), and we did not find products with 2-hydrazone moieties.

The amount of 27-oxo-3,11-dimethylheptacosan-2-one, estimated by comparison with known amounts of 29-oxo-3,11-dimethylnonacosan-2-one, was ~0.15 ng per female. Thus, the ratio of the C_{29} aldehyde to its C_{27} homolog is about 4:1. These oxo-dimethyl ketone components were about 40-fold less abundant than the respective alcohols (compounds **2** and **5**, Table 1).

The 20% ether fraction from flash column chromatography also contained a second active fraction that eluted several minutes after the 27-oxo- and 29-oxo-dimethyl ketones in normal phase HPLC. It may represent additional sex pheromone components whose compositions are currently under investigation.

Biological Activity of the Synthetic New Pheromone Components The biological activities of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one were tested at various doses on 3–8 groups of 10 individually housed males. 27-Hydroxy-3,11-dimethylheptacosan-2-one (**5**) was about tenfold more active than the other two C_{27} components (Fig. 5). The estimated doses to which 50% of the males responded for each component (RD_{50}) are presented in Table 2. The RD_{50} values for compounds **4** and **6** were similar, but significantly higher than for compound **5**, as demonstrated by both logistic analysis and non-overlapping 95% fiducial limits.

The activity of 3,11-dimethylheptacosan-2-one was compared in a dose-response study to the activity of a blend of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one, and 27-oxo-3,11-dimethylheptacosan-2-one in a 100:10:1 ratio. No synergism between the different C_{27} compounds was observed: the activity of a mixture of 100:10:1 methyl ketone/alcohol/aldehyde was not significantly different from that of the methyl ketone alone (Fig. 6).

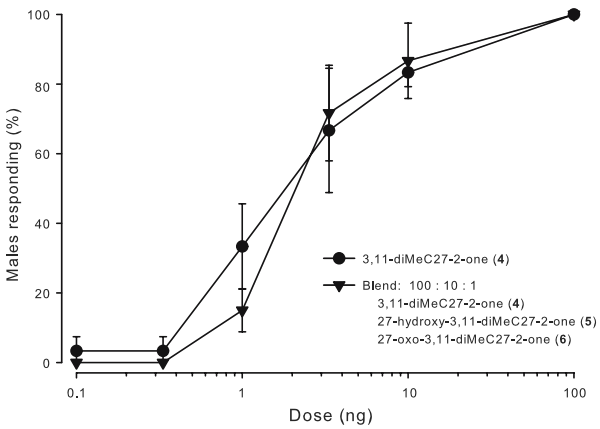

Fig. 6 Results of behavioral assays showing interaction among C_{27} sex pheromone components. 3,11-Dimethylheptacosan-2-one (**4**) was compared to a blend of 3,11-dimethylheptacosan-2-one, 27-hydroxy-3,11-dimethylheptacosan-2-one (**5**), and 27-oxo-3,11-dimethylheptacosan-2-one (**6**) in a ratio of 100:10:1, respectively. Data show mean percentage of males responding (±SE; N=30–60 per compound/dose combination)

Discussion

Semiochemicals are generally identified by the well-established approach of bioassay-guided sequential fractionation of extracts followed by chemical analysis, structure elucidation, and confirmation of biological activity with synthesized authentic compounds. A complementary approach predicts possible pheromone components based on an understanding of the chemistry and biochemistry of pheromone production coupled with knowledge of the evolutionary relationships among taxa. For example, Bjostad et al. (1984) predicted the structures of sex pheromone components of the cabbage looper, *Trichoplusia ni*, based on knowledge of biosynthetic pathways and fatty acid precursors. The pheromone gland of this noctuid moth utilizes acetate to produce octadecanoic and hexadecanoic acids which undergo Δ11-desaturation to produce Z11-18: acid and Z11-16:acid. However, the major component of the sex pheromone is (*Z*)-7-dodecen-1-yl acetate (Z7-12:OAc). When labeled Z11-16:acid was applied to glands, it was incorporated into both Z7-12:acid and the corresponding acetate ester pheromone. Because the main pheromone component was produced through a Δ11-desaturation followed by chain shortening, reduction, and acetylation to form the acetate ester, Bjostad et al. (1984) reasoned that other pheromone components would be produced from intermediates in the chain shortening sequence. Indeed, other acetate esters, including Z9-14:OAc, Z7-14:OAc, and Z5-12:OAc, were found to be pheromone components, the first derived from chain shortening of Z11-16:acid and the latter two from Z11-18:acid. In a similar manner, pheromone components of the spruce budworm (*Choristoneura fumiferana*) were predicted based on identification of fatty acids in the pheromone gland and their temporal variation in relation to pheromone production (Silk and Kuenen 1986), although in this case, the proposed components were not shown conclusively to be biologically active. Overall, few pheromone components have been identified from predictions based on biosynthetic pathways. In this paper, we confirmed that candidate compounds, predicted based on the biosynthetic pathways of related contact sex pheromone components, do indeed serve as pheromone components in the German cockroach.

In insects, cuticular hydrocarbons and hydrocarbon pheromones are formed through fatty acid elongation followed by decarboxylation (reviewed by Howard and Blomquist 2005). For example, in the housefly, the sex pheromone component (*Z*)-9-tricosene is first formed, and this alkene is then metabolized by a cytochrome-P450 enzyme to the corresponding epoxide and ketone, which also serve as sex pheromone components (reviewed by Blomquist 2003). This conversion of hydrocarbons to methyl ketone and epoxide pheromone components in the housefly served as a guide for our research on pheromone biosynthesis in the German cockroach.

The major cuticular lipids in all life stages of the German cockroach consist of an isomeric mixture of 3,7-, 3,9-, and 3,11-dimethylnonacosane (Jurenka et al. 1989). However, the dimethyl ketone fraction of cuticular extracts of adult females, which contains the pheromone 3,11-dimethylnonacosan-2-one, comprises only the 3,11-isomer. This led Jurenka et al. (1989) to propose a biosynthetic relationship between the hydrocarbon and its dimethyl ketone pheromone analog. Using radiolabeled compounds coupled with radio-GC, Chase et al. (1992) determined that the dimethyl ketone sex pheromone arises from the insertion of an oxygen into the preformed 3,11-dimethyl alkane. Radioactivity from the alkane was detected in both 3,11-dimethylnonacosan-2-ol and 3,11-dimethylnonacosan-2-one, but only in adult females, whereas when tritiated 3,11-dimethylnonacosan-2-ol was applied to the cuticle, it was readily and highly efficiently converted to the corresponding dimethyl ketone pheromone not only in females but also in males. These results confirmed that the dimethyl ketone sex pheromone of *B. germanica* arises via a female-specific hydroxylation of 3,11-dimethylnonacosane and a subsequent non-sex-specific oxidation to 3,11-dimethylnonacosan-2-one.

The same mechanism presumably converts 3,11-dimethylheptacosane, a component of the cuticular hydrocarbon profile of the German cockroach, to 3,11-dimethylheptacosan-2-one. Dose-response studies with both natural and synthetic 3,11-dimethylheptacosan-2-one confirmed its biological activity, although it was found to be approximately tenfold less active than its C_{29} homolog (Schal et al. 1990; Eliyahu et al. 2004).

Chase et al. (1992) previously proposed that a similar hydroxylation and oxidation at the C-29 position of 3,11-dimethylnonacosan-2-one might give rise to 29-hydroxy- and 29-oxo-3,11-dimethylnonacosan-2-one, the other components of the contact sex pheromone blend, and that the same mechanism might convert 3,11-dimethylheptacosan-2-one to its 27-hydroxy- and 27-oxo- analogs.

Identification of the C_{27} alcohol and aldehyde as sex pheromone components was fraught with difficulties, and it is not surprising that earlier studies with cuticular extracts of more than 200,000 females did not reveal these compounds. The two active homologs in each class (dimethyl ketones, alcohols, aldehydes) differ only in chain length and can only be separated by high performance fractionation methods, such as preparative GC. Furthermore, the aldehydes are thermally unstable, and derivatization is advisable before GC analysis. Also, silylation of the hydroxy group in the alcohols renders the analysis easier because the derivatives are less polar, and hence, less susceptible to adsorption by active sites on the column or in the injector. Moreover, both the C_{27} alcohol and aldehyde

occur in minute amounts that are largely obscured by other compounds in the extracts. Therefore, predictions based on the biosynthetic pathway of the C_{29} components were crucial for the location, recognition, and identification of the three C_{27} components.

Our finding of the predicted C_{27} compounds in the cuticular pheromone blend, and in a similar blend ratio to that of the C_{29} components, further corroborates the biosynthetic pathway proposed and partly demonstrated by Chase et al. (1992; reviewed by Schal et al. 2003). Furthermore, our results provide motivation for a reexamination of other hydrocarbons that might serve as precursors for methyl ketones and their oxidation derivatives that might also mediate behavioral responses. A prime candidate for consideration is 3,11-dimethylhentriacontane, which occurs on the cuticular surface in tiny amounts (Carlson and Brenner 1988). Structure–activity studies on 3,11-dimethylnonacosan-2-one indicate that the apparently promiscuous pheromone receptor of *B. germanica* males accepts chain lengths that are slightly shorter or longer than the optimal 29-carbon alkyl chain (Nishida and Fukami, 1983). Because the C_{27} homolog elicited behavioral responses, we predict that the C_{31} homolog will also show biological activity.

Although the 3,11-dimethyl structure is important for eliciting behavioral response, structure–activity studies have shown that both the 3-monomethyl and 11-monomethyl compounds are also biologically active, albeit at much higher doses (Nishida and Fukami 1983). The epicuticular lipids of *B. germanica* contain 11-methylheptacosane, 11-methylnonacosane, 11-methyltriacontane, 3-methylheptacosane, and 3-methylnonacosane, as well as dimethylalkanes that include an 11-methyl branch, including 11,15-dimethylheptacosane, 11,15-dimethylnonacosane, 5,11-dimethylheptacosane, and 5,11-dimethylnonacosane (Augustynowicz et al. 1987; Carlson and Brenner 1988; Jurenka et al. 1989). It is possible that the same mechanism that inserts a C-2 carbonyl into the preformed dimethylalkane might do the same with monomethyl alkanes of the proper chain length. We recently discovered that oxidation metabolites of 11-methylheptacosane are indeed biologically active. Nishida and Fukami (1983) found that when antennae of the Oriental cockroach, *Blatta orientalis*, stroked against the antennae of male German cockroaches, courtship was elicited in the latter species. Through fractionation and bioassays, we have recently identified two more active compounds: 11-methylheptacosan-2-one and 27-oxo-11-methylheptacosan-2-one (Eliyahu et al. 2008). 11-Methylheptacosane is abundant on the cuticular surface of the Oriental cockroach (Lockey and Dularay 1986), and it is likely that in this cockroach too, the methyl ketone and aldehyde are oxidation products of the hydrocarbon. 3-Methyl and 11-methyl hydrocarbons of the appropriate chain length are present in the cuticular lipids of German cockroach females, and they similarly could be converted to biologically active methyl ketones, alcohols, and aldehydes.

Our dose-response results with synthetic C_{27} components are in agreement with similar results from the C_{29} components (Nishida and Fukami 1983). Both the C_{29} and C_{27} primary alcohols were about tenfold more active than the respective methyl ketones, whereas the aldehydes were not significantly more active than the respective methyl ketones of the same chain length. Unlike most insect pheromone blends, especially in the case of volatile pheromones, each of the 6 contact sex pheromone components can independently elicit the complete repertoire of sexual responses. Schal et al. (1990) found that the 2 methyl ketone components of the pheromone interacted additively, not synergistically. We show here that there is also no synergism between different component classes. Therefore, it appears that the total semiochemical activity might be achieved by additive effects, with low amounts of the more polar compounds being compensated for by their lower thresholds for eliciting courtship responses.

Further studies are necessary to determine the definitive number of components in the contact sex pheromone blend of the female German cockroach. Two points of caution are worth mentioning. First, the sex pheromone reception system of the male German cockroach exhibits a highly promiscuous response, responding to compounds that deviate significantly from the native pheromone. Indeed, it represents one of the few cases where the native pheromone can be significantly improved upon, for example by changing the C-2 carbonyl to a C-2 hydroxy group. This might explain in part the frequent observations of interspecific courtship in cockroaches, as described above. It also highlights the important point that for a pheromone component to be considered as such, it must not only elicit a response from the receiver but also must be present on the producer.

Second, the relative behavioral value to the male of each female pheromone component must be considered. The dimethyl ketone generally constitutes 10% of the abundance of its precursor hydrocarbon analog (Chase et al. 1992), and the alcohol and aldehyde occur at ~5 and ~0.1% of the respective parent dimethyl ketone (Table 1). Assuming that other, yet to be identified, semiochemicals occur at similar ratios, it seems that if they arise from relatively poorly represented hydrocarbons, they might occur in subnanogram amounts. Moreover, these compounds are presumably spread over the total cuticular surface and possibly buried within hundreds of micrograms of cuticular hydrocarbons. It remains to be determined how much of each component is "operationally functional", that is, perceived by the male when he briefly strokes the female's antennae. We previously determined that each female antenna contains about 1 ng of 3,11-dimethylnonacosan-2-one and 0.4 ng of

3,11-dimethylheptacosan-2-one, closely matching the minimal amounts required to elicit courtship behavior from males as determined from dose-response studies (Eliyahu et al. 2004). It is conceivable that many minor components that could potentially act as pheromones at appropriate doses are represented on the female's cuticle and antennae at amounts substantially below the male's threshold of detection, and thus, they may represent "noise" arising from biosynthetic processes that are not completely selective. We suggest that such compounds should only be labeled as pheromone components if they elicit responses in dose ranges within which they are naturally found on the producer.

Acknowledgments We thank Ritsuo Nishida for providing synthetic 29-hydroxy-3,11-dimethylnonacosan-2-one, and Takuya Tashiro for helping with an earlier synthesis of 29-oxo-3,11-dimethylnonacosan-2-one. We appreciate Wittko Francke's assistance in interpreting MS data and his critique of the manuscript, and Cavell Brownie for help with statistical analyses. We thank Jinping Sun, Jeffrey Spontak, and Richard Santangelo for maintaining insect colonies, Edward Vargo, Charles Apperson, and Robert Anholt for useful comments on an early draft of this manuscript. This work was supported in part by the Blanton J. Whitmire Endowment at North Carolina State University.

References

AUGUSTYNOWICZ, M., MALINSKI, E., WARNKE, Z., SZAFRANEK, J., and NAWROT, J. 1987. Cuticular hydrocarbons of the German cockroach, *Blattella germanica* L. *Comp. Biochem. Physiol. B.* 86:519–523.

BJOSTAD, L. B., LINN, C. E., DU, J.-W., and ROELOFS, W. L. 1984. Identification of new sex pheromone components in *Trichoplusia ni*, predicted from biosynthetic precursors. *J. Chem. Ecol.* 10:1309–1323.

BLOMQUIST, G. J. 2003. Biosynthesis and ecdysteroid regulation of housefly sex pheromone production, pp. 231–252, in G. J. Blomquist, and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London.

CARLSON, D. A., and BRENNER, R. J. 1988. Hydrocarbon-based discrimination of three North American *Blattella* cockroach species (Orthoptera: Blattellidae) using gas chromatography. *Ann. Entomol. Soc. Am.* 81:711–723.

CHARLTON, R. E., WEBSTER, F. X., ZHANG, A., SCHAL, C., LIANG, D., SRENG, I., and ROELOFS, W. L. 1993. Sex pheromone for the brownbanded cockroach is an unusual dialkyl-substituted α-pyrone. *Proc. Natl. Acad. Sci. U. S. A.* 90:10202–10205.

CHASE, J., TOUHARA, K., PRESTWICH, G. D., SCHAL, C., and BLOMQUIST, G. J. 1992. Biosynthesis and endocrine control of the production of the German cockroach sex pheromone 3,11-dimethylnonacosan-2-one. *Proc. Natl. Acad. Sci. U. S. A.* 89: 6050–6054.

ELIYAHU, D. 2007. Chemical communication in the German cockroach: pheromones and heterospecific courtship eliciting compounds. Ph.D. dissertation. North Carolina State University, Raleigh.

ELIYAHU, D., MORI, K., TAKIKAWA, H., LEAL, W. S., and SCHAL, C. 2004. Behavioral activity of stereoisomers and a new component of the contact sex pheromone of female German cockroach, *Blattella germanica*. *J. Chem. Ecol.* 30:1839–1848.

ELIYAHU, D., NOJIMA, S., CAPRACOTTA, S. S., COMINS, D. L., and SCHAL, C. 2008. Identification of cuticular lipids eliciting interspecific courtship in the German cockroach, *Blattella germanica. Naturwissenschaften* doi:10.1007/s00114-007-0339-7.

GEMENO, C., and SCHAL, C. 2004. Sex pheromones of cockroaches, pp. 179–247, in R. T. Cardé, and J. G. Millar (eds.). Advances in Insect Chemical Ecology. Cambridge University Press, Cambridge.

HOWARD, R. W., and BLOMQUIST, G. J. 2005. Ecological, behavioral and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–393.

JURENKA, R. A., SCHAL, C., BURNS, E., CHASE, J., and BLOMQUIST, G. J. 1989. Structural correlation between cuticular hydrocarbons and female contact sex pheromone of German cockroach *Blattella germanica* (L.). *J. Chem. Ecol.* 15:939–949.

KUGIMIYA, S., NISHIDA, R., KUWAHARA, Y., and SAKUMA, M. 2002. Phospholipid composition and pheromonal activity of nuptial secretion of the male German cockroach, *Blattella germanica*. *Entomol. Exp. Appl.* 104:337–344.

LOCKEY, K. H., and DULARAY, B. 1986. Cuticular methylalkanes of adult cockroaches, *Blatta orientalis* and *Periplaneta americana*. *Comp. Biochem. Physiol. B.* 85:567–572.

MCNEIL, J. N. 1991. Behavioral ecology of pheromone-mediated communication in moths and its importance in the use of pheromone traps. *Annu. Rev. Entomol.* 36:407–430.

MORI, K., MASUDA, S., and SUGURO, T. 1981. Stereocontrolled synthesis of all of the possible stereoisomers of 3,11-dimethylnonacosan-2-one and 29-hydroxy-3,11-dimethylnonacosan-2-one. *Tetrahedron* 37:1329–1340.

NISHIDA, R., and FUKAMI, H. 1983. Female sex pheromone of the German cockroach, *Blattella germanica*. *Mem. College Agric., Kyoto Univ.* 122:1–24.

NISHIDA, R., FUKAMI, H., and ISHII, S. 1974. Sex pheromone of the German cockroach (*Blattella germanica* L.) responsible for male wing-raising: 3,11-dimethyl-2-nonacosanone. *Experientia* 30:978–979.

NOJIMA, S., NISHIDA, R., KUWAHARA, Y., and SAKUMA, M. 1999a. Nuptial feeding stimulants: a male courtship pheromone of the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae). *Naturwissenschaften* 86:193–196.

NOJIMA, S., SAKUMA, M., NISHIDA, R., and KUWAHARA, Y. 1999b. A glandular gift in the German cockroach, *Blattella germanica* (L.) (Dictyoptera: Blattellidae): the courtship feeding of a female on secretion from male tergal glands. *J. Insect Behav.* 12:627–640.

NOJIMA, S., KUGIMIYA, S., NISHIDA, R., SAKUMA, M., and KUWAHARA, Y. 2002. Oligosaccharide composition and pheromonal activity of male tergal gland secretions of the German cockroach, *Blattella germanica* (L.). *J. Chem. Ecol.* 28: 1483–1494.

NOJIMA, S., SCHAL, C., WEBSTER, F. X., SANTANGELO, R. G., and ROELOFS, W. L. 2005. Identification of the sex pheromone of the German cockroach, *Blattella germanica*. *Science* 307:1104–1106.

ROTH, L. M., and WILLIS, E. R. 1952. A study of cockroach behavior. *Am. Midl. Nat.* 47:66–129.

SAS INSTITUTE 2003. The SAS System for Windows, release 9.1. SAS Institute Inc., Cary, NC.

SCHAL, C., BURNS, E. L., JURENKA, R. A., and BLOMQUIST, G. J. 1990. A new component of the female sex pheromone of *Blattella germanica* (L.) (Dictyoptera: Blattellidae) and interaction with other pheromone components. *J. Chem. Ecol.* 16:1997–2008.

SCHAL, C., FAN, Y., and BLOMQUIST, G. J. 2003. Regulation of pheromone biosynthesis, transport, and emission in cockroaches, pp. 283–322, in G. J. Blomquist, and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular Biology. Elsevier Academic Press, London.

SILK, P. J., and KUENEN, L. P. S. 1986. Spruce budworm (*Choristoneura fumiferana*) pheromone chemistry and behavioral responses to pheromone components and analogs. *J. Chem. Ecol.* 12:367–383.

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Identification, Synthesis, and Bioassay of a Male-Specific Aggregation Pheromone from the Harlequin Bug, *Murgantia histrionica*

Deane K. Zahn · Jardel A. Moreira · Jocelyn G. Millar

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Abstract Sexually mature male harlequin bugs produced a sex-specific compound, identified as one of the stereoisomers of the sesquiterpene epoxyalcohol 4-[3-(3,3-dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol (henceforth murgantiol), a compound with four chiral centers and 16 possible stereoisomers. Production of the compound was highest during the middle of the day. Individual virgin male bugs in separate containers produced the compound at a higher rate than virgin males in groups. The carbon skeleton was verified by synthesis of several mixtures which, in total, contained all possible isomers, one of which matched the insect-produced compound. The relative and absolute configurations of the insect-produced compound remain to be determined. In laboratory bioassays, insect-produced and synthetic murgantiol attracted harlequin bugs of both sexes, suggesting that murgantiol is a male-produced aggregation pheromone, analogous to those found in a number of other phytophagous bug species.

Keywords Aggregation pheromone · Heteroptera · Hemiptera · 4-[3-(3,3-dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol · Murgantiol

Introduction

The harlequin bug, *Murgantia histrionica* (Hahn), is an important pest of cabbage, broccoli, and other cole crops in the United States (McPherson 1982). Methods for monitoring this and other stink bug species, such as sampling the crop by using a sweep net or a beating tray, or by visual inspection of damage or presence of insects, are typically labor-intensive. Sampling at frequent intervals may be required to detect immigration of bug populations into crops, so that control measures can be applied before significant damage occurs. Consequently, attractive sex or aggregation pheromones used as baits in monitoring traps could prove of value in integrated pest management programs.

To date, pheromones have been identified for only a few stink bug species. For all phytophagous pentatomids for which sex or aggregation pheromones have been identified, the compounds were produced by males (reviewed in McBrien and Millar 1999; Ho and Millar 2001a, b; Millar 2005). In addition to using sex-specific pheromones, phytophagous stink bugs use species- and sex-specific substrate-borne vibrations for intraspecific communication over relatively short distances (Čokl and Doberlet 2003). The vibrational signals of *M. histrionica* were recently described (Čokl et al. 2004), with males producing five different vibrational songs and females producing only one song. Contrary to those found for most stink bugs investigated, the vibrational signals did not seem to play a role in orientation toward the source of the vibration (i.e., mate location). Rather, the vibrational signals appeared to function in close-range mate recognition and courtship, suggesting that signals other than vibrational signals may be important in bringing the two sexes together over greater distances.

Harlequin bugs produce or sequester a variety of chemicals. For example, the metathoracic scent gland secretion of adult *M. histrionica* consists of (2*E*,6*E*)-octadienedial and (2*E*,6*E*)-octadiene-1,8-diol diacetate (Aldrich et al. 1996). Additionally, when adults are

D. K. Zahn · J. A. Moreira · J. G. Millar (✉)
Department of Entomology, University of California, Riverside, CA 92521, USA
e-mail: Jocelyn.millar@ucr.edu

squeezed, a sticky froth is expelled from the margins of the prothorax. This fluid contains aglycones of glucosinolates, *sec*-butyl isothiocyanate, methylthiopropyl nitrile, and methylthiobutyl nitrile, as well as two strong-smelling alkylmethoxypyrazines, 2-*sec*-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine, which have been hypothesized to serve as warning odors (Aldrich et al. 1996). These compounds are likely derived from glucosinolates sequestered from host plants, providing the vividly colored aposematic bugs with a formidable chemical defense against predation. The bugs can retain these compounds for an extended period of time (Aliabadi et al. 2002). However, none of these compounds has been reported to have a role as pheromones.

We describe herein the collection and analysis of volatile compounds released by live adult *M. histrionica* of both sexes. In addition to a number of compounds common to both sexes, one major sex-specific compound was found in the odors released by sexually mature adult males. This compound functions as an aggregation pheromone component for the harlequin bug.

Materials and Methods

Insect Colonies Adults and nymphs of *M. histrionica* were collected from bladderpod, *Isomeris arborea* Nutt. (Capparaceae), from one site each in Riverside and San Diego, CA, USA. A colony was started in the laboratory in 2003 and augmented every year since with approximately 100 males, females, and nymphs from each location. Voucher specimens were submitted to the University of California, Riverside Entomology Research Museum (UCRC 145863–145882). Insects were reared at 26±1°C, approximately 45% RH, with a photoperiod of 16:8 h (L:D) provided by fluorescent lights. Immature insects were held separately from adults in cylindrical plastic containers (20×15 cm), with two 4-cm holes on opposite sides of each container covered with brass screening. Adults were held in 30×17×18 cm glass-topped rearing cages. Insects were provided with Napa cabbage, cauliflower, and broccoli three times per week. Upon eclosion, adults were sexed and maintained for 14 d with florets of broccoli in 175 ml transparent plastic cups, or in groups in 3.8-l cardboard ice-cream cartons with ventilated lids, before use.

Collection of Insect-Produced Compounds from Live Bugs A system for collecting volatile compounds from live organisms was set up as previously described (Millar and Sims 1998). Ten to fifteen virgin adult bugs and two large cauliflower florets were put into a two-part, 400 ml cylindrical glass aeration chamber, lined with screen upon which bugs could perch. Humidified, charcoal-filtered air (6–14 mesh activated charcoal granules) was drawn through the chamber by vacuum at 250 ml per min. Volatile chemicals were collected overnight on activated charcoal traps made from 4 mm ID glass tubes loaded with a 0.4-cm bed of 50–200 mesh activated charcoal (Cat. # 05–690A, Fisher Scientific, Pittsburgh, PA, USA), precleaned by heating at 200°C under a flow of clean N_2 (~100 ml per minute). Aerations of groups of bugs were conducted continuously for 2–3 weeks at ~25°C and 50% RH with cohorts of male and female bugs of known age, changing the food and the collectors every other day. Aerations of individual bugs were conducted in the same way, but using smaller, 200-ml chambers. All aeration chambers were within the main colony rearing room. Additional cohorts of sexed virgin bugs, of the same age as those in the chamber, were maintained and used to replace any bugs in the chamber that died. As a control, two to three cauliflower florets were put in a 1-l aeration chamber and aerated for 20 h with an air flow rate of 1 l per minute. Trapped volatiles were stripped off the activated charcoal by elution with methylene chloride (500 µl; Optima grade, Cat. # D151–4, Fisher Scientific, Pittsburgh PA, USA), and the resulting extracts stored in glass vials with Teflon-lined screw caps at ~−20°C until needed.

Single-bug and group (5, 10, and 15) aerations were set up simultaneously to determine whether the male-specific compound was released at different rates by individuals or groups. For this, bugs were either held individually (N=20) or in groups (N=20 each group) for 2 weeks before use. Traps were changed every other day and eluted with 500 µl of CH_2Cl_2. The amounts of male-specific compound per bug hour were analyzed by one-way ANOVA and means were separated by Games–Howell tests.

To determine the diurnal cycle of release of the male-specific compound, five cohorts of five virgin, sexually mature, adult males (14–16 days old), and three florets of cauliflower were aerated in chambers as described above. After a cohort of bugs was confirmed to be producing the male-specific compound, the traps were changed every 2 h during the photophase. A single trap was used to collect volatiles produced between 23:00 and 07:00. An external standard of hexadecane in CH_2Cl_2 (1.6 µg/ml) was used to estimate the amount of male-specific compound present in each extract. The total amounts of the male-specific compound produced during each 24-h period were calculated along with the percentage of the total produced during each 2-h interval. For the 8-h period between 23:00 and 07:00, the average amount per 2 h was calculated. The percentage data from all cohorts were averaged, analyzed by ANOVA, and means were separated by Student–Newman–Keuls tests.

General Procedure for Y-tube Bioassays Bioassays were conducted with a vertical glass Y-tube (5 cm ID; bottom arm=18 cm long, top arms=15 cm long, Y angle=90°) with female ground glass joints, each fitted with male joints terminating in a hose nipple. The Y-tube was illuminated from above with a 1.2-m long light bank containing a Sylvania Octron 3500K F032/735 32 W fluorescent light and a GE F40PL/AQ Plant and Aquarium wide spectrum fluorescent light, providing light intensities of 350 lux at the bottom to 600 lux at the top of the Y-tube. Charcoal-purified air was pulled through the apparatus (2.5 liter per minute) by vacuum. Bugs were tested individually and used only once. Each bug was gently placed inside the bottom male joint of the Y-tube, which was then inserted into the female joint. *M. histrionica* are positively phototactic and negatively geotactic, and generally walked up toward the light. A positive response was classified as a bug crossing a line 12 cm up either arm from the junction. If a bug did not move within 10 min of the start of the bioassay, it was discarded. Each Y-tube and joint was used once, then washed, acetone rinsed, and oven-dried at 140°C. Bioassays were conducted in a temperature (22–25°C) and humidity (40–50% RH) controlled room, 4 to 8 h after the onset of photophase. Positions of treatments were alternated every second replicate. Choice data were analyzed with chi-square tests with one degree of freedom and $\alpha=0.05$.

The Y-tube olfactometer was also used to determine which sex of adult *M. histrionica* produced pheromone. The top joints of the Y-tube were connected to 200 ml glass aeration chambers that contained five sexually mature virgin bugs and two cauliflower florets, or two cauliflower florets alone. Chambers were positioned above the Y-tube, behind a cardboard screen so contents were not visible to the bugs in the Y-tube. Ten minutes after the chambers were set up, a test bug was introduced into the bottom of the Y-tube and its response recorded by an observer and on video tape (Panasonic high-density recording, time lapse video recorder, model AG-6740p). A fresh group of five unmated, sexually mature bugs was used as a pheromone source for each day of bioassay. Four separate experiments were conducted, testing the responses of females to odors from males, females to odors from females, males to odors from females, and males to odors from males. The responses of 40 bugs were tested in each experiment. Because females did not attract males, but males attracted females, only females were used as responders in the subsequent experiments, except where indicated otherwise.

The numbers of virgin female *M. histrionica* attracted to 5 bug-hour equivalents (1 bug-hour equivalent=the volatiles collected from 1 bug in 1 h) of crude extract in CH_2Cl_2 vs a CH_2Cl_2 blank were compared. Aliquots of extract or solvent were loaded on half-circular filter papers (1.5 cm in diameter) mounted on wires in the top of each arm of the Y-tube. Five bug-hours of extract contained ~30 ng of the major male-specific component, as determined by gas chromatography.

A pooled extract of male-produced odors was separated into eight fractions (see below), and the fraction containing the major male-specific compound (5 bug-hour equivalents) was bioassayed against a CH_2Cl_2 control. Equal aliquots of the remaining seven fractions were combined and tested against a CH_2Cl_2 control. Having determined that only the fraction containing the major male-specific compound was attractive to females, this fraction was then tested against an equivalent amount of crude extract.

Mixtures containing synthetic enantiomers (see below) of the major male-specific compound were tested using 14-day-old sexually mature adults of both sexes. The mixture derived from (*S*)-citronellal was tested vs a solvent control, and both synthetic mixtures were tested vs the crude extract. Finally, equal doses of the two synthetic mixtures were tested against each other. The mixtures (240 ng of each mixture of four stereoisomers, ~60 ng/isomer), in CH_2Cl_2, and solvent controls, were loaded onto filter paper discs as described above.

Gas Chromatography (GC) and Mass Spectrometry (MS) Analyses of Extracts Extracts were concentrated under a gentle stream of nitrogen, and analyzed by splitless gas chromatography with a Hewlett–Packard (H–P) 5890 GC fitted with a DB-5 column (30 m×0.32 mm ID×0.25 μm film, J&W Scientific, Folsom, CA, USA), using a temperature program of 40°C for 1 min, then 15°C min^{-1} to 250°C. The injector and detector temperatures were 250°C and 280°C, respectively, and helium was used as carrier gas. Extracts were also analyzed by GC-MS, using an HP 5890 GC, fitted with a DB5-MS column (20 m×0.2 mm ID×0.33 μm film), interfaced to an HP 5970B mass selective detector (electron impact ionization, 70 eV). The same temperature program described above was used, with injector and transfer line temperatures of 250 and 280°C, respectively. Injections (1 μl) were made in splitless mode. Previously known compounds were tentatively identified from their mass spectra, and from matches with the NBS-NIH mass spectral database. Identifications were confirmed by matching retention times and mass spectra with those of authentic standards (see below).

An aliquot of the purified male specific compound was also analyzed by chemical ionization mass spectrometry (NH_3 reagent gas) by direct insertion on a VG-7070B instrument.

Fractionation of Extracts of Volatiles Collected from Sexually Mature Male Bugs Thirty aeration extracts of

sexually mature male bugs, prepared from May 2003 to August 2005, were combined and concentrated to ~1 ml by fractional distillation of the solvent through an 8-cm long Vigreux column. The concentrated extract was transferred to a vial and the solvent removed under a gentle stream of nitrogen. The sample was reconstituted to ~250 µl with pentane, and then loaded onto a 2.8-ml solid phase extraction (SPE) cartridge (J.T. Baker silica gel SPE, stock #7086–01, precleaned by flushing with 3× 1 ml diethyl ether, then 3×1 ml pentane). The extract was rinsed onto the SPE bed with two to three drops of pentane, and eluted with 2×1 ml pentane, 2×1 ml 10% ether in pentane, and 4×1 ml ether, collecting each elution as a separate fraction.

Aliquots (100 µl) of crude extract were subjected to a series of microchemical tests to ascertain characteristics of the male-specific compound.

a) To test for the presence of a conjugated diene, an aliquot was treated with two drops of a solution of the dienophile, 4-methyl-1,2,4-triazolin-3,5-dione (MTAD; Young et al. 1990) in CH_2Cl_2 (2 mg/ml), producing a pale pink solution which was analyzed immediately by GC-MS.
b) To test for an ester function, an aliquot was concentrated just to dryness, and then treated with three drops of 1 M NaOH in ethanol to water (95:5). After standing at room temperature for 4 h, 200 µl of water were added and the mixture extracted with 500 µl pentane. The pentane extract was dried over Na_2SO_4, concentrated under a stream of nitrogen, and analyzed by GC-MS.
c) To test for an alcohol function, an aliquot was treated with one drop each of pyridine and acetyl chloride. The resulting mixture was stirred for 2 h, treated with 200 µl of saturated aqueous $NaHCO_3$, and extracted with 0.5 ml pentane. The pentane extract was concentrated and analyzed as above.
d) To test for reducible functional groups, an aliquot in diethyl ether was treated with ~1 mg of $LiAlH_4$, with stirring at room temperature for 2 h. The mixture was quenched with 1 M HCl and extracted with 0.5 ml pentane. The pentane extract was dried over Na_2SO_4, concentrated, and analyzed.
e) To test for unsaturation, an aliquot of extract and ~1 mg of 5% Pd on carbon were placed in a 1.5-ml screwcap vial with a Teflon-lined septum. The septum was punctured with a syringe needle attached to a balloon filled with hydrogen, the lid was loosened for 10 s to flush the vial with hydrogen and resealed, and the mixture stirred for 2 h. The reactant mixture was filtered through a small plug of Celite and analyzed.

NMR Analysis of the Male-Specific Compound Forty-two extracts were combined, concentrated, and fractionated as described above, and the fraction containing the male-specific was compound concentrated under a gentle stream of nitrogen, taking the solution just to dryness. Approximately 200 µl of CD_2Cl_2 were added, and the solution was concentrated just to dryness again to remove traces of non-deuterated solvent. The residue (estimated at >100 µg) was then taken up in CD_2Cl_2 and transferred to a 3-mm NMR tube. ^{13}C, ^{1}H, and ^{1}H-^{1}H COSY spectra were obtained with both a Varian INOVA-400 and a Brucker 600 MHz NMR spectrometer; the latter instrument was also used to obtain NOESY, ^{13}C-DEPT 135, HMBC, and HSQC spectra.

Chemicals Cineole, limonene, myrcene, dodecane, and dimethyldisulfide were purchased from Aldrich Chemical Co. (Milwaukee, WI, USA). Dimethyltrisulfide was obtained from Alfrebro (Monroe, OH, USA). The bisabolene epoxide pheromones of *Acrosternum hilare* were available from previous studies (McBrien et al. 2001). The sex-specific compound produced by male harlequin bugs was synthesized as described below.

For the syntheses, unless stated otherwise, all reactions were carried out under argon. Tetrahydrofuran (THF) used in the synthesis was distilled from sodium/benzophenone ketyl under argon. ^{1}H- and ^{13}C-NMR spectra were taken as $CDCl_3$ or CD_2Cl_2 solutions, recorded on a Varian INOVA-400 (400 and 100 MHz, respectively) spectrometer. Chemical shifts are expressed in parts per million (ppm) (δ-scale) relative to $CDCl_3$ (7.26 and 77.23 ppm) and CD_2Cl_2 (5.32 and 54.00 ppm) signals for ^{1}H and ^{13}C NMR, respectively. Routine GC analyses were performed with an HP 5890 series II GC, equipped with a DB-5 column (30 m×0.25 µm ID, 0.25 µm film thickness, J&W Scientific, programmed from 100°C for 1 min, then at 15° C min^{-1} to 250°C) or a DB-WAX column (30 m×0.25 µm ID, 0.25 µm film thickness, J&W Scientific), programmed from 40°C for 1 min, then at 5°C min^{-1} to 250°C).

*Preparation of (3S)-α-Methylene-3,7-dimethyl-6-octen-1-al (**2**)* Piperidine (5.39 g, 63.3 mmol) was added dropwise over ~5 min to a three-necked, round-bottomed flask, containing (*S*)-citronellal **1** (4.69 g, 30.4 mmol; Aldrich Chem. Co., Cat. #37375-3, 96%), attached to a condenser. The temperature was increased to 44°C and the mixture became cloudy, and then cleared again. Formaldehyde (37% aqueous solution, 7.8 ml) was added dropwise, keeping the temperature under 40°C, followed by glacial acetic acid (0.78 ml) dropwise. The mixture was stirred overnight at room temperature and then heated to 80°C for 1.5 h. The mixture was cooled to room temperature, diluted

with hexanes (80 ml), then washed sequentially with HCl (2 M in water, 2×30 ml), saturated aqueous $NaHCO_3$ (20 ml), and brine (20 ml). The organic phase was separated and dried with anhydrous Na_2SO_4 and concentrated under vacuum. The crude product was Kugelrohr distilled (64–67°C, 1.6 mm Hg), affording 4.01 g of aldehyde **2** (79.3% yield). ^{1}H NMR (400 MHz, $CDCl_3$): δ 1.07 (d, 3H, J=7.2 Hz), 1.33–1.44 (m, 1H), 1.48–1.56 (m, 1H), 1.57 (s, 3H), 1.67 (s, 3H), 1.86–2.00 (m, 2H), 2.70 (sext, 1H, J=7.0 Hz), 5.08 (br t, 1H, J=7.0 Hz), 5.99 (s, 1H), 6.23 (s, 1H), 9.53 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 17.85, 19.75, 25.89, 25.96, 31.18, 35.79, 124.34, 131.88, 133.25, 155.71, 194.89. MS (*m/z*, rel. abundance): 166 (M^+, 8), 151 (8), 137 (2), 135 (3), 133 (4), 124 (4), 123 (8), 121 (1), 110 (8), 109 (41), 107 (3), 106 (2), 105 (4), 96 (3), 95 (18), 93 (9), 91 (5), 84 (10), 83 (15), 81 (18), 69 (28), 67 (26), 56 (12), 55 (51), 53 (16), 43 (16), 41 (100).

*Preparation of 4-[(1S)-1,5-Dimethylhex-4-enyl]cyclohex-2-en-1-one (**3**)* Sodium (177 mg, 7.7 mmol) was added to a 200-ml, three-necked, round-bottomed flask containing methanol (23 ml) and attached to a condenser. After the sodium had dissolved, a solution of methyl acetoacetate (2.74 g, 23.6 mmol) in methanol (35 ml) was slowly added (~20–25 min) and the mixture was stirred 30 min at room temperature. A solution of aldehyde 2 (3.92 g, 23.6 mmol) in methanol was added dropwise over 40 min. The resulting mixture was stirred 2 h at room temperature and then refluxed for 2 h. After cooling, the methanol was removed under vacuum, the residue diluted in hexanes (100 ml), washed with saturated aqueous NH_4Cl, water and brine, dried over anhydrous Na_2SO_4, and concentrated under vacuum. The crude product was purified by vacuum flash chromatography on silica gel, eluting with hexane/ethyl acetate (97:3). The resulting product was purified further by Kugelrohr distillation (112–117°C, 0.35 mm Hg), affording 2.19 g of cyclohexenone **3** (45.1% yield) as a ~1:1 mixture (estimated from ^{1}H and ^{13}C NMR spectra) of inseparable diastereomers. ^{1}H NMR ($CDCl_3$): δ 0.90 (d, 1.5H, J= 6.8 Hz), 0.94 (d, 1.5H, J=7.2 Hz), 1.20–1.32 (m, 1H), 1.38–1.48 (m, 1H), 1.62 (s, 3H), 1.70 (s, 3H), 1.90–2.15 (m, 4H), 2.30–2.56 (m, 4H), 5.06–5.14 (m, 1), 6.02 (dt, 1H, J=3.2 and 10.2 Hz), 6.82–6.90 (m, 1H). ^{13}C NMR ($CDCl_3$): d 16.23, 16.75, 17.91, 24.21, 25.92, 26.07, 34.14, 34.34, 36.29, 36.35, 37.74, 37.93, 41.30, 41.85, 124.34, 124.37, 129.91, 130.17, 132.03, 132.04, 154.54, 155.64, 200.34. Several of these peaks corresponded to composite signals from carbons in both diastereomers. MS (*m/z*, rel. abundance) 206 (M^+, 9), 191 (4), 177 (1), 163 (5), 149 (4), 136 (6), 135 (5), 124 (5), 123 (36), 122 (30), 121 (16), 109 (13), 107 (10), 96 (18), 95 (13), 94 (16), 91 (4), 79 (16), 77 (6), 69 (61), 67 (25), 55 (31), 53 (15), 43 (10), 41 (100).

*Preparation of 4-[(1S)-1,5-Dimethylhex-4-enyl)-1-methylcyclohex-2-en-1-ol (**4**)* A solution of cyclohexenone **3** (2.18 g, 10.6 mmol) in diethyl ether (20 ml) was cooled to −78°C, and methyl lithium (26.4 ml of a 1.2-M solution in hexanes, 31.7 mmol) added dropwise. The resulting mixture was stirred 4 h, warmed to room temperature, and stirred overnight. The reaction was quenched with dilute aqueous NH_4Cl and extracted with ethyl acetate (4×20 ml). The combined organic phase was washed with brine, dried over anhydrous Na_2SO_4, and concentrated by rotary evaporation. The residue was purified by flash chromatography on silica gel, eluting with 5% ethyl acetate in hexane, yielding alcohols **4** in two fractions (less polar fraction, higher Rf, 0.681 g; more polar fraction, lower Rf, 1.272 g; 83.1% yield). Each fraction consisted of mixtures of two diastereomers that were inseparable by flash chromatography; each mixture eluted as a single peak on a DB-5 GC column. Less polar diastereomers: ^{1}H NMR ($CDCl_3$): δ 0.84 (d, 1.5H, J=7.2 Hz), 0.89 (d, 1.5H, J=6.8 Hz), 1.28 (s, 3H), 1.61 (s, 3H), 1.69 (s, 3H), 1.14–1.60 (m, 7H), 1.80–2.08 (m, 4H), 5.07–5.14 (m, 1H), 5.59–5.70 (m, 2H). MS (*m/z*, rel. abundance): 222 (M^+, 3), 207 (10), 204 (M^+-18, 7), 189 (5), 179 (2), 161 (8), 151 (4), 148 (6), 147 (4), 138 (12), 137 (17), 133 (7), 123 (15), 121 (12), 119 (39), 109 (22), 108 (4), 107 (10), 105 (9), 95 (23), 94 (22), 93 (24), 91 (12), 82 (12), 81 (13), 79 (19), 77 (14), 69 (91), 67 (25), 55 (37), 53 (15), 43 (80), 41 (100). More polar diastereomers: ^{1}H NMR: ($CDCl_3$): δ 0.82 (d, 1.5H, J=6.4 Hz), 0.85 (d, 1.5H, J=7.2 Hz), 1.10–1.22 (m, 1H), 1.27 (s, 3H), 1.32–1.55 (m, 4H), 1.60 (s, 3H), 1.68 (s, 3H), 1.60–1.75 (m, 2H), 1.83–2.14 (m, 4H), 5.06–5.13 (m, 1H), 5.48–5.65 (m, 2H). MS (*m/z*, rel. abundance): 222 (M^+, 2), 207 (11), 204 (M^+-18, 10), 189 (3), 179 (2), 161 (8), 151 (4), 148 (3), 147 (3), 138 (14), 137 (20), 133 (6), 123 (20), 121 (14), 119 (38), 109 (19), 108 (4), 107 (10), 105 (9), 95 (23), 94 (11), 93 (23), 91 (12), 82 (13), 81 (13), 79 (14), 77 (14), 69 (86), 67 (24), 55 (37), 53 (15), 43 (83), 41 (100).

*Preparation of 4-[3S-(3,3-Dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol ((S)-murgantiol—**5**)* *m*-Chloroperbenzoic acid (ca. 70%, 0.333 g, ~1.35 mmol) was added in small portions over 30 min to a well-stirred suspension of the less polar, higher Rf, fraction of alcohols **4** (0.300 g, 1.35 mmol) in an aqueous solution of $NaHCO_3$ (0.5 M, 10 ml) in an ice-bath (Fringuelli et al. 1992). The mixture was stirred 3 h at 0°C, then brine (50 ml) added, and the mixture was extracted with hexanes (4×20 ml). The combined organic phases were dried with anhydrous Na_2SO_4 and concentrated by rotary evaporation. The residue was purified by vacuum flash chromatography (hexanes/ethyl acetate 5:1) to yield 0.240 g of epoxyalcohols **5** (74.5%) as an inseparable mixture of diastereomers. ^{1}H NMR (400 MHz, CD_2Cl_2): δ 0.86 (d, 1.5H, J=6.8 Hz),

0.90 (d, 1.5H, J=6.0 Hz), 1.24 (S, 6H), 1.27 (S, 3H), 1.35–1.70 (m, 9H), 1.76–1.87 (m, 1H), 1.99–2.10 (M, 1H), 2.63–2.70 (M, 1H), 5.58–5.70 (m, 2H). The ^{13}C spectrum was complex because of the mixture of stereoisomers, but contained peaks that matched those of insect-produced murgantiol. MS (m/z, rel. abundance) 205 (2), 165 (9), 147 (6), 138 (10), 135 (4), 134 (13), 132 (10), 123 (10), 122 (4), 121 (12), 119 (11), 109 (15), 107 (9), 106 (6), 105 (9), 95 (15), 94 (19), 93 (17), 91 (11), 81 (15), 79 (18), 77 (11), 71 (27), 69 (12), 67 (15), 59 (20), 55 (21), 43 (100), 41 (47).

*Preparation of 4-[3R-(3,3-Dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol ((R)-murgantiol—**5**)* An analogous mixture of diastereomers containing the other enantiomer of murgantiol was prepared in identical fashion, and in similar yield, using (*R*)-citronellal (Aldrich Chem. Co, technical grade, 90%) as the starting material.

Results

Y-tube Bioassays with Odors from Live Insects, or Insect Extracts Sexually mature females were significantly attracted to odors from live males in Y-tube bioassays (Fig. 1). Males were also significantly attracted to odors from other males. In contrast, odors from live females were not attractive to either sex (Fig. 1).

Crude extracts of male-produced odors were significantly more attractive to female *M. histrionica* than a solvent control (Fig. 2). Fractionation of the extract and bioassay of the fractions showed that the fraction containing the male-specific component (eluted from the silica gel cartridge with the first aliquot of 100% ether) was attractive to females (Fig. 2), whereas the recombination of the remaining seven fractions appeared to be repellent (Fig. 2). In a follow-up bioassay, the active fraction was as attractive as

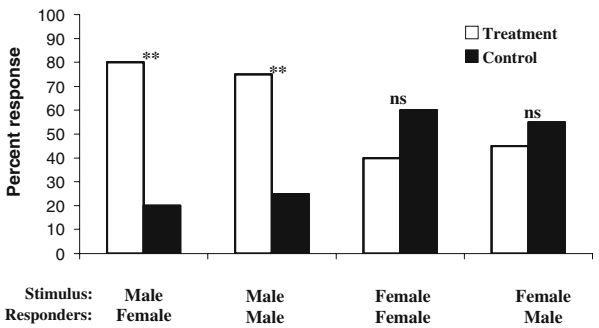


Fig. 1 Results of vertical Y-tube bioassays that tested attraction of sexually mature virgin *Murgantia histrionica* to odors from live, sexually mature virgin conspecifics on a cauliflower floret vs odors from a cauliflower floret control (N=24). Data were analyzed with χ^2 tests. **P<0.001; *ns* not significant, P>0.05

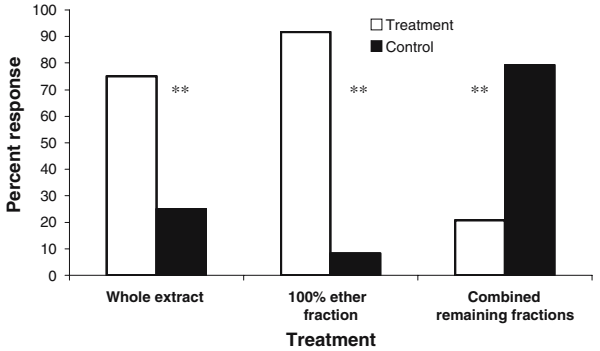


Fig. 2 Results of vertical Y-tube bioassays testing attraction of sexually mature, virgin female *Murgantia histrionica* to fractions of an extract of odors from *M. histrionica* males vs a solvent control (N=24). Each experiment was analyzed with χ^2 test. **P<0.001

the crude extract (N=24, χ^2=0.22, P>0.05), suggesting that the fraction containing the male-specific compound, and possibly the compound itself, may be responsible for all the attractiveness of the extract.

Production of the Male-Specific Compound by Individual Males or Males in Groups Males that were aerated individually, or in groups of only five, produced more murgantiol per bug than males aerated in groups of ten or more (Fig. 3). Individual bugs and bugs aerated in groups of five produced the male-specific compound at similar rates (Games–Howell test, P=0.41; Games and Howell 1976).

Diurnal Cycle of Pheromone Production The production of the male-specific compound followed a defined diurnal cycle (Fig. 4), with production increasing from midmorning and peaking in the early afternoon.

Analysis of Extracts of Odors from Adult Bugs Typical gas chromatograms of extracts of odors from groups of 10–15 sexually mature, virgin adult bugs of both sexes are shown in Fig. 5. Several compounds appeared in extracts from both females and males, including dodecane, dimethyldisulfide, dimethyltrisulfide, *S*-methylmethanethiosulfonate, myrcene, limonene, and cineole. With the exception of *S*-methylmethanethiosulfonate, for which a standard was not available, the identifications of these compounds were verified by comparison of their retention times and mass spectra with those of authentic standards. Myrcene, limonene, and cineole were also found in cauliflower odor controls, whereas the sulfur-containing compounds were only seen in the insect odor extracts.

Comparison of extracts collected from sexually mature virgin males and females (>14 day old) by gas chromatography (Fig. 5) revealed the presence of two male-specific compounds. The first was identified by retention time and

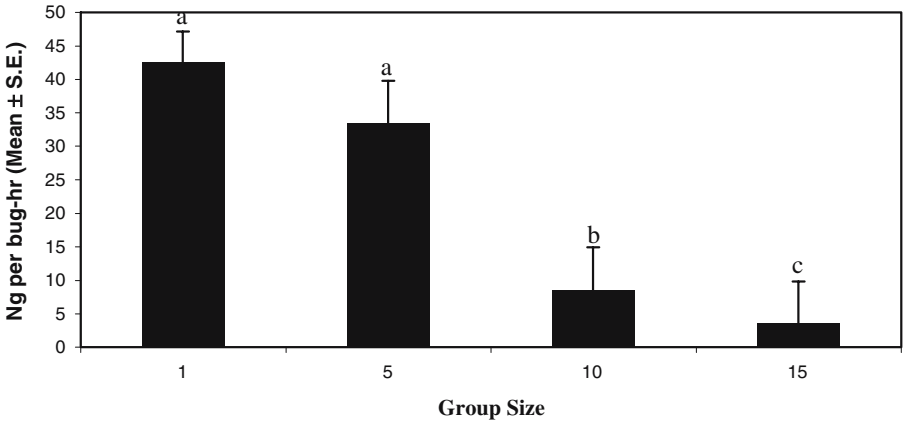


Fig. 3 Production of the male-specific compound by groups of different numbers of *Murgantia histrionica* virgin males. One-way ANOVA for group size, $F=11.48$, $df=3$, $P<0.001$. Bars surmounted by the same letter are not significantly different (Games–Howell test, $P<0.05$)

mass spectral matches as tridecane, a common component of stink bug odors (e.g., Ho and Millar 2001a, b). The second compound (Kovats index, KI, 1743 on DB-5 column; Kovats 1965) was not found in aerations of sexually immature males, nor was it a component of the frothy defensive emissions (2-*sec*-butyl-3-methoxypyrazine and 2-isopropyl-3-methoxy-pyrazine; (Aldrich et al. 1996)) or one of the two components reported from the metathoracic glands of male bugs ((2*E*,6*E*)-octadienedial and (2*E*,6*E*)-octadien-1,8-diol diacetate; Aldrich et al. 1996). The spectrum also gave no meaningful matches with any literature spectra in the NBS-NIH mass spectral database.

Electron impact ionization GC-MS analysis (70 eV) of the compound gave an apparent molecular ion at $m/z=220$, with an ion at $m/z=202$ probably due to loss of water (Fig. 6), for a possible molecular formula of $C_{15}H_{24}O$. The base peak at m/z 93 suggested a methylcyclohexadiene fragment. The spectrum bore some similarity to the mass spectra of sesquiterpenoids, and the overall complexity of the spectrum suggested that the compound was not aromatic. Based upon comparison of the retention indices of known compounds with the KI for the *Murgantia* compound, it seemed unlikely that the molecular weight was actually 220 amu. For example, the bisabolene epoxide pheromone components ($m/z=220$) of *Nezara viridula* and *Acrosternum hilare* had retention indices on a DB-5 GC column of 1609 (*trans* isomer) and 1616 (*cis* isomer) vs the *Murgantia* compound with KI 1743. Thus, we suspected that the loss of 18 amu from $m/z=220$ to give an ion at $m/z=202$ in the 70 eV EI mass spectrum of the *Murgantia* compound was not the first loss of water but the second, and that the actual molecular ion at m/z 238, corresponding to a molecular formula of $C_{15}H_{26}O_2$, was too unstable to be seen under EI-MS conditions. This molecular formula requires three rings or sites of unsaturation. A molecular ion at m/z 238 also was not observed with chemical ionization mass spectrometry (NH_3 reagent gas) using a direct insertion probe.

Microchemical tests provided further information. The compound did not react with the dienophile 4-methyl-1,2,4-triazoline-3,5-dione, ruling out the presence of a conjugated diene. The compound was not changed by treatment with NaOH in aqueous alcohol (ruling out an ester), by acetyl chloride and pyridine in ether (ruling out primary and

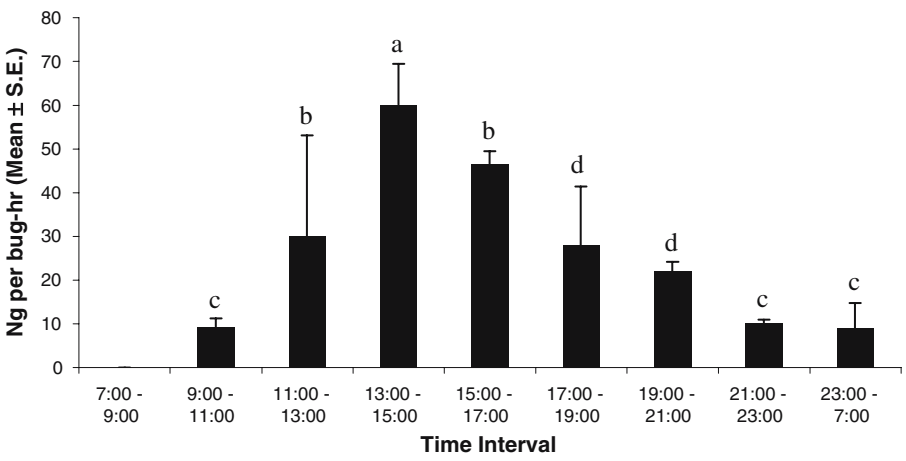


Fig. 4 Diurnal rhythm of release of the male-specific compound by *Murgantia histrionica* males ($N=5$ cohorts). Two-way ANOVA for cohort effect, $F=3.08$, $df=4, 39$, $P=.03$; for time interval effect $F=107.1$, $df=7, 39$, $P<0.001$. Bars surmounted by the same letter are not significantly different (Student–Newman–Keuls test, $P<0.05$)

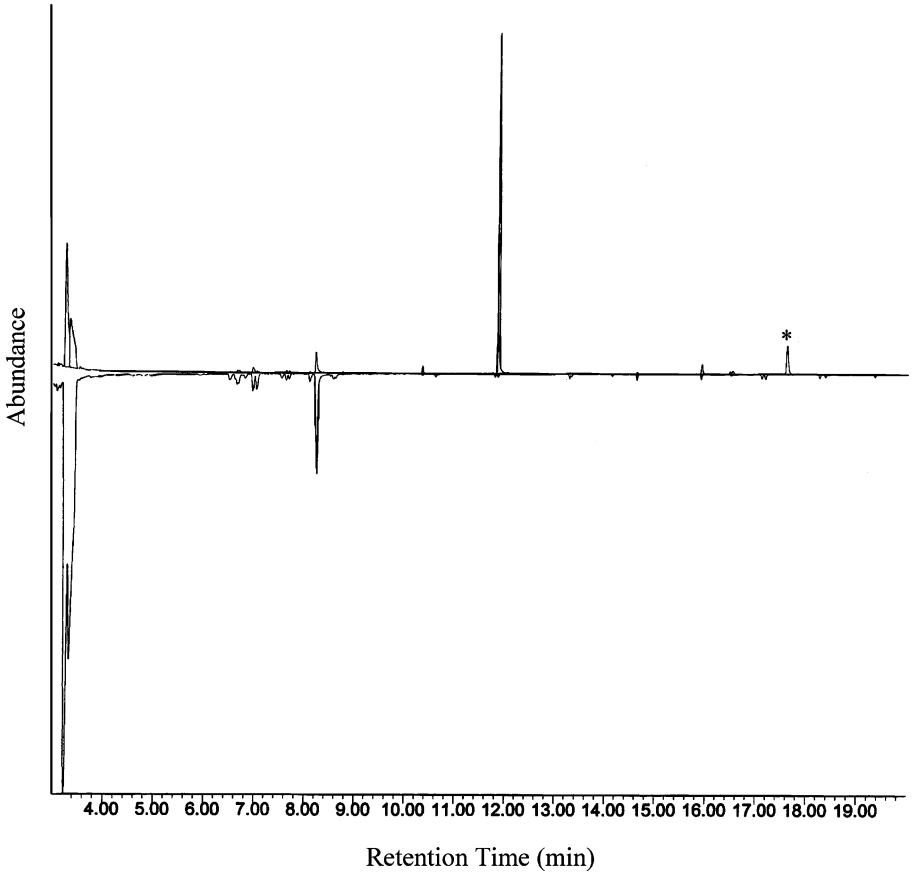


Fig. 5 Representative gas chromatograms of crude extracts (males on top, females on bottom) from sexually mature adult *Murgantia histrionica*. Murgantiol is indicated with an *asterisk*. The large peak in the male extract is tridecane

secondary alcohols), or by treatment with $LiAlH_4$ at room temperature (ruling out aldehydes, ketones, or esters). This suggested that the two postulated oxygen functionalities were probably present as ethers or tertiary alcohols. The presence of at least one polar functional group was suggested by fractionation of the crude extracts on silica gel: the compound appeared in the first fraction eluted with 100% diethyl ether.

Reduction of the compound by catalytic hydrogenation gave a mixture of compounds with apparent molecular

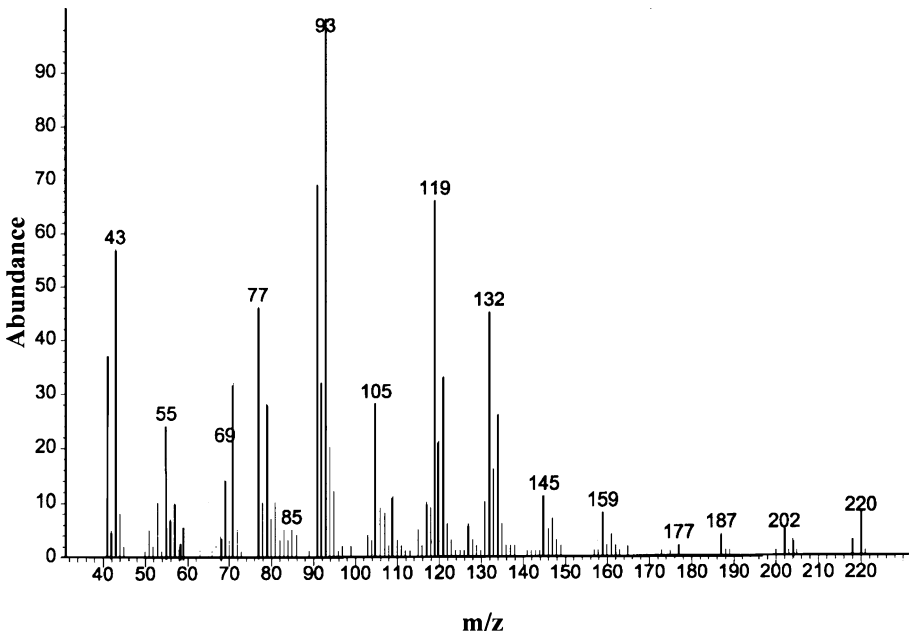


Fig. 6 Electron impact ionization (70 eV) mass spectrum of the *Murgantia histrionica* male-specific compound (murgantiol)

weights of 224 or 226 by EI GC-MS, corresponding to loss of water from the actual molecular ions, indicative of two or three double bonds or other functionalities susceptible to catalytic hydrogenation. Most of these derivatives had a base peak or large fragment at *m*/*z* 95 or 97, suggestive of a methylcyclohexane substructure, such as that found in bisabolane-type sesquiterpenoids. Furthermore, males of several stinkbug species are known to produce pheromones with bisabolene skeletons, such as the bisabolene epoxides from *Nezara viridula* (Aldrich et al. 1987; Baker et al. 1987) and *Acrosternum hilare* (McBrien et al. 2001), zingiberene and sesquiphellandrene from *Thyanta* spp. (McBrien et al. 2002), and zingiberenol from *Tibraca limbativentris* (Borges et al. 2006). In total, the evidence supported a bisabolene-type sesquiterpenoid structure, with two oxygens present as ethers or tertiary alcohols.

With this fragmentary information about the possible structure of the unknown, crude aeration extracts from multiple cohorts of virgin males were combined to obtain sufficient material (>100 μg) for NMR analysis, and fractionated by liquid chromatography. The fraction enriched in the male-specific compound was concentrated, and taken up in CD_2Cl_2 rather than the usual $CDCl_3$ solvent to avoid possible decomposition of the sample by the traces of DCl commonly found in $CDCl_3$.

The ^{13}C spectrum showed the expected fifteen carbons (Table 1). The three carbons with chemical shifts between δ 58.5 and 67.6 ppm were in the correct position for carbons with an attached oxygen, indicating that one of the two oxygens was bonded to two carbons as a straight-chain or cyclic ether. The two carbons at 134.2 and 134.3 ppm were in the correct range for alkene carbons, indicative of a single C–C double bond and, by default, indicating that there were two rings in the structure. The ten other carbons ranging from 16.1 to 41.2 ppm were within the expected range for aliphatic carbons with no directly attached heteroatoms (Table 1).

A DEPT experiment, in combination with HSQC data (see below) confirmed that there were four methyl carbons (δ 16.1, 19.0, 25.2, and 30.1), four methylene carbons (δ 20.7, 27.7, 31.3 and 37.8), five methine carbons (δ 37.1, 41.2, 64.9, 134.2, and 134.3), and two quaternary carbons (δ 58.5 and 67.6) (Table 1). The fact that both alkene carbons were methines showed that the single alkene present was 1,2-disubstituted. Combining the information from the ^{13}C and DEPT experiments suggested the presence of a tertiary alcohol (67.6 ppm) and a trisubstituted epoxide (δ 64.9 and 58.5), accounting for all three carbons bonded to oxygens and one ring. It was noteworthy that treatment with $LiAlH_4$ during the microchemical tests did not open

Table 1 Summary of ^{13}C and ^{1}H NMR spectral data from the male-specific compound from *Murgantia histrionica*

Carbon Number	^{13}C (ppm)	^{1}H, ppm (*J*)	^{1}H-^{1}H COSY	HMBC Correlations	NOESY Correlations
1	41.2 (CH)	2.04 (m)	H6a	C4, C6	H2, H6, H7, H14
2	134.2 (CH)	5.61 (br d, 10.2 Hz)	H3	C1, C3, C4, C6, C7	H1, H3, H14, H15
3	134.3 (CH)	5.65 (br d, 10.2 Hz)	H2	C2, C4	H2, H14, H15
4	67.6	-			
5a	37.8 (CH_2)	1.51(m)	H5b		H5b, H15
5b		1.80 (m)	H5a, H6b	C4, C6	H5a, H6, H15
6a	20.7 (CH_2)	1.46 (m)	H1		–[a]
6b		1.54 (m)			–[a]
7	37.1 (CH)	1.60 (m)	H14		H1, H8a, H8b, H14
8a	31.3 (CH_2)	(m)[b]			H7, H9, H14
8b		(m)[b]			H7, H9, H14
9a	27.7 (CH_2)	1.48 (m)			–[a]
9b		1.55 (m)			–[a]
10	64.9 (CH)	2.66 (t, 6.6 Hz)	H9a, H9b	C9	H12, H13
11	58.5	-			
12	25.2 (CH_3)	1.27 (s)		C10, C11, C13	H10, H13
13	30.1 (CH_3)	1.23 (s)		C9, C10, C11, C12	H9, H12
14	16.1 (CH_3)	0.85 (br d, 6.8 Hz)	H7	C1, C7, C8	H1, H7, H8
15	19.0 (CH_3)	1.23 (s)		C4, C5, C6	H2, H3, H5b

For the ^{13}C data, the number of protons attached to each carbon is indicated in parentheses. For the ^{1}H data, the splitting pattern and the coupling constants (*J*) in hertz are indicated in parentheses. The assignments of protons to specific carbons were confirmed with an HSQC experiment.
[a] Extensive overlap prevented conclusive assignment of signals.
[b] Four peaks instead of two were seen in the HSQC, at 1.56, 1.46, 1.35, and 1.25 ppm.

the epoxide ring, presumably due to the mild conditions used.

This also confirmed the presence of the two postulated oxygens and, with the evidence for 15 carbons, the likely molecular weight of 238 amu and molecular formula of $C_{15}H_{26}O_2$. Thus, the single remaining site of unsaturation had to be a second ring which, from the mass spectral and NMR data, was probably a methyl-substituted cyclohexene with one oxygen attached as a tertiary alcohol.

The ^1H spectrum corroborated much of the above information (Table 1). There were three, three-proton singlets at 1.23, 1.23, and 1.27 ppm, from three methyl groups attached to quaternary carbons. The fourth methyl group appeared as a doublet at 0.85 ppm, indicative of a methyl group attached to a tertiary carbon. There was a one-proton triplet at 2.66 ppm (J=6.6 Hz), consistent with a proton attached to an epoxide carbon, and a possible allylic hydrogen as a complex multiplet centered around 2.04 ppm. The alkene configuration was *Z*, based on the coupling constant (10.2 Hz) between the two broadened single-proton doublets at 5.65 and 5.61 ppm. The remaining protons were accounted for by overlapped multiplets between about 1.6 and 1.25 ppm.

The ^1H-^1H COSY spectrum indicated that the two alkene hydrogens (5.61 and 5.65 ppm) on C_2 and C_3, respectively, were coupled to each another, as expected. The possible allylic proton (2.04 ppm) on C_1 was coupled to one of the two protons on C_6 (~1.46 ppm). The single epoxide proton (2.66 ppm) on C_{10} was correlated with the two protons on C_9 in the overlapped multiplets between 1.47–1.60 ppm, and one of the two protons on C_5 (1.80 ppm) was coupled with multiple protons in the overlapped 1.47–1.60 ppm range.

Further information on connectivity through two and three bonds was obtained from the HMBC experiment (Table 1). The HMBC experiment showed correlations between the quaternary C_4 (67.6 ppm) and the alkene proton H_3 (5.65 ppm), the methyl protons at 1.23 ppm (H_{15}), and one of the protons (1.80 ppm) of the C_5 methylene carbon at 37.8 ppm, indicative of a tertiary allylic alcohol flanked by a CH_2 group. The H_5 proton at 1.80 ppm showed a further correlation to the C_6 methylene carbon at 20.7 ppm. To finish establishing the connectivity within the cyclohexene ring, the remaining olefin proton at 5.61 ppm on C_2 was correlated with the ring junction carbon C_1 at 41.2 ppm and with the methylene carbon at C_6.

The epoxide carbons at 64.9 and 58.5 ppm showed interactions with protons of two methyl groups (singlets) at 1.23 (H_{13}) and 1.26 (H_{12}) ppm, with the two methyl groups being directly bonded to the C_{11} quaternary carbon (58.5 ppm), confirming the trisubstituted epoxide fragment in the side chain. The epoxide proton H_{10} at 2.66 ppm was correlated with the C_9 methylene carbon at 27.7 ppm. The methyl protons at 0.85 ppm (H_{14}) showed correlations with C_1 (41.2 ppm), C_7 (37.1 ppm), and C_8 (31.3 ppm), establishing the rest of the connectivity in the side chain, and between the side chain and the ring junction.

The NOESY spectrum (summarized in Table 1) provided further corroboration of several parts of the structure, with correlations between the epoxide proton and the adjacent methylene protons and the C_{12} methyl, between one of the protons (1.80 ppm) on C_5 and the C_{15} methyl group, between the alkene proton H_2 at 5.61 ppm and the bridgehead proton on C_1 (2.04 ppm), and between the bridgehead proton H_1 and the C_{14} methyl.

In total, the evidence unequivocally supported the bisabolane-type skeleton shown in Fig. 7, with one double bond in the cyclohexene ring, a tertiary alcohol at position 4 of the ring, and a trisubstituted epoxide in the side chain. This structure has four chiral centers, for a total of 16 possible isomers. The gross structure was confirmed by synthesis as described below but, to date, we have not been able to assign unequivocally the relative and absolute configurations of the insect-produced compound, henceforth referred to as murgantiol.

Synthesis of Murgantiol (Scheme 1) Because it was not possible to determine the relative stereochemistry of the insect-produced murgantiol, a short synthetic route was developed that would produce all possible stereoisomers of the compound, one of which should then match the natural

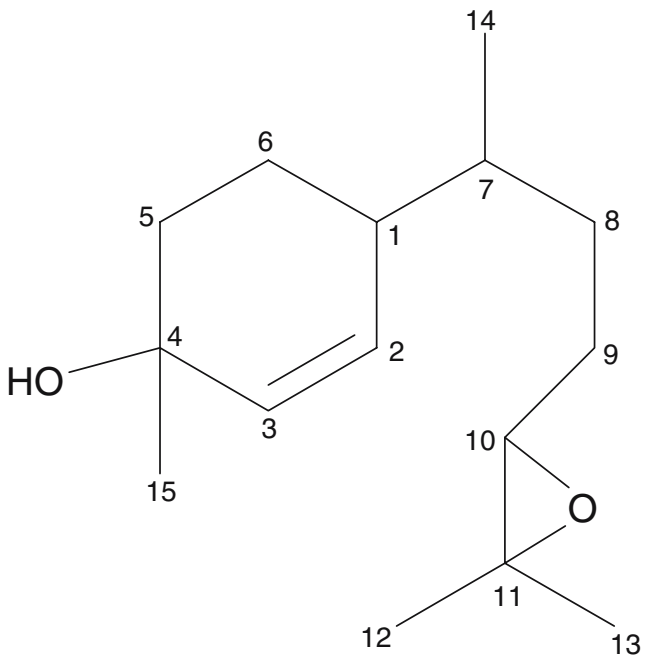
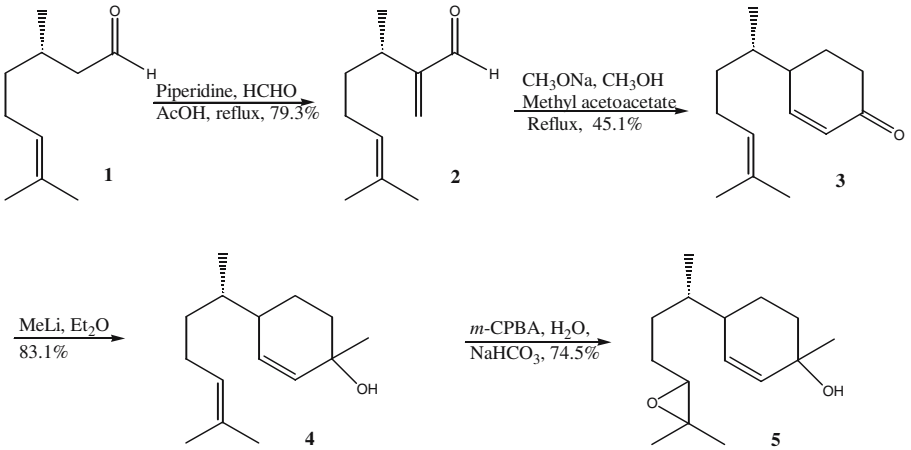


Fig. 7 Structure of murgantiol



Scheme 1 Synthesis of murgantiol as a mixture of diastereomers, from (*S*)-citronellal

compound (Scheme 1). Citronellal, available in both enantiomeric forms, was chosen as the starting material, so that one chiral center in the molecule would be known. Two parallel syntheses were carried out, using (*R*)- and (*S*)-citronellal as starting materials, respectively, to ensure that all 16 possible stereoisomers would be represented. Thus, citronellal 1 was converted into α,ß-unsaturated aldehyde 2 by α-methylenation with formaldehyde (79.3% yield) (Chavan et al. 1997). Treatment with methyl acetoacetate and sodium methoxide then produced the 4-(1,5-dimethyl-hex-4-en-1-yl)cyclohex-2-en-1-one 3, presumably by conjugate addition followed by an aldol condensation, as a mixture of diastereomers (~1:1, determined by NMR) at the ring junction (Chavan et al. 1997). The mixture was not resolved on capillary GC (DB-5). Addition of methyl lithium to enone 3 afforded a mixture (38:62 – less/more polar isomer) of diastereomers of alcohol 4 in 83.1% yield (Hagiwara et al. 2002). The diastereoisomeric mixture was separated by flash chromatography into two fractions, and both were epoxidized individually with buffered *m*-chloroperbenzoic acid. Each diastereomeric mixture thus yielded a further mixture of diastereoisomeric epoxyalcohols 5. NMR and mass spectra, and GC retention times on two columns (DB-5 and DB-Wax) of the epoxidation products 5 were compared with those of the insect-produced compound. One of the stereoisomers in the product mixture 5, from epoxidation of the less polar, higher Rf diastereomer of 4, matched the retention times of the insect-produced compound on DB-5 and DB-Wax GC columns. Comparisons of the mass, ^{1}H, and ^{13}C NMR spectra of the synthetic mixture from the less polar alcohol 4 with the natural pheromone gave good agreement, confirming the gross structure of the insect-produced compound as one of the stereoisomers of 4-[3-(3,3-dimethyloxiran-2-yl)-1-methylpropyl]-1-methylcyclohex-2-en-1-ol (5). The relative and absolute stereochemistry of the insect-produced compound remain to be determined.

Bioassays of Synthetic Murgantiol The mixtures of murgantiol diastereomers prepared from (*R*)- and (*S*)-citronellal were both attractive to female bugs in Y-tube bioassays. Thus, 21 of the 24 females tested were attracted to murgantiol derived from (*S*)-citronellal vs three females attracted to the crude extract control (χ^2 test, $P<0.001$), whereas 18 of 24 females tested were attracted to murgantiol derived from (*R*)-citronellal vs the control (χ^2 test, $P<0.014$). When the mixtures of murgantiol diastereomers derived from (*R*)- and (*S*)-citronellal were tested against one another, 17 of 24 females tested were attracted to the mixture derived from (*S*)-citronellal (χ^2 test, $P<0.04$). Similarly, when these mixtures were tested against each other with sexually mature virgin males as the responding animals, 19 of 24 males tested were attracted to the mixture derived from (*S*)-citronellal (χ^2 test, $P<0.004$).

Discussion

Both female and male bugs were attracted to odors from live males in Y-tube bioassays, indicating that mature males produce an aggregation pheromone, as previously observed for other phytophagous stink bug species including *Nezara viridula* (Harris and Todd 1980), several *Euschistus* spp. (Aldrich et al. 1991), *Biprorulus bibax* (James et al. 1994), and *Plautia stali* (Moriya and Shiga 1984; Sugie et al. 1996). In contrast, two other pentatomid species [*Thyanta pallidovirens* (Millar 1997) and *Acrosternum hilare* (McBrien et al. 2001)] have been reported to produce compounds attractive to females only.

Single males released significantly more murgantiol than males aerated in groups of ten or more, suggesting that males in groups may detect each other's presence influencing their release of pheromone. An analogous situation was recently reported for the predatory stink bug *Eocanthecona furcellata*, with single individuals producing orders of

magnitude more pheromone than groups of bugs (Ho et al. 2005). Although the full biological significance of these results has not been determined, for practical purposes, they suggest that collection of pheromone from individual bugs or small groups of bugs will be most efficient for analysis or identification of the pheromone.

The quantity of pheromone released peaked between 13:00–15:00, mirroring the peak of reproductive activity of the bugs (Zahn et al. 2007). Murgantiol was detected throughout most of the 24 h-sampling period, but the pattern of release suggested that the compound was released primarily during the photophase. The small amounts of murgantiol detected during the scotophase may have been due to the slow release of murgantiol absorbed in the insects' cuticular lipids or on the glass walls of the aeration chamber. Insects of both sexes were dissected to look for possible pheromone glands, but no obvious macroscopic male-specific glands that may be the site of pheromone production were found. Other phytophagous pentatomid species produce pheromones from sex-specific unicellular glands on the ventral surface of the abdomen (Pavis and Malosse 1986; Lucchi 1994).

The diurnal peak in pheromone production also corresponded with the general activity cycle of the insect. *M. histrionica* are aposematic, with effective chemical defenses, which protect them from predation during daylight hours (Aliabadi et al. 2002). Unlike other stink bug species that are heavily parasitized by Diptera and Hymenoptera which use the bugs' pheromone for location, no parasitoids of nymphal and adult *M. histrionica* are known, although there are several egg parasitoids (Chittenden 1920; Mitchell and Mau 1971; Ludwig and Kok 1998; Amarasekare 2000; Millar et al. 2001). For other phytophagous pentatomids, the majority of reproductive activity occurs in the late afternoon or around dusk, which allows them to evade predation or parasitization, and to avoid unfavorable temperature conditions. In particular, the risk of desiccation may limit the activity period of bug species such as *Thyanta* spp., *Nezara viridula*, and *Chlorochroa* spp., that typically feed on seeds rich in oils and proteins but with little water content (Hall and Teetes 1982; Panizzi et al. 1995; Wang and Millar 1997; Zalom et al. 1997; Ho and Millar 2001a, b; Nuessly et al. 2004; Numata 2004). In contrast, *M. histrionica* typically feeds on a wide range of plant tissues that are high in water, and thus the risk of desiccation through being active during the hottest part of the day is probably low.

Murgantiol contains four chiral centers at carbons 1, 4, 7, and 10 (Fig. 7), for a total of 16 possible stereoisomers (eight diastereomeric pairs of enantiomers). Although we have been able to match the insect-produced compound to a component in a synthetic blend of stereoisomers, we have not yet determined which isomer this is. The difficulty in trying to determine exactly which stereoisomer the bugs produce was exacerbated by our synthesis of murgantiol not being stereoselective. We separated alcohol mixture 4 into two fractions, each containing two stereoisomers, with the alcohol in either the axial or the equatorial orientation. However, after epoxidation of each of these fractions, we were unable to separate the resulting mixtures, which may contain as many as four stereoisomers, varying in the configurations of C_1 at the ring junction and C_{10} in the side chain. Similar problems have been reported in the separation of diastereomers of less functionalized, but related, sesquiterpenes such as zingiberene and its ring junction epimer (Breeden and Coates 1994). Furthermore, because the epoxide in murgantiol is several carbons removed from the other chiral centers in the molecule, diastereomers differing only in the configuration of the chiral epoxide carbon might not be expected to separate easily by chromatographic methods. It is also noteworthy that epoxidation under buffered conditions at 0°C (Fringuelli et al. 1992) was highly selective for the trisubstituted double bond in the sidechain rather than the 1,2-disubstituted double bond in the cyclohexene ring.

The exact identification of the insect-produced compound was further complicated by the HSQC spectrum showing four, rather than two, signals from the protons on C_9, as well as several very close, paired signals from carbons in the 1D ^{13}C spectrum, suggesting that the molecule may exist as more than one conformer. It seems less likely that two isomers are present because of the clarity of the ^{1}H signals in the 1D proton spectrum, and only the protons on a single carbon gave an HSQC signal that appeared anomalous.

Several interesting points emerged from the bioassays of the insect extracts and of the synthetic murgantiol mixtures. First, the insect fraction containing murgantiol was as attractive as crude extract, whereas the mixture of the remaining fractions was repellent. This suggests that the crude extract may contain defensive compounds or other repellent stimuli, the effects of which are overcome when murgantiol is present. Second, test bugs responded to the synthetic mixtures of murgantiol isomers, indicating that the bugs were not deterred by the presence of stereoisomeric impurities in the blends. Remarkably, the insects also responded to synthetic murgantiol mixtures prepared from either enantiomer of citronellal. Whether this result was an artifact of testing mixtures of stereoisomers, some of which are attractive, or indicative of a general response to all stereoisomers, will only be resolved when the pure stereoisomers are available for testing.

The structural similarity between murgantiol and a number of other sesquiterpenoid pheromones isolated from various pentatomid species is noteworthy. For example, epoxides of bisabolene constitute the male-produced pher-

omone components of *Nezara viridula* (Aldrich et al. 1987; Baker et al. 1987) and *Acrosternum hilare* (McBrien et al. 2001), and zingiberene and sesquiphellandrene form part of the pheromone blend of several *Thyanta* spp. More recently, zingiberenol 4 (the penultimate intermediate in our syntheses of murgantiol, Scheme 1), has been identified from males of the rice stalk stink bug, *Tibraca limbativentris* (Borges et al. 2006). As with murgantiol, the relative configuration of this compound, which has three chiral centers and hence eight possible stereoisomers, has yet to be determined. Thus, the bisabolane skeleton is emerging as a recurring structural motif among pheromones of stink bugs.

Although we do not know which stereoisomer of murgantiol is produced by male harlequin bugs, for practical purposes, it is convenient that the bugs respond to mixtures of the murgantiol isomers, which are relatively easy to synthesize. Thus, it should be possible to proceed to field trials with the impure "technical grade" murgantiol, to determine the potential for use of this compound in developing pheromone-based monitoring methods for this pest.

References

ALDRICH, J. R., OLIVER, J. E., LUSBY, W. R., KOCHANSKY, J. P., and LOCKWOOD, J. A. 1987. Pheromone strains of the cosmopolitan pest *Nezara viridula* (Heteroptera: Pentatomidae). *J. Exp. Zool.* 244:171–175.

ALDRICH, J. R., HOFFMANN, M. P., KOCHANSKY, J. P., LUSBY, W. R., EGER, J. E., and PAYNE, J. A. 1991. Identification and attractiveness of a major pheromone component for Nearctic *Euschistus* spp. stink bugs (Heteroptera: Pentatomidae). *Environ. Entomol.* 20:477–483.

ALDRICH, J. R., AVERY, J. W., LEE, C., GRAF, J. C., HARRISON, D. J., and BIN, F. 1996. Semiochemistry of cabbage bugs (Heteroptera: Pentatomidae: *Eurydema* and *Murgantia*). *J. Entomol. Sci.* 31:172–182.

ALIABADI, A., RENWICK, J. A., and WHITMAN, D. W. 2002. Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. *J. Chem. Ecol.* 28:1749–1762.

AMARASEKARE, P. 2000. Spatial dynamics in a host-multiparasitoid community. *J. Anim. Ecol.* 69:201–213.

BAKER, R., BORGES, M., COOKE, N. G., and HERBERT, R. H. 1987. Identification and synthesis of (*Z*)-(1′S,3′R,4′)-(-)-2-(3′,4′-epoxy-4′-methylcyclohexyl)-6-methylhepta-2,5-diene, the sex pheromone of the southern green stink bug, *Nezara viridula* (L.). *J. Chem. Soc. Chem. Commun.* 1987:414–416.

BORGES, M., BIRKETT, M., ALDRICH, J. R., OLIVER, J. E., CHIBA, M., MURATA, Y., LAUMANN, R. A., BARRIGOSSI, J. A., PICKETT, J. A., and MORAES, M. C. B. 2006. Sex attractant pheromone from the rice stalk stink bug, *Tibraca limbativentris* Stal. *J. Chem. Ecol.* 32:2749–2761.

BREEDEN, D. C., and COATES, R. M. 1994. 7-Epizingiberene, a novel bisabolane sesquiterpene from wild tomato leaves. *Tetrahedron* 50:11123–11132.

CHAVAN, S. P., DHONDGE, V. D., PATIL, S. S., RAO, Y. T. S., and GOVANDE, C. A. 1997. Enantiospecific total synthesis of (+)-laevigatin. *Tetrahedron: Asymmetry* 8:2517–2518.

CHITTENDEN, F. H. 1920. Harlequin cabbage bug and its control. *USDA Farmers Bulletin* 1061:1–13.

ČOKL, A., and DOBERLET, M. V. 2003. Communication with substrate-borne signals in small plant-dwelling insects. *Annu. Rev. Entomol.* 48:29–50.

ČOKL, A., PRESERN, J., VIRANT-DOBERLET, M., BAGWELL, G. J., and MILLAR, J. G. 2004. Vibratory signals of the harlequin bug and their transmission through plants. *Physiol. Entomol.* 29:372–380.

FRINGUELLI, F., GERMANI, R., PIZZO, F., SANTIELLI, F., and SAVELLI, G. 1992. Regioselective epoxidation of allylic alcohols with monoperoxyphthallic acid in water. *J. Org. Chem.* 57:1198–1202.

GAMES, P. A., and HOWELL, J. F. 1976. Pairwise multiple comparison procedure with unequal n's and/or variances: A Monte Carlo Study. *J. Educ. Stat.* 2:113–125.

HAGIWARA, H., OKABE, T., ONO, H., KAMAT, V. P., HOSHI, T., SUZUKI, T., and ANDO, M. 2002. Total synthesis of bisabolane sesquiterpenoids, α-bisabol-1-one, curcumene, curcuphenol, and elvirol: utility of catalytic enamine reaction in cyclohexenone synthesis. *J. Chem. Soc., Perkin Trans.* 1:895–900.

HALL, D. G., and TEETES, G. L. 1982. Yield loss-density relationships of 4 Species of panicle-feeding bugs, *Oebalus pugnax* F., *Nezara viridula* L., *Chlorochroa ligata* Say (Heteroptera: Pentatomidae), and *Leptoglossus phyllopus* L. (Heteroptera, Coreidae) in sorghum. *Environ. Entomol.* 11:738–741.

HARRIS, V. E., and TODD, J. E. 1980. Male-mediated aggregation of male, female and 5th-instar southern green stink bugs and concomitant attraction of a tachinid parasite, Trichopoda pennipes. *Entomol. Exp. Appl.* 27:117–126.

HO, H. Y., and MILLAR, J. G. 2001a. Identification and synthesis of a male-produced sex pheromone from the stink bug *Chlorochroa sayi*. *J. Chem. Ecol.* 27:1177–1201.

HO, H. Y., and MILLAR, J. G. 2001b. Identification and synthesis of male-produced sex pheromone components of the stink bugs *Chlorochroa ligata* and *Chlorochroa uhleri*. *J. Chem. Ecol.* 27:2067–2095.

HO, H., HSU, Y., CHUANG, Y., and CHOW, Y. 2005. Effect of rearing conditions on production of sternal gland secretion, and identification of minor components in the sternal gland secretion of the predatory stink bug *Eocanthecona furcellata*. *J. Chem. Ecol.* 31:29–37.

JAMES, D. G., MORI, K., ALDRICH, J. R., and OLIVER, J. E. 1994. Flight-mediated attraction of *Biprorulus bibax* Breddin (Hemiptera: Pentatomidae) to natural and synthetic aggregation pheromone. *J. Chem. Ecol.* 20:71–80.

KOVATS, E. 1965. Gas chromatographic comparison of organic substances in the retention index system. *Adv. Chromatogr.* 1:229–247.

LUCCHI, A. 1994. The scent gland system of *Nezara viridula* (L.) (Heteroptera: Pentatomidae). III. Male ventral abdominal glands. *Redia* 77:1–10.

LUDWIG, S. W., and KOK, L. T. 1998. Phenology and parasitism of harlequin bugs, *Murgantia histrionica* (Hahn) (Hemiptera: Pentatomidae), in Southwest Virginia. *J. Entomol. Sci.* 33:33–39.

MCBRIEN, H. L., and MILLAR, J. G. 1999. Phytophagous bugs, pp. 277–304, in J. Hardie, and A. K. Minks (eds.). Pheromones of non-lepidopteran insects associated with agricultural crops. CABI Publishing, London, UK.

MCBRIEN, H. L., MILLAR, J. G., GOTTLIEB, L., CHEN, X., and RICE, R. E. 2001. Male-produced sex attractant pheromone of the green stink bug, *Acrosternum hilare* (Say). *J. Chem. Ecol.* 27:1821–1839.

MCBRIEN, H. L., MILLAR, J. G., RICE, R. E., MCELFRESH, J. S., CULLEN, E., and ZALOM, F. G. 2002. Sex attractant pheromone of the red-shouldered stink bug *Thyanta pallidovirens*: A pheromone blend with multiple redundant components. *J. Chem. Ecol.* 28:1797–1818.

MCPHERSON, J. E. 1982. The Pentatomoidea (Hemiptera) of northeastern North America with emphasis on the fauna of Illinois, pp. 1–240, The Pentatomoidea. University Press, Carbondale, IL, USA.

MILLAR, J. G. 1997. Methyl (2*E*,4*Z*,6*Z*)-deca-2,4,6-trienoate, a thermally unstable, sex-specific compound from the stink bug *Thyanta pallidovirens*. *Tetrahedron Lett.* 38:7971–7972.

MILLAR, J. G. 2005. Pheromones of true bugs, pp. 37–84, in S. Schulz (ed.). Topics in Current Chemistry 240. The Chemistry of Pheromones and Other Semiochemicals. Springer, Berlin.

MILLAR, J. G., and SIMS, J. J. 1998. Preparation, cleanup, and preliminary fractionation of extracts, pp. 1–37, in J. G. Millar, and K. F. Haynes (eds.). Methods in Chemical Ecology. Chapman and Hall, NY.

MILLAR, J. G., RICE, R. E., STEFFAN, S. A., DAANE, K. M., CULLEN, E., and ZALOM, F. G. 2001. Attraction of female digger wasps, *Astata occidentalis* Cresson (Hymenoptera: Sphecidae) to the sex pheromone of the stink bug *Thyanta pallidovirens* (Hemiptera: Pentatomidae). *Pan-Pac. Entomol.* 77:244–248.

MITCHELL, W. C., and MAU, R. F. L. 1971. Response of female southern green stink bug Hemiptera-Pentatomidae and its parasite, *Trichopoda pennipes*, to male stink bug pheromones. *J. Econ. Entomol.* 64:856–859.

MORIYA, S., and SHIGA, M. 1984. Attraction of the male brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae) for males and females of the same species. *Appl. Entomol. Zool.* 19:317–322.

NUESSLY, G. S., HENTZ, M. G., BEIRIGER, R., and SCULLY, B. T. 2004. Insects associated with faba bean, *Vicia faba* (Fabales: Fabaceae), in southern Florida. *Fla. Entomol.* 87:204–211.

NUMATA, H. 2004. Environmental factors that determine the seasonal onset and termination of reproduction in seed-sucking bugs (Heteroptera) in Japan. *Appl. Entomol. Zool.* 39:565–573.

PANIZZI, A. R., NIVA, C. C., and HIROSE, E. 1995. Feeding preference by stink bugs (Heteroptera: Pentatomidae) for seeds within soybean pods. *J. Entomol. Sci.* 30:333–341.

PAVIS, C., and MALOSSE, C. 1986. A sex pheromone produced by mature males in the southern green stink bug, *Nezara viridula* (L.). *C. R. Acad. Sci. Ser. 3 Sci. Vie* 303:273–276.

SUGIE, H., YOSHIDA, M., KAWASAKI, K., NOGUCHI, H., MORIYA, S., TAKAGI, K., FUKUDA, H., FUJIIE, A., YAMANAKA, M., OHIRA, Y., TSUTSUMI, T., TSUDA, K., FUKUMOTO, K., YAMASHITA, M., and SUZUKI, H. 1996. Identification of the aggregation pheromone of the brown-winged green bug, *Plautia stali* Scott (Heteroptera: Pentatomidae). *Appl. Entomol. Zool.* 31:427–431.

WANG, Q., and MILLAR, J. G. 1997. Reproductive behavior of *Thyanta pallidovirens* (Heteroptera: Pentatomidae). *Ann. Entomol. Soc. Am.* 90:380–388.

YOUNG, D. C., VOUROS, P., and HOLICH, M. F. 1990. Gas chromatography-mass spectrometry of conjugated dienes by derivatization with 4-methyl-1,2,4-triazoline-3,5-dione. *J. Chromatogr.* 522:295–302.

ZAHN, D. K., GIRLING, R. D., MCELFRESH, J. S., CARDÉ, R. T., and MILLAR, J. G. 2007. Biology and reproductive behavior of *Murgantia histrionica* (Heteroptera: Pentatomidae). *Ann. Ent. Soc. Am*., in press.

ZALOM, F. G., SMILANICK, J. M., and EHLER, L. E. 1997. Fruit damage by stink bugs (Hemiptera: Pentatomidae) in bush-type tomatoes. *J. Econ. Entomol.* 90:1300–1306.

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Individual and Geographic Variation of Skin Alkaloids in Three Species of Madagascan Poison Frogs (*Mantella*)

John W. Daly · H. Martin Garraffo · Thomas F. Spande · Lesley-Ann Giddings · Ralph A. Saporito · David R. Vieites · Miguel Vences

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Abstract Alkaloid profiles for 81 individual mantellid frogs, *Mantella baroni* (Boulenger 1988) (*N*=19), *M. bernhardi* (*N*=51), and *M. madagascariensis* (Grandidier 1877) (*N*=11), from six different populations from Madagascar were examined. Marked individual differences in alkaloid composition (number, type, and amount) were observed between different species and between populations of the same species. Disjunct populations of each of the three species differed significantly in alkaloid composition. Sympatric populations of *M. baroni* and *M. madagascariensis* also differed significantly in alkaloid composition. In *M. bernhardi*, differences in alkaloid composition were marginally associated with different sexes. A total of 111 alkaloids, including isomers, were detected in analysis of the individuals from the three species. The majority (47%) appear likely to be obtained from dietary mites, whereas many of the others (18%) are presumed to be from ants, and a few (4%) are from millipedes. Putative dietary sources for the remaining alkaloids are generally unknown, but beetles are probably the source of at least some of the tricyclic alkaloids (6%). In addition, alkaloid compositions from extracts of groups of individuals from five additional populations of *M. baroni* and from one population of *M. bernhardi* (Vences et al. 1994) and one population of *M. cowanii* (Boulenger 1882) were examined. An additional 50 alkaloids, including isomers, were detected in the combined samples, bringing the total number of alkaloids identified from these four species of mantellid frogs to 161. Alkaloid compositions in mantellid poison frogs are diverse and highly dependent on geographic location that appear to be largely determined by the nature and availability of alkaloid-containing prey items.

Keywords Alkaloids · Ants · Chemical defense · Chemical sequestration · Mantellid frogs · Mites · Trophic relationships · Vertebrate

Abbreviations

ANOSIM	analysis of similarity
FAME	fatty acid methyl ester
GC–MS	gas chromatography–mass spectrometry
3,5-I; 5,8-I	disubstituted indolizidine
5,6,8-I	trisubstituted indolizidine
nMDS	nonmetric multidimensional scaling
PTX	pumiliotoxin
aPTX	allopumiliotoxin
hPTX	homopumiliotoxin

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J. W. Daly (✉) · H. M. Garraffo · T. F. Spande · L.-A. Giddings
Laboratory of Bioorganic Chemistry, NIDDK, NIH, HHS,
Bethesda, MD 20892, USA
e-mail: jdaly@nih.gov

R. A. Saporito
Department of Biological Sciences,
Florida International University,
Miami, FL 33199, USA

D. R. Vieites
Museum of Vertebrate Zoology and Department of Integrative Biology, University of California,
Berkeley, CA 94720-3160, USA

M. Vences (✉)
Division of Evolutionary Biology, Zoological Institute,
Technical University of Braunschweig,
Spielmannstrasse 8,
38106 Braunschweig, Germany
e-mail: m.vences@tu-bs.de

3,5-P	3,5-disubstituted pyrrolizidine
1,4-Q	1,4-disubstituted quinolizidine
SVL	snout-to-vent length
Spiro	spiropyrrolizidine
Tri	tricyclic alkaloid
ZCMV	zoological collection Miguel Vences

Introduction

The wide variety of lipophilic alkaloids present in skin extracts of poison frogs of the Neotropics (Dendrobatidae), subtropical South America (Bufonidae, *Melanophryniscus*), and Madagascar (Mantellidae, *Mantella*) appears to be directly sequestered from dietary arthropods (Daly et al. 1994a, b, 1997; Daly 1998). The putative arthropod sources of such alkaloids are as follows: (1) The widespread pumiliotoxins (PTXs) appear to be derived from oribatid mites (Takada et al. 2005; Saporito et al. 2006, 2007a), although two such PTX alkaloids have been reported from Panamanian formicine ants (Saporito et al. 2004). (2) The several classes of izidines with branch points in their carbon skeleton also appear likely to be derived from oribatid mites (Takada et al. 2005; Saporito et al. 2006, 2007a). However, one such izidine, a 5,8-disubstituted indolizidine, has been reported from a Madagascan myrmicine ant (Clark et al. 2005). (3) The izidines without branch points in their carbon skeleton appear to be derived from myrmicine ants, as are (4) the unbranched pyrrolidines and piperidines (Jones et al. 1999). Recently, two unbranched 3,5-disubstituted pyrrolizidines were reported from a formicine ant, and a different 3,5-disubstituted pyrrolizidine was reported from a ponerine ant (Clark et al. 2006). A 2,5-disubstituted pyrrolidine also was reported from the same species of formicine ant (Clark et al. 2006). In addition, one unbranched 3,5-disubstituted indolizidine and two unbranched pyrrolidines have been detected in oribatid mites (Saporito et al. 2007a). It seems likely that the ultimate source of alkaloids with branching in the carbon skeletons will be oribatid mites and the source of those with unbranched structures will be myrmicine ants (Saporito et al. 2007a). (5) Spiropyrrolizidine alkaloids appear to be derived from siphonotid millipedes (Saporito et al. 2003; Clark et al. 2005); however, one such alkaloid was recently reported from an oribatid mite (Saporito et al. 2007a). (6) Tricyclic alkaloids, such as precoccinelline, appear to be derived from coccinellid beetles (Daloze et al. 1995); however, precoccinelline and other tricyclic alkaloids were also recently reported from oribatid mites (Takada et al. 2005; Saporito et al. 2007a).

Variation in alkaloid composition (the number, type, and amount) within and among species has been reported for Neotropical dendrobatid poison frogs (Myers and Daly 1976; Daly et al. 1987, 1992, 2000, 2002; Myers et al. 1995; Saporito et al. 2006, 2007b), bufonid poison frogs (Garraffo et al. 1993a; Mebs et al. 2005; Daly et al. 2007), and mantellid poison frogs (Garraffo et al. 1993b; Daly et al. 1996; Clark et al. 2005, 2006). Individual variability in alkaloid composition has been reported for dendrobatids (Daly et al. 1994a; Myers et al. 1995; Saporito et al. 2006), bufonids (Mebs et al. 2005; Daly et al. 2007), and mantellids (Clark et al. 2006). The literature indicates that alkaloid compositions are strongly dependent on geographic location and that compositions change with time. Differences in habitat among locations and changes in habitat over time (succession) are likely responsible for determining the availability of alkaloid-containing prey arthropods, which is reflected as geographic and temporal variation in alkaloid composition of poison frogs (Daly et al. 1987, 1996, 2002; Saporito et al. 2006, 2007b).

This study was designed to provide further insight into the factors that are involved in alkaloid composition variability within and among populations of poison frogs of the mantellid genus *Mantella*. The poison frogs of this genus consist currently of 16 described and, probably, at least one undescribed species that are found in a variety of habitats in Madagascar (Vences et al. 1999; Glaw and Vences 2006). All of these species are small, diurnal frogs that have aposematic coloration, practice microphagy, and accumulate dietary alkaloids that act as a defense against predators (Daly et al. 1996, 1997; Vences and Kniel 1998; Vences et al. 1998; Schaefer et al. 2002). Alkaloid compositions have been reported for 11 of the species (see Garraffo et al. 1993a; Daly et al. 1996; Clark et al. 2005, 2006). In this paper, we report our findings of individual alkaloid composition for 81 individuals, comprising six populations of three different species of *Mantella* from Madagascar (*Mantella baroni* of the *M. cowanii* group, *M. madagascariensis* of the *M. madagascariensis* group, and *M. bernhardi*, the sole member of the *M. bernhardi* group [Pintak et al. 1998; Vences et al. 1999, 2004]). Individuals were sampled from two sympatric populations of *M. baroni* and *M. madagascariensis* and from two geographically distant populations of *M. bernhardi*. Profiles of the major, minor, and trace alkaloids are presented for the 81 individuals. In addition, we also report on a number of extracts from groups of *M. baroni*, *M. bernhardi*, and *M. cowanii*. Alkaloid composition for mantellid frogs was examined for possible relationships with geographic location (habitat), species, size, and sex. Geographic location (and associated habitat) appears to be the primary determinant of variation in alkaloid composition; however, differences among sympatric species were also observed.

Methods and Materials

Mantellid Frog Collections—Individual Frog Analyses A total of 81 mantellid frogs of three species were collected: *M. baroni* (*N*=19), *M. bernhardi* (*N*=51), and *M. madagascariensis* (*N*=11). Each of the three species was collected at two different locations (Fig. 1), and therefore, a total of six different populations were examined. All frogs sampled from one site were usually collected during the same day, sometimes during a time span of 2–3 days, by opportunistic searching that involved observation and removal of leaf litter and low vegetation or by precisely targeting calling males, especially in the case of *M. baroni*. All frogs were collected on relatively small plots of a maximum of 0.5 ha, often much smaller. For instance, all *M. bernhardi* from Vevembe were found within an area of 20×20 m and all *M. madagascariensis* from Ranomafana within an area of 10×10 m, in a small degraded area under *Eucalyptus* trees near primary rainforest.

Individual frogs were sexed and measured for snout-to-vent length (SVL) to the nearest 0.1 mm. Collection localities included Ranomafana, a relatively large National Park in southeastern Madagascar, which contained several specific collecting localities for *Mantella* (e.g., Ranomafanakely, Mangevo, Vatoharanana, Vohiparara; Fig. 1). Individual skin extracts reported herein to originate from Ranomafana all came from a specific collection site, locally known as "Ranomafanakely," along National Road 45 from the village of Vohiparara toward the town of Fianarantsoa. The combined skin extracts reported herein to originate from Vohiparara came from the Kidonavo Bridge. Besariaka is located far to the north of Ranomafana, whereas Manombo and Vevembe are far to the south (Fig. 1). At two sites, *M. baroni* and *M. madagascariensis* were collected in syntopy. At Ranomafana, *M. madagascariensis* was found close to the road in a tiny patch of degraded forest dominated by *Eucalyptus* spp., whereas *M. baroni* was found at a distance of less than 50 m in primary rainforest. At Besariaka, the two species were fully mixed and, apparently, were using exactly the same microhabitat.

Collection dates and the total number of frogs sampled at each location are provided in table headings. The global positioning system (GPS) coordinates and nature of each site and voucher identification numbers are tabulated in the Supplementary Information. Voucher specimens are deposited at the Zoological Museum Amsterdam, the Université d'Antananarivo, Département de Biologie Animale (UADBA), and the Zoologische Staatssammlung München.

Mantellid Frog Collections—Combined Frog Analyses In January–February, 2003, 29 mantellid frogs from three species, *M. baroni* (*N*=19), *M. bernhardi* (*N*=8), and *M.*

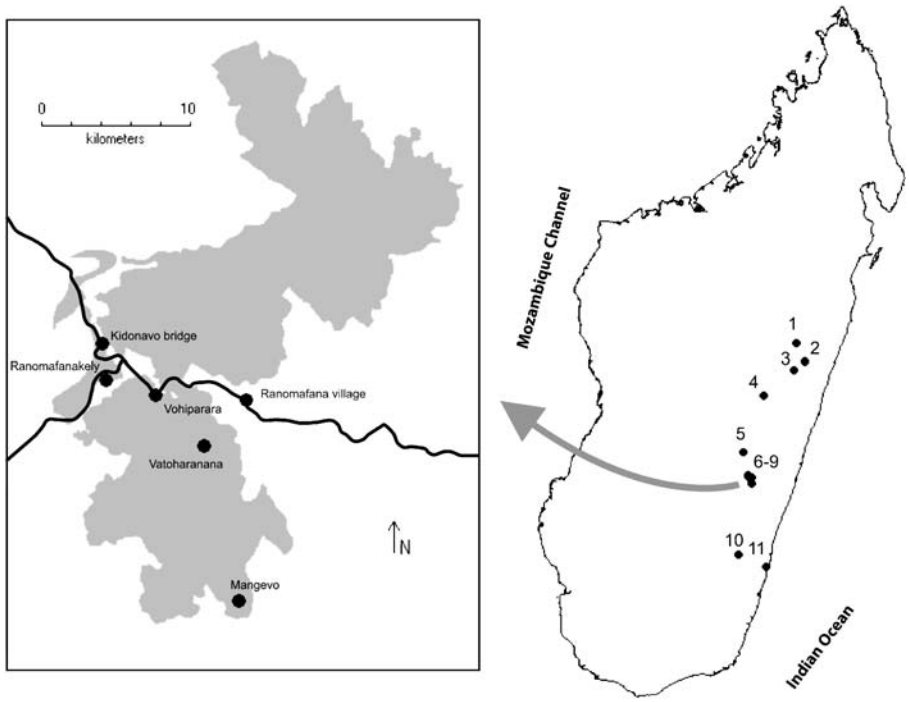

Fig. 1 Map of localities in Madagascar where *Mantella* frogs were collected including Andriabe (*1*), Vohindrazana (*2*), Besariaka (*3*), Tsinjoarivo (*4*), Antoetra (5), Vatoharanana (*6*), Vohiparara (*7*), Mangevo (*8*), Ranomafanakely (*9*), Vevembe (*10*), and Manombo (*11*). The *map on the left* shows detailed geographical location of localities 6–9. Ranomafana National Park is indicated in *gray*

cowanii (*N*=2), were also collected for extracting alkaloids from samples of pooled individuals. *M. baroni* was collected from five different populations, whereas *M. bernhardi* and *M. cowanii* were collected from one population each (Fig. 1). Individual frogs of the same species from each site were combined for alkaloid analyses, and therefore, alkaloid composition is based on combined skin samples. Collection dates and the total number of frogs sampled at each location are provided in table headings. The GPS coordinates and nature of each site are tabulated in the Supplementary Information. Voucher specimens are deposited as described above for the frogs that were extracted individually.

Spectral Analyses A Finnigan–Thermoelectron gas chromatography–mass spectrometry (GC–MS) (GCQ) was used to obtain all mass spectral data reported here. The GC was fitted with a Restek-5MS (Bellefonte, PA, USA) fused silica column (30 m×0.25 mm inside diameter [i.d.], 0.25-μm film thickness) and used a temperature program of 100 to 280°C at 10° per min with a final hold time of 5 min. The injector temperature was 280°C. The carrier (He) flow was controlled at 1 ml/min. The gas chromatography–Fourier transform infrared spectrometry (GC-FTIR) spectral analyses were obtained with an HP-5 (Hewlett-Packard, USA) fused silica-bonded capillary column (25 m× 0.32 mm i.d., ×0.17 μm film thickness) programmed from 100 to 280° at a rate of 10° per min, interfaced with an HP model 5971 Mass Selective Detector and an HP Model 5965B IRD detector (narrow band 4,000–750 cm^{-1}). An HP ChemStation was used to generate MS and FTIR spectra. For additional details of spectral analyses, see Saporito et al. 2006 and 2007b.

Individual Frog Skin Analyses Individual mantellids were examined for alkaloids by using GC–MS. In many cases, chemical ionization-mass spectrometry (ND_3) was used to confirm molecular ions and the number of exchangeable hydrogens. Alkaloids were identified by comparison of spectral and chromatographic properties to previously detected and identified poison frog alkaloids (see Supporting Information of Daly et al. 2005 for a complete listing). The single skin samples were stored in small plastic vials sealed with a silicone rubber O-ring with approximately 0.5–1.5 ml of methanol, which unfortunately led to contamination of each sample with a series of silicone polymers and dibutyl phthalate. Because of the large number of samples examined in this study and the length of time necessary for complete alkaloid partitioning (see below for combined skin analyses), we developed a fairly rapid "semi-purification" of the samples for GC–MS analysis. The method is as follows: To 50 μl of the methanolic frog skin extract, 50 μl of a 0.1 M solution of HCl in methanol was added and swirled well. Then, the methanol was immediately blown off with a stream of nitrogen gas. The HCl in methanol had been prepared by addition of acetyl chloride to methanol, to form a 1 M solution, which was then diluted 1:10 with additional methanol. After evaporation, the residue consisted of hydrochlorides of any frog skin alkaloid and nonvolatile contaminants. It was redissolved in a small volume of methanol, followed by re-evaporation. This process was repeated twice. Then, 50 μl of reagent grade octane was added. The octane was blown off with nitrogen to remove any residual methanol. Then, a small portion of octane was added, swirled with the sample residue, and removed with a pipette, thereby removing the neutral silicones, phthalates, and fatty acid methyl esters (FAMEs). This was repeated twice, and the final traces of octane were removed with a nitrogen stream leaving a whitish residue of amine hydrochlorides. The residue was dissolved in 50 μl of methanol and the vial tightly capped with a Teflon-lined APC (Alltech, Deerfield, IL, USA) cap for later alkaloid analysis. One microliter was injected for the GC–MS analysis (see above). The octane washes were occasionally combined from a group of samples and checked for any dissolved amine hydrochlorides. Traces of the lower molecular weight amine hydrochlorides were detected in some extracts, but the large majority of materials were silicone polymers, dibutyl phthalate, and FAMEs. Each sample could be prepared in less than 10 min. Only one sample was processed at a time to ensure minimum contact between the methanolic HCl and alkaloid mixtures to avoid any acid-catalyzed reactions. We cannot rule out the possibility that some GC peaks represent acid-catalyzed artifacts. To check for possible artifacts, aliquots from each set of individual methanolic skin extracts were combined, and an alkaloid fraction was obtained by the standard partitioning under mild conditions and analyzed (see below). The volumes of the skin extracts varied by a factor of two to three, and no attempt was made to apply a correction, as the weights of frog skin, while probably fairly uniform, were unknown. Thus, quantitation is an approximation, but there were large differences in the overall amounts, ranging in MS total ion currents by 10^2 (see legends to tables). Some samples had no detectable or barely detectable levels of alkaloids. Combined samples consisting of aliquots from all individuals of the same species and collection sites were also subjected to alkaloid partitioning. For example, 50 μl of each individual extract of *M. baroni* from the Ranomafana site was combined for standard alkaloid-partitioning as previously described (Daly et al. 1994a). Such combined samples, much more concentrated than the single skin samples, were used to help establish retention times and alkaloid compositions for the single skin samples as well as an aid in ruling out any

acid-catalyzed reactions potentially occurring during the individual skin protocols. Such artifacts, not detected in the partitioned alkaloid fraction, proved to be rare and minor and are not reported.

Despite the individual skin protocols having problems of not excluding completely neutrals like silicones, phthalates, and FAMEs and possibly generating artifacts arising from the inadvertent acid catalysis of unwanted reactions with methanol, there may be an advantage in retaining very volatile alkaloids by virtue of having converted them to hydrochlorides. Some extracts (no. 113 of Table 3) had low molecular weight alkaloids (e.g., **197D**, **199B**), not observed after using the standard partitioning protocol.

Combined Frog Skin Analyses An alkaloid fraction was prepared from the methanol extract of the combined skins by using the partitioning methodology as described in Daly et al. (1994a, b). The resultant alkaloid fractions were analyzed spectrally by GC–MS and, in some cases, by GC-FTIR [for details of the GC–MS and GC-FTIR spectral analysis, see Saporito et al. (2006, 2007b)].

Statistical Analyses—Individual Frogs Variation in alkaloid composition within and among mantellid populations was visualized graphically by using nonmetric multidimensional scaling (nMDS). In nMDS plots, individuals/populations that have greater similarity in alkaloid compositions will be plotted closer to each other than individuals/populations with very different alkaloid compositions [see Saporito et al. (2006, 2007b) for further examples and discussions on the use of these techniques]. Differences in alkaloid composition among these populations were analyzed with a one-way analysis of similarity (ANOSIM). Alkaloid composition is a simultaneous measure of the number, type, and amount of alkaloids, and therefore, the use of nMDS plots in association with ANOSIM provides a more biologically meaningful view of alkaloid variation in poison frogs as compared to individual analyses of the number and amount of alkaloids. Differences in alkaloid composition between sexes for *M. bernhardi* from Mandombo were also visualized by using nMDS, and differences were analyzed with a one-way ANOSIM. All nMDS plots and ANOSIM results are based on Bray–Curtis dissimilarity matrices. All nMDS and ANOSIM statistical analyses were performed by using the software program PRIMER (version 5; Clarke and Warwick 2001).

Linear regression was used to determine if the total number of alkaloids (a measure of alkaloid diversity) varied with size of the frog (measured as SVL) within and among the three different mantellid species. The statistical program SPSS (version 11.5 for Microsoft Windows) was used to perform these statistical analyses.

Results

A total of 111 alkaloids, including isomers, were identified from skin extracts of individuals of *M. baroni* (*N*=19; Tables 1 and 2), *M. bernhardi* (*N*=51; Tables 5 and 6), and *M. madagascariensis* (*N*=11; Tables 3 and 4). Representatives of all classes of alkaloids noted in "Introduction" were present in at least one species or population (Table 7). Twenty representative alkaloids were detected relatively frequently in this study [Fig. 2, see Daly et al. (2005) for details concerning structures of the more than 800 alkaloids reported to date from alkaloid-containing amphibians]. A total of 82 alkaloids, including isomers, were detected in skin extracts of the combined samples (ranging from one to eight skins per sample) of *M. baroni*, *M. bernhardi*, and *M. cowanii* (Tables 8, 9, 10, and 11). Representatives of all classes of alkaloids noted in "Introduction" were present in at least one species or population (Table 11). Fifty alkaloids that were detected in these combined frog samples, mainly 5,8-disubstituted indolizidines and alkaloids of undefined structure (unclassified alkaloids), were not detected in any of the individual frog samples (cf. Tables 7 and 11). Thus, 161 alkaloids were detected in this study of four mantellid species (Tables 7 and 11).

Previously unreported new alkaloids are indicated by asterisks within the text and tables. Their GC retention times, mass spectral data, and other data are presented in the Supplementary Information. Tentative structures for some of these previously unreported new alkaloids are proposed in the Supplementary Information.

Individual Skin Alkaloid Analyses—M. baroni from Ranomafana Many of the alkaloids identified in 15 skin extracts of *M. baroni* from Ranomafana were of the PTX group (Table 1). The dominant PTX alkaloids in most of the extracts were PTX **251D** and **309A** and homoPTX **265N**. Other alkaloids of the homoPTX class (**251R**, **281K**) were detected in trace amounts. Only one alloPTX was detected (**325A**), which also usually occurred in trace amounts. Several extracts had trace or minor levels of PTX **237A** (a C-15 analog of the C-16 PTX **251D**). A keto-PTX **307F′** (characterized by an enhanced *m*/*z* 194 ion) was detected in most of the extracts, but only as a trace or minor alkaloid. PTX **267C** and deoxyPTX **251H** occurred in skin extracts rarely and usually as trace alkaloids. In addition to alkaloids of the PTX group, many individuals of *M. baroni* from Ranomafana contained significant amounts of 1,4-disubstituted quinolizidines **217A** and **231A**, along with 5,8-disubstituted indolizidine **217B**. The 5,6,8-trisubstituted indolizidine **273A** was present in large amounts in nearly every extract, often accompanied by a minor diastereomer. The PTX group of alkaloids (PTX, alloPTX, and homoPTX) and the branched chain indolizidine and

Table 1 Alkaloid profiles for *Mantella baroni* (15 individuals) from Ranomafana, 22 January 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins						
			Mites						
			237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**251R** (hPTX)	**265N** (hPTX)	**267C** (PTX)	**281K** (hPTX)
126f	27.5	+++	2	3		1	3		
127m	25.0	+++	1	2		1	3		1
128s	20.4	+		1				1	
129m	24.7	+++	1	2			3		
130m	23.6	++		2			2		
131m	23.6	+++	1	2		1	2		
132m	21.7	+++	1	2			2		
133f	27.1	+++	*1*	3		1	3/1		
134m	24.7	+++	1	2			2		
135m	22.3	++	2	2			3		
136m	22.7	+++	1	3		1	2		
137m	24.6	+++	1	3	1		2	1	
138m	23	+++	1	3		1	3		
139m	23	++		3			3		
140m	23.5	+++	1	2			3		

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins				Izidines				
	Mites								
	293D (deoxyPTX)	**307F′** (PTX)	**309A** (PTX)	**325A** (aPTX)	**205L**[c] (dehydro -5,8-I)	**217A** (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**233A** (1,4-Q)
126f		2	2			1	1	1	1
127m		2	3	1		2	2	2	1
128s									
129m		1	2				2	3	1
130m		1	2			2	1	1	
131m		1	3			2/1	1	1	
132m		1	3			2/2	1	2	
133f			3	1		2	2	3	
134m		1	3			1	1	2	
135m		1	3		1	2	2	3/1	
136m		2	2			2	1	2	
137m	1	2	2	1		1	1		1
138m			2	1	1	1	1	1	
139m		1	2			3		2	
140m			1	1	1	1	2	2	

ZCMV# + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites			Ants				Millipedes/Beetles/Unknown
	251N (5,8-I)	**273A** (5,6,8-I)	**293O**[c] (dehydro-5,8-I)	**249A** (3,5-I)	**249I** (3,5-P)	**251O** (3,5-P)	**275C** (3,5-I)	Other
126f		2/1		1	1		/2/	
127m		2/1					/2/2	
128s								
129m		2/1				2/1	/2/	
130m		1		1			1/3/	
131m		2/1				1	/1/2	
132m		2/2					/2/	Spiro **236** (1)

Table 1 (continued)

ZCMV# + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites			Ants				Millipedes/Beetles/Unknown
	251N (5,8-I)	**273A** (5,6,8-I)	**293O**[c] (dehydro-5,8-I)	**249A** (3,5-I)	**249I** (3,5-P)	**251O** (3,5-P)	**275C** (3,5-I)	Other
133f		2/1		1			/2/2	
134m		2					/1/	Spiro **236** (1)
135m	1	2					/2/	Spiro **236** (1)
136m		2					/2/2	
137m	1	2/1					1//1	Spiro **236** (1)
138m		2/1	1	1		1	1/2/	Spiro **236** (1); Unclass **207N** (1)
139m		2					//2	
140m		3/2				2	//2	Spiro **236** (1)

Probable class and dietary source of each alkaloid are indicated in the headings (see abbreviations).
[a] Sex (*m* male; *f* female, *s* subadult) is indicated.
[b] Total content of alkaloids [major (+++), minor (++), trace (+)] are based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor; $\leq 10^3$ = trace. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two or three intensities are tabulated, two or three isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid was not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

quinolizidine alkaloids of *M. baroni* from Ranomafana are likely derived from oribatid mites (Saporito et al. 2007a). Izidines with unbranched carbon skeletons, which likely are sequestered from myrmicine ants (Jones et al. 1999), were rare in *M. baroni* from Ranomafana. The only exception was the presence of diastereomers of the 3,5-disubstituted indolizidine **275C**, which were present in all but one individual and often in large amounts. The spiropyrrolizidine **236** of millipede origin (Saporito et al. 2003; Clark et al. 2005) occurred in six of the 15 individuals of *M. baroni* from Ranomafana.

Individual Skin Alkaloid Analyses—M. baroni from Besariaka The skin extracts of four *M. baroni* from Besariaka (all adult males; Table 2) differed from the same species collected from Ranomafana (Table 1). However, alkaloid composition among individuals was again characterized by many alkaloids of the PTX group, including PTXs **251D**, **307F′**, **307G**, and **309A**, which were found in all frogs. No homoPTXs were detected. Two alloPTXs (**323J*** and **325A**) were identified in large amounts in most frogs. Keto-PTXs **307F″** and **307F‴** (characterized by an enhanced *m*/*z* 193 fragment ion) occurred in one extract along with the more common **307F′**. Similar to the *M. baroni* from Ranomafana, frogs from Besariaka contained large amounts of 1,4-disubstituted quinolizidine **217A**. Frogs from Besariaka had a somewhat greater diversity of putative mite izidine alkaloids (*N*=13), as compared to frogs from Ranomafana (*N*=11). The 5,6,8-trisubstituted indolizidine **223A** and dehydro-5,8-disubstituted indolizidine **245F**, both of which are putative mite alkaloids, occurred in three of the four Besariaka frogs. Neither of these two alkaloids was detected in Ranomafana frogs. Putative ant alkaloids in the Besariaka frogs were represented by trace amounts of the 3,5-disubstituted pyrrolizidine **223M** in one frog and by the 3,5-disubstituted indolizidine **251O** in two frogs. Interestingly, the 3,5-disubstituted indolizidine **275C**, common in *M. baroni* from Ranomafana, was not detected in *M. baroni* from Besariaka.

Alkaloid Variation Within and Among Populations of M. baroni As previously documented for *M. baroni* (Daly et al. 1996; Clark et al. 2006), alkaloid composition within and among populations of this species can differ markedly (see Tables 1 and 2 for extracts of individuals from two populations and Tables 8 and 9 for extracts of groups from five populations). Alkaloid compositions among the 15 individual frogs from Ranomafana were more similar to each other than were the compositions of the four individual frogs from Besariaka (see nMDS plot of Fig. 3a). Alkaloid composition of *M. baroni* was significantly different between Ranomafana and Besariaka (Global R=0.99; P<0.001; Fig. 3a).

Individual Skin Alkaloid Analyses—M. madagascariensis from Ranomafana Alkaloid composition of six extracts of *M. madagascariensis* from Ranomafana (Table 3) differed from that of *M. baroni* from Ranomafana (Table 1). However, it should be noted that the two species were not in exactly the same microhabitat at the Ranomafana site (see "Methods and Materials"). Most individuals of *M.*

Table 2 Alkaloid profiles for *Mantella baroni* (four individuals) from Besariaka, 15 February 2004

ZCMV[#] + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins							
			Mites							
			251D (PTX)	**265X** (deoxyPTX)	**267C** ((PTX)	**281N** (deoxyPTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307F″/F‴** (PTX)	**307G** (PTX)
911 m	24.9	+++	1					1		1
912 m	27.1	+++	3					1		2
913 m	26.2	+++	1					2		1
914 m	26.0	+++	1	1	1	1	1	1	1/1	1

ZCMV[#] + sex[a]	Pumiliotoxins			Izidines						
	Mites									
	309A (PTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223A** (5,6,8-I)	**231A** (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247E** (5,8-I)	**249BB**[c] (5,6,8-I)
911 m	2	2	3	3	1	2		1	1	
912 m	2	2	3	2	1			2		1
913 m	2	2	3	2	2		1			
914 m	1/2		1/2	3/1		1		1	1	

ZCMV[#] + sex[a]	Izidines							Spiros/tricyclics/unclass
	Mites					Ants		Millipedes/beetles/unknown
	251N (5,8-I)	**265F** (dehydro5,8-I)	**265U** (5,6, 8-I)	**267E** (5,8-I)	**267S** (5,8-I)	**223M** (3,5-P)	**251O** (3,5-I)	Other
911 m		1						Tri **245J** (2); Unclass **249AA**[c] (1)
912 m	1	1	1		1	1	1/1	
913 m							1	Tri **245J** (1)
914 m		1		1	1			Tri **245J** (2); Unclass **307L**[c] (1)

The probable class and dietary source of the alkaloid are indicated in the heading (see abbreviations).
[a] Sex (*m* male) is indicated.
[b] Total content of alkaloids [major (+++)] is based upon total ion chromatogram intensities with 10^4 or greater. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

madagascariensis had substantial amounts of homoPTX **265N**, as was the case for the *M. baroni*. However, in three of the six *M. madagascariensis*, homoPTX **281K** was present as a dominant or substantial alkaloid, whereas the same alkaloid was present in only one of the 15 *M. baroni* from Ranomafana and then only as a trace alkaloid. PTXs **237A** and **309A**, abundant in *M. baroni*, occurred only as trace alkaloids in *M. madagascariensis*. PTX **251D**, a dominant or substantial alkaloid in all but one of the 15 *M. baroni*, occurred in substantial amounts in only one of the six *M. madagascariensis* while being a trace alkaloid in another four. PTX **267C**, which was detected only twice as a trace alkaloid in 15 *M. baroni*, occurred in three of the six *M. madagascariensis*, once as a major alkaloid, once as a minor alkaloid, and once as a trace alkaloid. There were considerable differences between *M. madagascariensis* and *M. baroni* in the izidine alkaloids that are of putative mite origin. The 1,4-disubstituted quinolizidines **217A** and **231A**, common in large amounts in *M. baroni*, were less common in *M. madagascariensis*. Similarly, the 5,8-disubstituted indolizidine **217B** was much less common in *M. madagascariensis*. The six *M. madagascariensis* frogs contained eight 3,5-disubstituted pyrrolizidines and indolizidines, which are of probable ant origin. This is in marked

Table 3 Alkaloid profiles for *Mantella madagascariensis* (six individuals) from Ranomafana, 22 January 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins								
			Mites								
			237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**251R** (hPTX)	**265N** (hPTX)	**267C** (PTX)	**281K** (hPTX)	**309A** (PTX)	**323A** (PTX)
109f	22.2	+++		2	1		3	3	3/1	1	1
110m	18.2	+++		1			3		2		1
111m	19.4	+		1			2	1	1	1	
112m	19.2	n.d.									
113m	20.6	+++		1							
114f	22.4	+++	1	1		1	3	2	3/1	1	

ZCMV# + sex[a]	Izidines										
	Mites										
	203A (5,8-I)	**207W**[c] (dehydro5,8-I)	**217A** (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**251N** (5,8-I)	**267S** (5,8-I)	**273A** (5,6,8-I)	**289G**[c] (5,6,8-I)	**293O**[c] (dehydro5,8-I)	**309K**[c] (izidine)
109f	1		3	1	1		1	3	1		
110m	1		3		1	1	1	1	1		
111m					1			1			
112m											
113m		1			1			1		1	1
114f	1		2					2/2	1		

ZCMV# + sex[a]	Izidines									Spiros/tricyclics/unclass
	Ants									Millipedes/beetles/unknown
	197J[c] (3,5-P)	**239K** (3,5-P)	**249A** (3,5-I)	**251O** (3,5-P)	**263S**[c] (3,5-P)	**265W** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	**291J** (izidine)	Other
109f	1	1	2		1			2	1	Spiro **236** (2/1); Tri **263T**[c] (1);
110m				1				1/1		Spiro **222** (1), **236** (2/1); Tri **247N**[c] (1), **263M** (1)
111m								1		Spiro **236** (1)
112m										
113m			1	1			3	1/1		Spiro **222** (1), **236** (3/1), **252B** (1); unclass **183C** (1),**197D**(1), **199B**[c] (1),
114f				1		1	2	1	1	Spiro **236** (2/1)

The probable class and dietary source of the alkaloid are indicated by the headings.

[a] Sex (*m* male; *f* female) is indicated.

[b] Total content of alkaloids [major (+++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major, $\leq 10^3$ = trace; n. d. = none detected. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

contrast to the 15 *M. baroni* frogs, where only four such alkaloids were detected. Both species had the presumed ant-derived 3,5-disubstituted indolizidine **275C** as a common constituent. In addition, the millipede alkaloid **236** was identified in 6 of the 15 *M. baroni* and in 5 of the 6 *M. madagascariensis*. Interestingly, in *M. madagascariensis*, a minor isomer of the spiropyrrolizidine **236** with a slightly longer retention time occurred in four of the frogs. Unfortunately, a GC-FTIR spectrum of this minor isomer could not be obtained. A minor isomer of **236** has been previously reported from *M. baroni* (Clark et al. 2005) and has been found to occur in siphonotid millipedes

Table 4 Alkaloid profiles for *Mantella madagascariensis* (five individuals) from Besariaka, 15 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins							Izidines
			Mites							
			251D (PTX)	**305A** (aPTX)	**305C** (aPTX)	**309A** (PTX)	**321C** (aPTX)	**323B** (aPTX)	**325A** (aPTX)	**211B** (izidine)
915 f	23.6	+++	1	1	1	1	1	1	1	
916 m	20.9	+++		1		1	2	1	1	1
917 f	25.0	+++		1			2	1/1		
918 m	21.8	+						1	1	
919 f	24.9	+						1	1	

ZCMV# + sex[a]	Izidines								
	Mites							Ants	
	217A (1,4-Q)	**223A** (5,6,8-I)	**239C** (5,6,8-I)	**239Z**[c] (5,6,8-I)	**267E** (5,8-I)	**267S** 5,8-I)	**269J**[c] (5,6,8-I)	**195B** 3,5-I)	**211E** (3,5-I)
915 f	1	1	1	3/1	1	1		1	
916 m	1	1	1	3					1
917 f	1	1	1	3/1			1		
918 m	1			1					
919 f	1			1					

The probable class and dietary source of the alkaloid are indicated in the headings (see abbreviations).
[a] Sex (*m* male; *f* female) is indicated.
[b] Total content of alkaloids [major (+++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major; $\leq 10^3$ = trace. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

(Clark et al. 2005; Saporito et al., unpublished data). One *M. madagascariensis* from Ranomafana had two additional millipede alkaloids, the spiropyrrolizidines **222** and **252B**.

Individual Skin Alkaloid Analyses—M. madagascariensis from Besariaka Alkaloid composition of five extracts of *M. madagascariensis* from Besariaka (Table 4) was markedly different than those from *M. baroni* (Table 2) of the same site and from the *M. madagascariensis* from Ranomafana (Table 3). Alkaloid composition in *M. madagascariensis* of Besariaka was dominated by a previously unreported 5,6,8-trisubstituted-indolizidine **239Z** presumed to be of mite origin. A GC-FTIR spectrum was obtained. The Bohlmann band pattern indicated an indolizidine of the 5*Z*,9*Z* configuration with a non-hydrogen-bonded hydroxyl group (3,666 cm^{-1}). A tentative structure is presented in the Supplementary Information. Two minor isomers also were detected. Other putative mite alkaloids that were present in three of the five *M. madagascariensis* from Besariaka were the 1,4-disubstituted quinolizidine **217A** and the 5,6,8-trisubstituted indolizidines **223A** and **239C**. The 5,6,8-trisubstituted indolizidine **239Z** was not detected in *M. baroni* of Besariaka; however, both **217A** and **223A** were present. Accompanying the "izidine" alkaloids in *M. madagascariensis* of Besariaka were alloPTXs **305A**, **321C**, **323B**, and **325A**. On the basis of differences in the pattern of mass spectral fragmentation, it appears that some of the alloPTXs may prove to be 16-hydroxyl isomers of the usual 15-hydroxyl alloPTXs. Methoxy alloPTX alkaloids of molecular weight 337 were detected in three extracts. However, upon further analysis, these compounds appear to be artifacts of a chemical reaction of certain alloPTXs (probably **323B**) with methanol during exposure to HCl-methanol in the fractionation process. These apparent methoxy alkaloids were not detected when combined skin extracts of the five *M. madagascariensis* were partitioned using the standard method of Daly et al. (1994a, b) to yield an alkaloid fraction. Two indolizidines, namely the 3-butyl-5-methylindolizidine **195B** and the 3-hydroxybutyl analog **211E**, were detected in *M. madagascariensis* from Besariaka. These alkaloids are likely derived from myrmicine ants. Neither of these indolizidines was detected in the *M. baroni* from Besariaka.

Table 5 Alkaloid profiles for *Mantella bernhardi* (26 individuals) from Manombo, 1 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins				Izidines				
			Mites								
			251D (PTX)	**307F′** (PTX)	**309A** (PTX)	**325A** (aPTX)	**203A** (5,8-I)	**207I** (1,4-Q)	**217A** (1,4-Q)	**217B** (5,8-I)	**223X** (5,6,8-I)
502f	18.4	++							3	1	
503f	18.0	+++					1		3/1		
504f	17.8	++	1						1		
505m	15.6	++	3						2	1	
506f	18.1	+							3		
507m	16.0	++	3						2		
501m	14.9	+	3						2		
508m	16.5	++							3	2	2
509m	16.1	++	1						3		
510m	15.7	++	2	1	1				2		
520f	18.3	+++	1								
521f	19.2	+	3						3		
522f	18.1	++							2	2	
523f	17.3	+	1	1	2				2		
524f	18.0	++			3	2					
620f	18.3	++				1					3
526m	14.3	+	2						3/2		
527f	19.6	++	2					1	3/2		
528f	18.8	+++	2						3/2		
529m	16.6	+							3/2		
621f	17.8	++		1		1			1/2	1	
525m	15.1	+							2/1		
530m	16.3	++							3		
531f	17.2	++							2		
532m	15.6	++							3		
413s[d]	?	+++	3						1/1		

ZCMV# + sex[a]	Izidines								Spiros/tricyclics/unclass
	Mites				Ants				Millipedes/beetles/unknown
	231A (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**249A** (3,5-I)	**251O** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	Other
502f			3		2				
503f			3	2	1				Tri **217H**[c](1); **231N** (1)
504f			1			3	2		Unclass **323H** (2)
505m			3			3			Tri **265CC**[c] (3)
506f			3		2	2			
507m	2		2			1			Unclass **237V**; **323H** (2)
501m	2		2			1			
508m	2	1	3		2	3			
509m	2		2	1		1	2		
510m	2		3		2	2			Unclass **235Q**(1); **237V** (1); **323H** (1)
520f					3			3	
521f			3		2	3			
522f					3	3		3	Unclass **323H** (3)
523f			2		2	1		1	Unclass **297F**[c] (3); **323H** (2)
524f			2		2	2		2	
620f					2	3	3	2	Unclass **323H** (2)
526m		1	1	1		2			

Table 5 (continued)

ZCMV# + sex[a]	Izidines								Spiros/tricyclics/unclass
	Mites				Ants				Millipedes/beetles/unknown
	231A (1,4-Q)	**233A** (1,4-Q)	**245F** (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**249A** (3,5-I)	**251O** (3,5-P)	**267H** (3,5-P)	**275C** (3,5-I)	Other
527f			3		2	3			Unclass **151C**[c] (1); **235Q**(1)
528f		1	3	1		2			
529m			3		2	1			
621f						1			
525m			2		2	1			
530m			3		3	1		3	
531f	1		3		3	1		3	
532m	2		3	1		2/1			Unclass **231J** (1)
413s[d]			1			1			Unclass **235Q** (1); **323H** (1)

The probable class and dietary source of the alkaloid are indicated in the headings. The class of each alkaloid is indicated (see abbreviations).
[a] Sex (*m* male; *f* female) is indicated (*s* subadult).
[b] Alkaloid amounts [major (+++), minor (++), trace (+)] are based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor; $\leq 10^3$ = trace. The amounts of each alkaloid are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted and the intensities are in the order of elution from the GC column.
[c] Alkaloids reported for the first time. See Supplementary Information for characterization.
[d] *UABD* uncataloged

Alkaloid Variation within and among Populations of M. madagascariensis With the exception of the one individual that contained no detectable alkaloids, alkaloid compositions among the six *M. madagascariensis* from Ranomafana were quite similar (see nMDS plot of Fig. 3b). The alkaloid compositions among the five individuals of *M. madagascariensis* from Besariaka were also similar to each other (see nMDS plot of Fig. 3b). Indeed, the alkaloid composition for two of the individual frogs was identical. However, alkaloid composition of the *M. madagascariensis* was significantly different between Ranomafana and Besariaka (Global R=1.0; P<0.008; Fig. 3b).

Individual Skin Alkaloid Analyses—M. bernhardi from Manombo The skin extracts of 26 *M. bernhardi* frogs collected from Manombo (Table 5) contained remarkably few alkaloids of the PTX group, with PTX **251D** as a dominant alkaloid in only five extracts and with PTX **309A** as a dominant alkaloid in only one extract. The alloPTX **325A** occurred as a minor alkaloid in one extract. The presumed mite alkaloids 1,4-disubstituted quinolizidines **217A** and **231A** and dehydro-5,8-disubstituted indolizidine **245F** were dominant alkaloids in most extracts. In addition, the putative ant 3,5-disubstituted pyrrolizidine **251O** and the 3,5-disubstituted indolizidines **249A** and **275C** occurred frequently as dominant alkaloids in many of the extracts. Interestingly, only one isomer of **275C** (5*Z*,9*Z* relative configuration) was observed in the extracts. This is in contrast to *M. baroni* (see Table 1), where frequently two or three diastereomers of **275C** were detected, with the 5,9*Z*-isomer usually being minor or absent. A minor diastereomer, accompanying 1,4-disubstituted quinolizidine **217A**, was seen in several extracts of the *M. bernhardi* from Manombo. This isomer of **217A** also occurred rarely in extracts of *M. baroni* (Tables 1 and 2). An isomer of alkaloid **217A** that previously had been detected in an extract of *Mantella betsileo* and based on comparison with synthetic material was shown to be the C-1-epimer of **217A** (unpublished results, cited in Michel et al. 2000).

The present collection of *M. bernhardi* from Manombo consisted of 11 males, 14 females, and 1 juvenile. This represents the only site in which numbers were large enough to compare alkaloid composition between sexes (see below). The 1,4-disubstituted quinolizidine **231A** appeared in six males, but in only one female. PTX **251D** was seen in 7 of the 11 males and only 4 of the 14 females. In the females, **251D** occurred as a trace alkaloid in three of these four individuals and as a minor alkaloid in only one individual. The 3,5-disubstituted indolizidine **275C** was observed in six females and in only one male. Thus, there appears to be a clear relationship between the occurrence of the putative mite alkaloid **231A** and of the putative ant alkaloid **275C** and the sex of the frog.

Individual Skin Alkaloid Analyses—M. bernhardi from Vevembe In contrast to the izidine-dominated alkaloid compositions for the 26 *M. bernhardi* from Manombo (Table 5), the compositions for the 25 *M. bernhardi* of Vevembe were dominated by alkaloids of the PTX group (Table 6). Nearly all individuals had PTXs **251D** and **309A**

Table 6 Alkaloid profiles for *Mantella bernhardi* (25 individuals) from Vevembe, 10 February 2004

ZCMV# + sex[a]	SVL (mm)	Amount[b]	Pumiliotoxins/homopumiliotoxins							
			Mites							
			251D (PTX)	**253A** (aPTX)	**253F** (PTX)	**267C** (PTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307F″/F‴** (PTX)	**307K**[c] (deoxyhPTX)
701m	15.8	+++						1		
702m	15.4	+++	3							
703m	15.1	+++	3							
704m	15.4	+++	3			1				
705m	15.5	+++	3					1	1/1	
706m	14.4	+++	1					2	1/1	
707m	14.2	+++	3							
708m	15.4	+++	3							
709m	15.7	+++	3							
710f	17.4	+	3							
711f	17.2	+++	3							
712m	16.0	+++	3							
713m	14.3	+++	3					2		
714m	15.5	++						2	1/1	
715m	14.0	+++	3			1				
901m	15.2	+++	3			1		1	1/1	
902m	14.6	+++	2					1	1/1	
903f?	18.2	+++	3							
904f?	18.6	+++	3					1		
905m	15.4	+++	3			1				
906m	15.4	+++						1		1
907m?	16.1	+++	3			1				
908m	16.3	+++	3				1	1		1
909m	14.8	+++	3	1	1	1				
m	15.9	+++	3			1				

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins						Izidines			
	Mites									
	309A (PTX)	**321B** (hPTX)	**323A** (PTX)	**323E** (hPTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223X** (5,6,8-I)	**231A** (1,4-Q)	**235B″** (5,8-I)
701m	3			3		3				
702m			1			3				
703m						2				
704m					1	2				
705m	2	1/1		1	1	3		1		
706m	3	1		2	1	2		2		
707m	1					2				
708m					1	2				
709m		1			1	3			2	
710f					1	3				
711f					1	2		1		
712m					1	2				
713m	2			2	1	2				
714m	2	1		2		2				
715m	1					2		2		
901m					1	2				1
902m	3			2		1	1			2
903f?					1	1/2				
904f?	2			1	1	2				

Table 6 (continued)

ZCMV# + sex[a]	Pumiliotoxins/homopumiliotoxins						Izidines			
	Mites									
	309A (PTX)	**321B** (hPTX)	**323A** (PTX)	**323E** (hPTX)	**323J**[c] (aPTX)	**325A** (aPTX)	**217A** (1,4-Q)	**223X** (5,6,8-I)	**231A** (1,4-Q)	**235B″** (5,8-I)
905m						2				
906m	3			3		3				
907m?	1			2	1	2				
908m	3			2		2		3		
909m	1					2		1		
m	1					2				

ZCMV# + sex[a]	Izidines							Spiros/tricycles/unknown
	Mites		Ants					Millipedes/beetles/ unclass
	245F (dehydro5,8-I)	**247O**[c] (dehydro5,8-I)	**237R** (3,5-P)	**239BB**[c] (3,5-P)	**249A** (3,5-I)	**251K** (3,5-P)	**251O** (3,5-P)	Other
701m					2			
702m	2	2					2	
703m								
704m			1				1	
705m			1	1			1	
706m							1	
707m								
708m								
709m	2							
710f							2	
711f							1	
712m								
713m			1					
714m								
715m						1		
901m								
902m								
903f?				2/1			1/1	Unclass **231J** (1)
904f?				1			2/2	
905m							2	
906m				1				
907m?				1				
908m				1			2/1	
909m				1/1			1	
m				1/1			1	Unclass **231J** (1)

The probable class and dietary source of alkaloids are indicated (see abbreviations).

[a] Sex: *m* male; *f* female; *?* indicates uncertainty as to sex.

[b] Total content of alkaloids [major (+++), minor (++), trace (+)] is based upon total ion chromatogram intensities with 10^4 or greater = major, 10^3–10^4 = minor, $\leq 10^3$ = trace. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicated that the alkaloid was not detected.

[c] Alkaloids reported for the first time. See Supplementary Information for characterization.

and alloPTX **325A** as significant or dominant alkaloids. However, three frogs had no PTX **251D**. HomoPTX **323E** was present in eight frogs as a significant alkaloid. A previously unreported alloPTX **323J** was present in trace amounts in 13 frogs. Another previously unreported alkaloid, deoxy-homoPTX **307K**, was found in two frogs. In contrast to the *M. bernhardi* from Manombo, "izidine" alkaloids that are proposed to be derived from dietary mites occurred rarely in *M. bernhardi* from Vevembe. Thus, the 1,4-disubstituted quinolizidine **217A** and the dehydro-5,8-

Table 7 Summary of alkaloids detected in single-skin extracts of populations of *Mantella*

Table number		1	2	3	4	5	6
Mantella species		*M. baroni*		*M. madagascariensis*		*M. bernhardi*	
Alkaloids		Ran.	Bes.	Ran.	Bes.	Man.	Vev.
PTX	**237A**	x			x		
	251D	x	x	x	x	x	x
	253F						x
	267C	x	x		x		x
	307A		x				x
	307F	x	x/x/x			x	x/x/x
	307G		x				
	309A	x	x/x	x	x	x	x
	323A				x		x
aPTX	**253A**			x			
	305A			x			
	305C			x			
	321C			x			
	323B		x	x/x			x
	323J[a]		x				x
	325A	x	x/x			x	x/x
deoxy PTX	**251H**	x			x		
	265X		x				
	281N		x				
	293D	x					x
hPTX	**251R**	x			x		
	265N	x/x			x		
	281K	x			x/x		
	321B						x/x
	323E						x
d-hPTX	**307K**[a]						x
3,5-P	**197J**[a]				x		
	223M		x				
	237R						x
	239K				x		x
	239BB[a]						x/x
	249I	x					
	251K						x
	251O	x/x	x		x	x/x	x/x
	263S[a]				x		
	265W	x			x		
	267H	x			x	x	
3,5-I	**195B**			x			
	211E			x			
	249A	x			x	x	x
	275C	x/x/x			x/x	x	
5,8-I	**203A**				x	x	
	217B	x			x	x	
	235B″						x
	247E		x				
	251N	x	x		x		
	267E		x	x			
	267S		x	x	x		
dehydro 5,8-I	**205L**[a]	x					
	207W[a]				x		
	245F		x			x	x
	247O[a]					x	x
	265F		x				

Table 7 (continued)

Table number		1	2	3	4	5	6
Mantella species		*M. baroni*		*M. madagascariensis*		*M. bernhardi*	
Alkaloids		Ran.	Bes.	Ran.	Bes.	Man.	Vev.
	293O[a]	x			x		
5,6,8-I	**223A**		x	x			
	223X					x	x
	239C			x			
	239Z[a]			x/x			
	249C		x				
	249BB[a]		x				
	265L		x				
	265U		x				
	269J[a]			x			
	273A	x/x			x/x		
	289G[a]				x		
1,4-Q	**207I**					x	
	217A	x/x	x/x	x	x	x/x	x
	231A	x/x	x		x	x	x
	233A	x	x			x	
Spiro	**222**				x		
	236	x			x/x		
	252B				x		
Tricyclics	**217H**[a]					x	
	231N[a]					x	
	245J		x				
	247N[a]				x		
	263M				x		
	263T[a]				x		
	265CC[a]					x	
Izidines	**211B**			x			
	291J				x		
	309K[a]				x		
Unclass.	**151C**[a]					x	
	183C				x		
	197D				x		
	199B[a]				x		
	207N	x					
	231J					x	x
	235Q					x	
	237V[a]					x	
	249AA[a]		x				
	297F[a]					x	
	307L[a]		x		x		
	323H					x	

For detailed distribution and quantitation data, see Tables 1, 2, 3, 4, 5, and 6. Structures for selected alkaloids are shown in Fig. 2 (see also Daly et al. 2005). See abbreviations for alkaloid classes.
Ran. Ranomafama; *Bes.* Besariaka; *Man.* Manombo; *Vev.* Vevembe; *d-hPTX* deoxy-hPTX
[a] Previously undescribed alkaloids. Properties and tentative structures for some of these alkaloids are in the Supplementary Information.

disubstituted indolizidine **245F** were common and often dominant alkaloids in *M. bernhardi* from Manombo (Table 5), but these alkaloids occurred rarely in *M. bernhardi* from Vevembe, with **217A** being found as a trace alkaloid in one frog and **245F** being found in significant amounts in only two frogs (Table 6). "Izidine" alkaloids that are presumably derived from dietary ants were represented in the Vevembe frogs by four 3,5-disubstituted pyrrolizidines, namely **237R**, **239BB**, **251K**, and **251O** and by the 3,5-disubstituted indolizidine **249A** (diastereomers of these alkaloids were occasionally present). Of these 3,5-disubstituted pyrrolizidines, only **239BB**

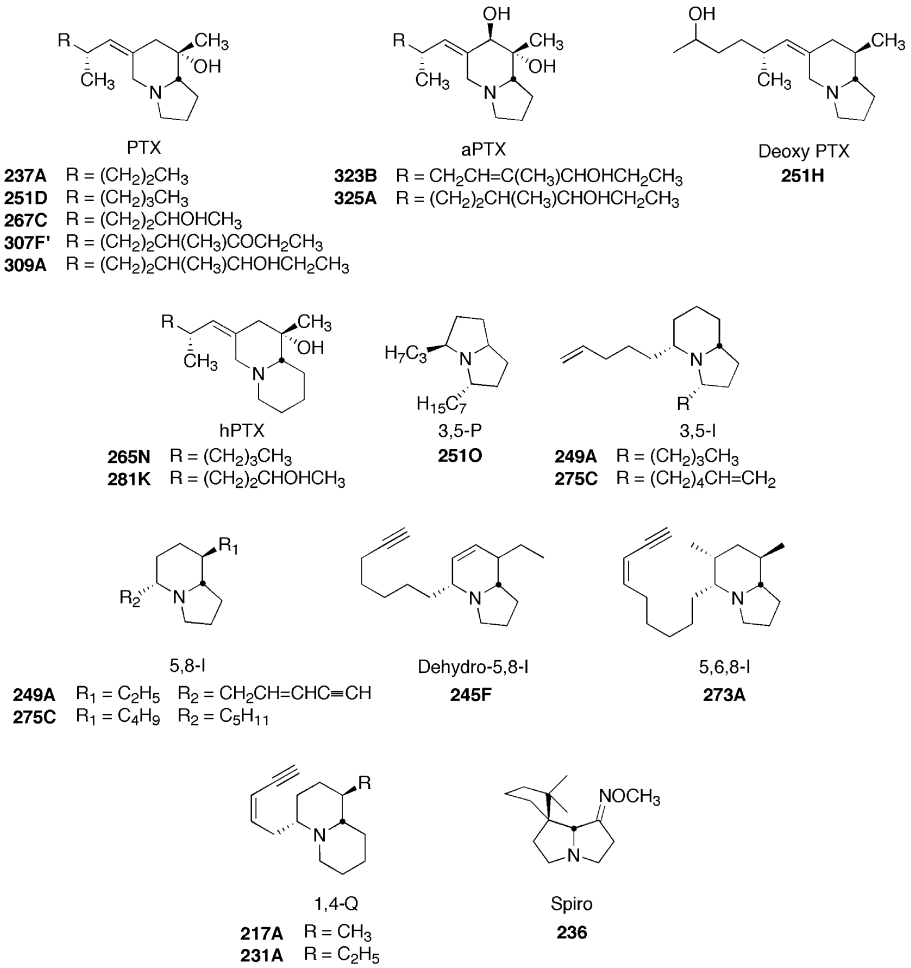

Fig. 2 Representative alkaloids of 11 of the structural classes detected in the present collections of mantellid species. These alkaloids occur relatively frequently or are major alkaloids in several extracts as are the following new alkaloids: deoxy-homoPTX **307K***, 3,5-disubstituted pyrrolizidine **239BB***, and 5,6,8-trisubstituted indolizidine **239Z*** (for tentative structures, see Supplementary Information)

and **251O** occurred in several frogs, and overall, such putative ant alkaloids were not very common in *M. bernhardi* from Vevembe. In the *M. bernhardi* from Manombo, the putative ant alkaloids **249A** and **251O** were present in almost all frogs (Table 5).

Alkaloid Variation Within and Among Populations of M. bernhardi There was a somewhat uniform variation in alkaloid composition among the 26 individuals of *M. bernhardi* from Manombo and also among the 25 individuals of *M. bernhardi* from Vevembe (see nMDS plot of Fig. 4a). There did appear to be a group of eight frogs from Vevembe (nos. 903 f to 910 m of Table 6) that were characterized by an alkaloid mix of PTX **309A** and izidines **239BB** and **251O** along with the very common PTX **251D** and alloPTX **325A**. The alkaloid composition of *M. bernhardi* was significantly different between Manombo and Vevembe (Global R=0.83; P<0.001; Fig. 4a). The alkaloid composition of *M. bernhardi* from Manombo was not significantly different between sexes (Global R=0.06; P= 0.20; Fig. 4b).

Alkaloid Variation among Species Alkaloid composition was significantly different between *M. baroni* and *M. madagascariensis* from Besariaka (Global R=1.0; P< 0.008; Fig. 5a). Alkaloid composition was also significantly different between *M. baroni* and *M. madagascariensis* from Ranomafana (Global R=0.96; P<0.001; Fig. 5b). A summary of variation is provided in Table 7.

Combined Skin Alkaloid Analyses—M. baroni A variety of PTXs (Table 8) and izidine alkaloids (Table 9) were detected among the five populations of *M. baroni*. The six combined skins from Mangevo (Fig. 1) and the one skin from Vohiparara (Kidonavo Bridge location of Fig. 1) are from the Ranomafana National Park. Alkaloid composition for the combined six skins of *M. baroni* from Mangevo was similar to those of the individual *M. baroni* frogs of

Table 8 Alkaloid profiles of pumiliotoxins/homopumiliotoxins for five populations of *M. baroni* from Mangevo (six skins), Andriabe (two skins), Tsinjoarivo (two skins), Vohidrazana (two skins), and Vohiparara (one skin), Jan–Feb 2003 (see Table 9 below for izidines and other alkaloids)

Species/site	Pumiliotoxins/homopumiliotoxins						
	Mites						
	Amount[a]	**237A** (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**265N** (hPTX)	**267C** (PTX)	**267N** (deoxyhPTX)
M.baroni/Mangevo #1	+++	3	2				
M. baroni/Andriabe	+++		2				1
M. baroni/Tsinjoarivo	+++						
M. baroni/Vohindrazana	+++	1	1				
M. baroni/Vohiparara	+++	1	3	1	2	1	

Species/site	Pumiliotoxins/homopumiliotoxins						
	Mites						
	279L[b] (desMePTX)	**281F** (H_2-PTX)	**291E** (deoxyPTX)	**293D** (deoxyPTX)	**295C** (deoxyPTX)	**307F′** (PTX)	**307F″/307F‴** (PTX)
M.baroni/Mangevo #1				1		2	
M. baroni/Andriabe						1	
M. baroni/Tsinjoarivo	1		2	1/1	2	1	1/1
M. baroni/Vohindrazana		2	2	1/2		1	1/1
M. baroni/Vohiparara						1	

Species/site	Pumiliotoxins/homopumiliotoxins					
	Mites					
	307G (PTX)	**309A** (PTX)	**323J** [b] (aPTX)	**323E** (hPTX)	**325A** (aPTX)	**337A** (hPTX)
M.baroni/Mangevo #1	2	3	1		2	1
M. baroni/Andriabe	1	3	2		3	
M. baroni/Tsinjoarivo	2	3		1	2	
M. baroni/Vohindrazana	3	3/1		1	1	
M. baroni/Vohiparara		2			1	

See Fig. 1 for sites and Supplementary Information for GPS coordinates, elevations, and exact dates of collection. The probable classes and dietary source are indicated in the heading.

[a] Total content of alkaloids is major (+++) in each case (see definition in legend to Table 6). The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[b] Alkaloids reported for the first time. See Supplementary Information for characterization.

Ranomafana (Ranomafanakely location of Fig. 1). The presence of PTXs **237A**, **251D**, **307F′**, and **309A** and alloPTX **325A** in the combined skins from Mangevo (Table 8) and individual skins (Table 1) reflect similarities in alkaloid composition for these two populations of *M. baroni*. The absence of homoPTX **265N** and the presence of PTX **307G** in substantial amounts only in the combined skins represent differences for these two populations of *M. baroni*. The differences in alkaloids among the populations of combined skins from Mangevo and individual skins of *M. baroni* from Ranomafana, particularly the lack of detection of **307G** and **223B** in the 15 individual skins, likely reflect differences in the geographic location of the two frog populations (Fig. 1). The alkaloids from five populations of *M. baroni* in Tables 8 and 9 that seem most common were the putative mite alkaloids PTXs **251D**, **307F′**, **307G**, and **309A**, alloPTX **325A**, and 1,4-disubstituted quinolizidines **217A** and **231A**, whereas the most

Table 9 Profiles of "izidines" and other alkaloids for five populations of *M. baroni* from Mangevo (six skins), Andriabe (two skins), Tsinjoarivo (two skins), Vohindrazana (two skins), and Vohiparara (one skin), Jan–Feb 2003

Species/site	Izidines								
	Mites								
	217A (1,4-Q)	**217B** (5,8-I)	**231A** (1,4-Q)	**231K** (5,6,8-I)	**233A** (1,4-Q)	**235B″** (5,8-I)	**241F** (5,8-I)	**243C** (5,8-I)	**245B** (5,8-I)
M. baroni/Mangevo #1	2		2						
M. baroni/Andriabe	2	2	2	1	2	2	1/2	2	2/2
M. baroni/Tsingoarivo	3								
M. baroni/Vohindrazana	2		2				1	2	
M. baroni/Vohiparara	1	1	1/2						

Species/site	Izidines								
	Mites								
	245H (dehydro-5,8-I-I)	**247E** (5,8-I)	**247F** (5,8-I)	**247J** (izidine)	**251N** (5,8-I)	**251V** (5,6,8-I)	**253B** (5,8-I)	**257D** (1,4-Q)	**273A** (5,6,8-I)
M. baroni/Mangevo #1	1			1					
M. baroni/Andriabe							1		
M. baroni/Tsingoarivo		2						1	
M. baroni/Vohindrazana			1			1		1	1
M. baroni/Vohiparara					1				1

Species/site	Izidines						Spiros/tricyclics/unclass
	Mites		Ants				Millipedes/beetles/unknown
	279D (5,8-I)	**281I** (5,8-I)	**223B** (3,5-P)	**249A** (3,5 I)	**251O** (3,5-P)	**275C** (3,5-I)	Other Alkaloids
M. baroni/Mangevo #1			2	1	2	1	
M. baroni/Andriabe					2	2/2	Tri **245J** (2); unclass **307M**[a] (*1*)
M. baroni/Tsingoarivo	1			2		3/2	Tri **243G** (2), **245J** (*2*); unclass **357B** (1)
M. baroni/Vohindrazana	3/1	1		3	1	2/1	Unclass **323G** (2)
M. baroni/Vohiparara						1/1	Unclass **231J** (1), **235R** (1), **249P** (1)

See Fig. 1 for sites and Supplementary Information for GPS coordinates, elevations, and exact dates of collection. The probable classes and dietary source are indicated in the heading.

Total content of alkaloids is major (+++) in each case (see definition in legend to Table 6). The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Where two intensities are tabulated, two isomers are noted, and the intensities are in the order of elution from the GC column. Blanks indicate the alkaloid is not detected.

[a] Alkaloids reported for the first time. See Supplementary Information for characterization.

common putative ant alkaloids were the pyrrolizidine **251O** and indolizidine **275C**.

Combined Skin Alkaloid Analyses—M. bernhardi PTXs **237A** and **309A**, homoPTX **337A**, 1,4-disubstituted quinolizidines **217A** and **231A**, 5,8-disubstituted indolizidine **245A**, dehydro-5,8-I **245H**, "izidine" **247J**, and 3,5-disubstituted pyrrolizidines **223B** and **251O** occurred both in *M. bernhardi* from Mangevo and in a nearby population of *M. baroni* also from Mangevo (Table 10, data on *M. baroni* is repeated from Tables 8 and 9). The *M. bernhardi* also contained a remarkable number of 5,8-disubstituted indolizidines, 1,4-disubstituted quinolizidines, and unclassified alkaloids, of which only a few were detected in the nearby population of *M. baroni*.

Combined Skin Alkaloid Analyses—M. cowanii Two skins of *M. cowanii*, a species closely related to *M. baroni* (Chiari

Table 10 Alkaloid profiles for *Mantella baroni* (six skins) and *M. bernhardi* (eight skins) both from the Mangevo area and *M. cowani* (two skins) from Antoetra, Jan–Feb 2003

Species/site	Amount[a]	Pumiliotoxins/homopumiliotoxins										
		Mites										
		237A (PTX)	**251D** (PTX)	**251H** (deoxyPTX)	**265G** (PTX)	**267C** (PTX)	**277B** (PTX)	**291G** (PTX)	**293D** (deoxyPTX)	**307F′** (PTX)	**307G** (PTX)	**309A** (PTX)
M. baroni/ Mangevo #1	+++	3	2						1	2	2	3
M. bernhardi/ Mangevo #2	+++	1										2
M. cowanii/ Antoetra	+++		3	1		1	1	1		1	1	2

Species/site	Pumiliotoxins/homopumiliotoxins					Izidines						
	Mites											
	323A (PTX)	**323J**[b] (aPTX)	**325A** (aPTX)	**337A** (hPTX)	339D (aPTX)	**205A** (5,8-i)	**217A** (1,4-Q)	**217B** (5,8-I)	**219B** (1,4-Q)	**231A** (1,4-Q	**221I** (5,8-I)	**241F** (5,8-I)
M. baroni/ Mangevo #1		1	2	1			2			2		
M. bernhardi/ Mangevo #2				1	2/2	1	3/2		1	1	1	1/1
M. cowanii/ Antoetra	1	1	2					2				

Species/site	Izidines										Tricycles/unclass
	Mites						Ants				Beetles/unknown
	243B (5,8-I)	**245A** (5,8-I)	**245H** (dehydro-5,8-I)	**247J** (izidine)	**249O** (5,8-I)	**279F** (5,6,8-I)	**223B** (3,5-P)	**249A** (3,5-I)	**251O** (3,5-P)	**275C** (3,5-I)	Other
M. baroni/ Mangevo #1		1	1	1			2	1	2	1	
M. bernhardi/ Mangevo #2	1	1	1	1	1	1	3		2		Tri. **235M** (1), unclass: **275J** (2/2), **293J** (2), **323H** (2), **325C** (1), **339E** (3/3),**341D** (1), **369** (2), **371** (2/2)
M. cowanii/ Antoetra		1									Unclass. **231J** (1) **249P** (1), **390** (1)

The probable class and dietary source of each alkaloid are indicated in the headings (see abbreviations).
[a] Total content of alkaloids is major (+++) in each case, indicating a total ion chromatogram intensity of 10^4 or greater. The amounts of each alkaloid in the table are relative to one another in each sample with 3≥50% in relative ion intensity, 2=8–50% relative ion intensity, and 1<8% relative ion intensity. Blanks indicate the alkaloid is not detected. Where two intensities are tabulated, two isomers are noted and the intensities are in the order of elution from the GC column.
[b] Alkaloids reported for the first time. See Supplementary Information for characterization.

et al. 2005), contained a variety of PTXs (Table 10). The dominant alkaloid was PTX **251D**. An earlier study of *M. cowanii*, frogs of which were presumably collected near Antoetra, reported PTX **251D** as the predominant alkaloid (Daly et al. 1996). The current *M. cowanii* had remarkably few izidines, consisting only of significant amounts of the 5,8-disubstituted indolizidine **217B** and trace amounts of the 5,8-disubstituted indolizidine **245A**.

Number of Alkaloids vs. Frog Size for Three Mantellid Species There was a positive significant relationship between the number of alkaloids and frog size (measured as

Table 11 Summary of alkaloids detected in extracts of populations of *Mantella*

Table number		8 and 9					10	10
Mantella species		*M. baroni*					*M. bernhardi*	*M. cowanii*
Alkaloids		Man. 1	And.	Tsi.	Vdr.	Vpa.	Man. 2	Ant.
PTX	**237A**	x			x	x	x	
	251D	x	x		x	x		x
	265G							x
	267C					x		x
	277B							x
	291G							x
	307F	x	x	x/x/x	x/x/x	x		x
	307G	x	x	x	x			x
	309A	x	x	x	x/x	x	x	x
	323A							x
aPTX	**323J**[a]			x				x
	325A	x	x	x	x	x		x
	339D						x/x	
deoxy PTX	**251H**					x		x
	291E			x	x			
	293D			x/x	x/x			
	295C			x				
dm-PTX	**279L**[a]			x				
H2-PTX	**281F**				x			
hPTX	**265N**					x		
	323E			x	x			
	337A	x					x	
dm-hPTX	**267N**		x					
3,5-P	**223B**	x					x	
	251O	x	x		x		x	
3,5-I	**249A**	x		x	x			
	275C	x	x/x	x/x	x/x	x/x		
5,8-I	**205A**						x	
	217B		x			x		x
	221I						x	
	235B″		x					
	241F		x/x				x/x	
	243B						x	
	243C		x					
	245B		x/x				x	
	247E			x				
	247F				x			
	249O						x	
	251N					x		
	253B		x					
	279D			x	x/x			
	281I					x		
dh-5,8-I	**245H**	x					x	
5,6,8-I	**231K**		x					
	251V				x			
	273A				x	x		
	279F						x	
1,4-Q	**217A**	x	x	x	x	x	x/x	
	219B						x	
	231A	x	x		x	x/x	x	
	233A		x					
	257D			x	x			
Tricyclics	**235M**						x	

Table 11 (continued)

Table number		8 and 9					10	10
Mantella species		*M. baroni*					*M. bernhardi*	*M. cowanii*
Alkaloids		Man. 1	And.	Tsi.	Vdr.	Vpa.	Man. 2	Ant.
	243G			x				
	245J		x	x				
Izidines	**247J**						x	
Unclass.	**231J**					x		x
	235R					x		
	249P					x		x
	275J						x	
	293J						x	
	307M[a]		x					
	323G				x			
	323H						x	
	325C						x	
	339E						x	
	341D						x	
	357B			x				
	369						x	
	371						x	
	390							x

Structures for selected alkaloids are shown in Fig. 2 (see also Daly et al. 2005). For detailed distribution and quantitation data, see Tables 8, 9 and 10. See abbreviations for alkaloid classes.

[a] Previously undescribed alkaloids. Properties and tentative structures for some of these alkaloids are in the Supplementary Information.

Man. 1 Mangevo 1; *Man. 2* Mangevo 2; *And.* Andriabe; *Tsi.* Tsinjoarivo; *Vdr.* Vohindrazana; *Vpa.* Vohiparara; *Ant.* Antoetra, *dm-PTX* desmethyl-PTX, *H2-PTX* dihydro-PTX, *dm-hPTX* desmethyl-hPTX, *dh-5,8-l* dehydro-5,8-l.

SVL), when all three species of mantellids were examined together (Fig. 6; $P<0.001$; $R^2=0.37$). However, when each species was examined independently, only *M. baroni* had a positive significant relationship between the number of alkaloids and frog size (Fig. 6; $P<0.02$; $R^2=0.27$). There was no relationship between the number of alkaloids and frog size for *M. madagascariensis* and *M. bernhardi* (Fig. 6; $P=0.838$ and $P=0.975$, respectively).

Discussion

Alkaloid composition varied within and among species of the mantellid frogs examined in this study (see MDS plots of Figs. 3, 4 and 5). Differences in alkaloid composition within species were largely related to geographic location, with the same species differing significantly in alkaloid composition between locations. These findings suggest that there are differences in the availability of alkaloid-containing arthropods based on geographic location, which are likely influenced by differences in habitat (i.e., vegetation, leaf litter, forest structure, etc.) at each location. Differences in alkaloid composition among locations have been reported previously for various poison frogs, including frogs of the genera *Mantella* (Garraffo et al. 1993a; Daly et al. 1996; Clark et al. 2005, 2006), *Melanophryniscus* (Garraffo et al. 1993b; Mebs et al. 2005; Daly et al. 2007), and *Pseudophryne* (Daly et al. 1990; Smith et al. 2002), as well as the dendrobatid frogs (Daly et al. 1987; Myers et al. 1995; Saporito et al. 2006, 2007b; and numerous references within). Within the same location, marked individual differences in alkaloid composition of the same species were also observed in the present study. Although these differences were not as great as differences between locations, they certainly suggest that location and availability of alkaloid-containing arthropods are also important on small spatial scales. Insights into the factors that result in differences in alkaloid composition among individuals from the same geographic location remain a challenge for further research.

The early studies on the alkaloid composition for collections of a mantellid species [e.g., *M. aurantiaca*, *M baroni*, *M. crocea*, and *M. pulchra*] at the same location indicated that there can be marked differences in alkaloid composition over time (Garraffo et al. 1993a; Daly et al. 1996). Recently, further examples of apparent temporal variation for populations of *M. baroni* from Sahavondrana and Vatoharanana locations in the Ranomafana region have been reported (Clark et al. 2006). Such findings indicate

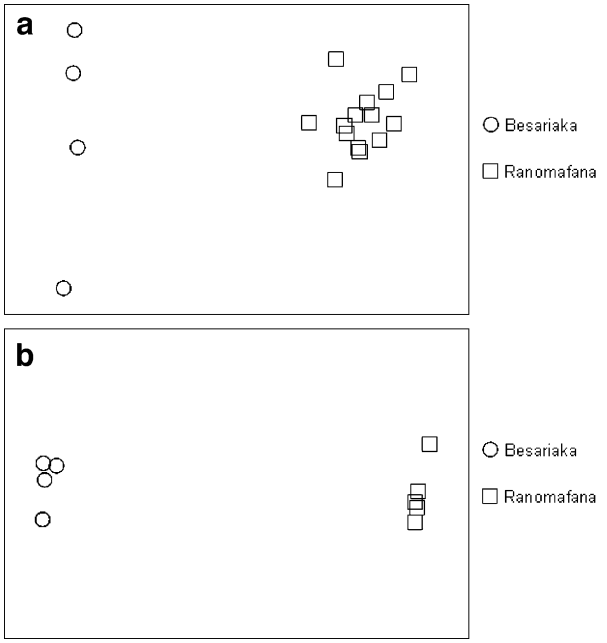

Fig. 3 **a** Multidimensional scaling plot of alkaloid composition in *M. baroni* from two different locations in Madagascar (stress=0.08). Alkaloid composition of *M. baroni* is significantly different between locations (Global R=0.99; P<0.001). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. One subadult frog from Ranomafana (Table 1, no. 128s) was removed from the analysis due to the small number of alkaloids (only two in trace amounts) detected relative to other frogs. **b** Multidimensional scaling plot of alkaloid composition in *M. madagascariensis* from two different locations in Madagascar (stress=0.01). Alkaloid composition of *M. madagascariensis* is significantly different between locations (Global R=1.0; P<0.008). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. One male frog from Ranomafana (Table 3, no. 112m) was removed from the analysis due to the absence of detectable alkaloids

that, in addition to geographic location, time also plays a prominent role in alkaloid variation of mantellid frogs. Temporal variation also has been reported for dendrobatid frogs (Daly et al. 1987, 2002; Saporito et al. 2006, 2007b) and bufonid toads (Daly et al. 2007). Such differences in alkaloid composition over time would likely be because of successional changes in habitat (i.e., vegetation, leaf-litter, forest structure, etc.), because of factors such as disturbance, which in turn lead to temporal shifts in alkaloid-containing arthropod availability (also see Daly et al. 2000; Saporito et al. 2006, 2007b).

Alkaloid profiles for three individuals of *M. baroni* collected in 2003 at Vatoharanana (see Fig. 1) were reported by Clark et al. (2005, 2006). Based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006), the major alkaloids in all three frogs (in order of elution from the GC column) were as follows: quinolizidine **217A**, indolizidine **217B**, PTX **237A** and PTX **309A**. Other alkaloids, present as significant constituents in one or two individuals, were quinolizidines **233A** and **257D**, PTXs **251D** and **307G**, indolizidine **275C**, and pyrrolizidine **251O**. Except for the last two putative ant alkaloids, all the major alkaloids are of putative mite origin. It should be noted that, often, the alkaloid peaks in the GC–MS traces are listed incorrectly in Table 1 of Clark et al. (2006). For example, PTX **309A**, a major alkaloid in the GC–MS traces for all three individuals, is listed as minor or trace in the table. The alkaloid composition for a combined sample of ten skins of *M. baroni* collected in Ranomafana in November of 1989 probably at or near the same Vatoharanana site was reported to consist mainly of PTX **309A** and 1,4-disubstituted quinolizidine **217A**, with significant amounts of PTX **237A**, 1,4-disubstituted quinolizidines **231A** and **233A**, and 5,8-disubstituted indolizidines **217B**, **243D**, and **245C** (Daly et al. 1996; see also Clark et al. 2006). Thus, with respect to the major alkaloid components, there did not appear to be major temporal changes.

A significant variation in alkaloid composition was observed among sympatric species sampled at the same location (see nMDS plots of Fig. 5a, b and Tables 1, 2, 3, and 4). *M. madagascariensis* and *M. baroni* were collected together at two different locations (Ranomafana [Ranoma-

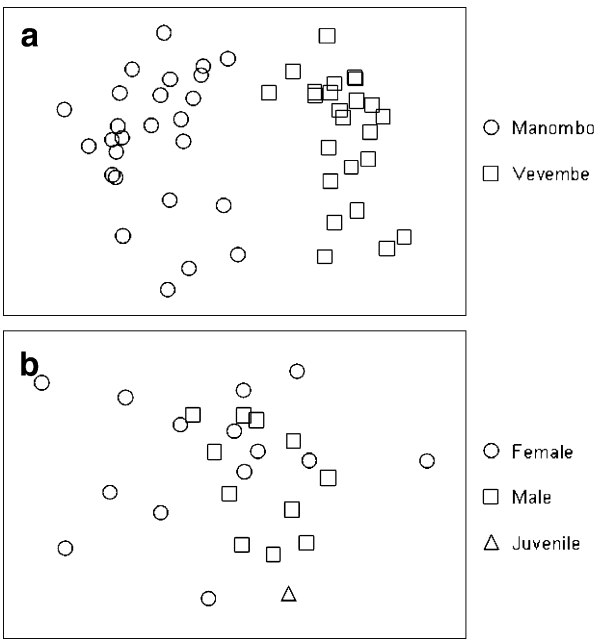

Fig. 4 **a** Multidimensional scaling plot of alkaloid composition in *M. bernhardi* from two different locations in Madagascar (stress=0.13). Alkaloid composition of *M. bernhardi* is significantly different between locations (Global R=0.83; P<0.001). *Each symbol* represents an individual frog from one of the two locations. The *distance between symbols* represents the difference in alkaloid composition. **b** Multidimensional scaling plot of alkaloid composition between sexes of *M. bernhardi* from Manombo (stress=0.17). Alkaloid composition is not significantly different between sexes (Global R=0.06; P=0.20). One of the frogs (Table 5, no. 413s) in the sample is a subadult. *Each symbol* represents an individual frog. The *distance between symbols* represents the difference in alkaloid composition

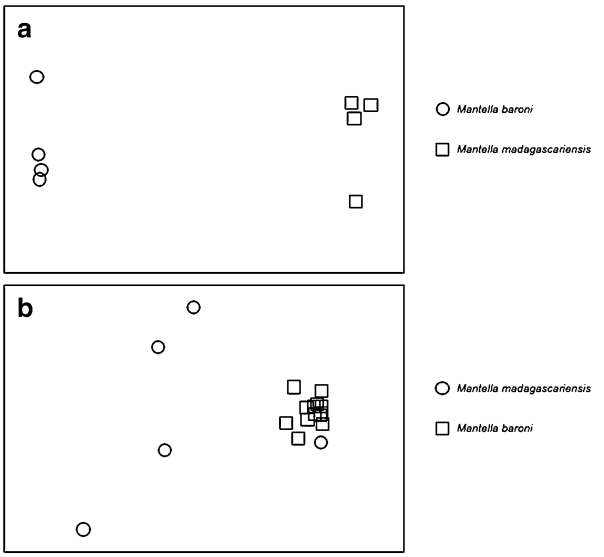

Fig. 5 **a** Multidimensional scaling plot of alkaloid composition in *Mantella baroni* and *M. madagascariensis* from Besariaka (stress= 0.01). Alkaloid composition is significantly different between *M. baroni* and *M. madagascariensis* (Global R=1.0; P<0.008). *Each symbol* represents an individual frog from one of the two species. The *distance between symbols* represents the difference in alkaloid composition. **b** Multidimensional scaling plot of alkaloid composition in *M. madagascariensis* and *M. baroni* from Ranomafana (stress= 0.1). Alkaloid composition is significantly different between *M. madagascariensis* and *M. baroni* (Global R=0.798, P<0.001). One male *M. madagascariensis* frog (Table 3, no. 112m) was removed from the analysis due to the absence of detectable alkaloids. One subadult *M. baroni* frog (Table 1, no. 128s) was removed from the analysis due to the small number of alkaloids (only two in trace amounts) detected relative to other frogs. *Each symbol* represents an individual frog from one of the two species. The *distance between symbols* represents the difference in alkaloid composition

fanakely] and Besariaka; see Fig. 1) and differed significantly between each other in alkaloid composition within each of the locations. As explained in "Materials and Methods," the two species may use partly different microhabitats at the Ranomafana site, but apparently not at the Besariaka site. Differences in alkaloid composition among these two sympatric species suggest that, at least in the case of Besariaka, there are either differences in feeding or in the sequestering systems responsible for uptake of alkaloids. Interestingly, these two sympatric species were originally considered different color morphs of the same species, but recent genetic analyses have suggested that they are separate, not even closely related species that belong to different species groups (Chiari et al. 2004, 2005). Certainly, the differences in alkaloid composition among these two sympatric species complement the findings that these are two different species. Future dietary analyses and alkaloid feeding experiments with these two species would provide more information as to whether or not alkaloid variation is because of differences in feeding or in the uptake system that sequesters dietary alkaloids. Whether there may also be changes in dietary habits with the age of the frogs is unknown.

Alkaloid composition for one individual of *M. madagascariensis*, which was collected in 2003 along with six *M. baroni* from Vohiparara (at Kidonavo bridge in Fig. 1), has been reported (Clark et al. 2005, 2006). Based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006), the major alkaloids in the *M. madagascariensis* sample (in order of elution from the GC column) appear to be the following: indolizidines **203A**, **205A**, and **217B**; quinolizidine **217A**; an unidentified (not listed by Clark et al. 2006) alkaloid, which is likely the dehydro-5,8-disubstituted indolizidine **205L**, reported as a

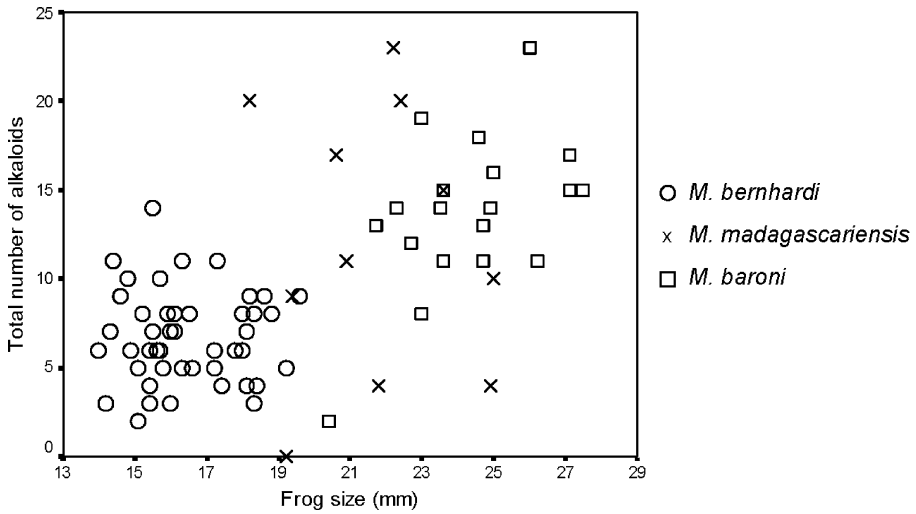

Fig. 6 The number of alkaloids versus frog size for all three mantellid species. When all three species are examined together, there is a positive significant relationship between number of alkaloids and frog size (P<0.001; R^2=0.37). However, when each species is examined independently, only *M. baroni* has a positive significant relationship between number of alkaloids and frog size (P value=0.02; R^2=0.27). There is no relationship between the number of alkaloids and frog size for *M. madagascariensis* and *M. bernhardi*

new alkaloid for the first time in the present study; homoPTX **265N**; indolizidine **275E**; and PTX **267C** (designated in error in the GC–MS legend of Clark et al. 2006 as deoxyPTX **267N**). All of these major alkaloids are probably of mite origin. The profiles of alkaloids in the six *M. baroni* from the same site were quite different from the one *M. madagascariensis* frog, based on the GC–MS chromatograms in the Supplementary Information of Clark et al. (2006). The dominant alkaloids were PTXs **251D**, **307G**, and **309A**, homoPTX **265N**, and for certain individuals pyrrolizidine **251O** and indolizidine **273A**. It should be noted that, often, minor or trace peaks in the GC–MS traces are listed in Table 1 of Clark et al. (2006) as major alkaloids (see critical comments on the Clark data in the Supplementary Information for the present report).

Differences in alkaloid composition between sexes have been suggested for other poison frogs (see Saporito et al. 2006, 2007b); however, because of small sample sizes and the confounding effects of location, this has not been specifically examined for any species. In the present study, an examination of alkaloid profiles in *M. bernhardi* from Manombo (Table 5: 11 males, 14 females) provided an opportunity to examine alkaloid composition between sexes. Alkaloid composition was not found to be significantly different between sexes (see nMDS plot in Fig. 4b; $P=0.20$), and therefore, males and females could not be distinguished on the basis of their alkaloid composition. However, although overall alkaloid composition was not significantly different between sexes, there do appear to be certain alkaloids that are related to sex in *M. bernhardi*. These alkaloids include the 1,4-disubstituted quinolizidine **231A** (present in six males in minor amounts, but in only one female and only in a trace amount), the 3,5-disubstituted indolizidine **249A** (present in 11 females, but in only 4 males), and the 3,5-disubstituted indolizidine **275C** (present in six females, but in only one male). The presence of **249A** and **275C** (mainly in females) is presumably because of sequestration from myrmicine ants, whereas the presence of **231A** (mainly in males) presumably is because of sequestration from oribatid mites. The differences in the presence of certain alkaloids observed between sexes of *M. bernhardi* may be explained by differences in diet (preference or availability based on behavior) between sexes. Breeding of this species occurs in swamps or near ponds in the forest, where males call from particular positions on the ground, close to the water, probably delimiting at least short-term territories. Consequently, females probably have larger home ranges than males, at least during the reproductive season, and are therefore presumably encountering a more diverse array of alkaloid-containing arthropods, whereas males are more subject to local availability of alkaloid-containing arthropods. In addition, female *Mantella* frogs are typically larger (SVL) than male frogs, which may suggest that they would consume more prey and/or have a larger capacity for storage of alkaloids. The tendency for larger females is particularly clear in *M. bernhardi*, where in the populations from Manombo and Vevembe, the males can be quite small (indeed being the smallest adult *Mantella* known) and the females distinctly larger (males 14–18 vs. females 17–20 mm SVL). In *M. bernhardi* from Manombo (Table 5), none of the 11 males had total alkaloid amounts classified as major (+++), whereas 11 of the 14 females did. However, the importance of location with respect to total amount of alkaloids is clear from a comparison to the *M. bernhardi* from Vevembe (Table 6), where of the 25 frogs (mostly males), all but one male and one female had alkaloid amounts classified as major (+++). Unfortunately, nothing is known as to whether any of the mantellid frogs have reached the maximum alkaloid storage capacity of the cutaneous (poison) glands. Furthermore, it should be noted that any general conclusions as to sex differences should be treated as tentative, because of the preponderance of males in all collections except *M. bernhardi* from Manombo, in which differences between sexes were detected with respect to certain alkaloids and perhaps with respect to amounts.

Poison glands are known to increase in diameter with increases in size of a poison frog, as has been shown for the dendrobatid poison frog *Oophaga pumilio* (Saporito et al., unpublished data), suggesting that larger frogs might have a larger storage capacity for alkaloids. The total amount of alkaloids is one measure of capacity, whereas the total number of alkaloids present in an individual frog can be thought of as a rough measure of the diversity of alkaloid-containing arthropods consumed by an individual over a lifetime. Within a species, larger poison frogs can be presumed to be older than smaller frogs. On the basis of these presumptions, it might be expected that the number of alkaloids (diversity) would be greater in larger mantellid frogs, both within and among species. When the three mantellid species were examined together, the number of alkaloids (including trace alkaloids) was positively correlated with frog size (Fig. 6; $P<0.001$; $R^2=0.37$), and in general, larger frog species tended to have a larger number of alkaloids. Clark et al. (2006) also reported a positive relationship between frog size (*M. baroni* vs. *M. bernhardi*) and the total number of alkaloids. However, in the present study, when each mantellid species is examined separately, size is related to the number of alkaloids only in *M. baroni* (Fig. 6; $P=0.002$; $R^2=0.27$). The lack of a strong relationship between frog size and the number of alkaloids within a mantellid species suggests that frog size (and probably age) is not the main determinant of total alkaloid number (alkaloid diversity). However, the differences between species may be at least partly explained by their different sizes and ages. Data on the age of specimens in

wild *Mantella* populations are needed to test this hypothesis. Inclusion of additional species and increased sample sizes are necessary to determine the extent by which size influences the diversity of alkaloids within a mantellid species. Analyses of alkaloids in individuals of two relatively small mantellid frogs, namely, *M. aurantiaca* and *M. milotympanum* are in progress.

The report by Clark et al. (2006) proposed that mantellid frogs from relatively pristine sites have a greater number of alkaloids than those from disturbed sites, suggesting that levels of disturbance are directly related to alkaloid diversity in poison frogs. Although differences in disturbance likely play a role in the abundance and distribution of alkaloid-containing arthropods, which likely influences alkaloid compositions in frogs, we do not think that the data presented in Clark et al. (2006) are compelling enough to make the claim that disturbance negatively influences alkaloid diversity in poison frogs. The proposal by Clark et al. (2006) is based on a comparison of the total number of alkaloids among very limited numbers of individuals of *M. baroni* from three different geographic locations (ranked as pristine, slightly disturbed, and disturbed). Given the amount of alkaloid variation within populations of poison frogs (illustrated in this study; also see Saporito et al. 2006), it is not clear what conclusions can accurately be made with such small sample sizes. In addition, Clark et al. 2006 were not justified in including four individuals from a disturbed site in which alkaloids were not obtained by extraction of the skin but solely by electrical stimulation of the skin (transcutaneous amphibian stimulation). Other critical comments on the data of Clark et al. (2006) are included in the present Supplementary Information.

Alkaloid compositions for *M. baroni* from seven sites, some relatively undisturbed and some disturbed, had been previously reported (Daly et al. 1996). A discussion of that study was neglected by Clark et al. (2006). The *M. baroni* from four disturbed areas had an average of 20 alkaloids per site, whereas those from three relatively undisturbed areas had an average of 17 alkaloids per site (Daly et al. 1996). Analyses were of combined skin samples from each site. In addition, the gas chromatograms presented in Daly et al. (1996) and Garraffo et al. (1993a) do not show any clear relationship between the amounts of alkaloids for *M. baroni* and the disturbance of a collection site. Of the four disturbed sites, two show major amounts of alkaloids, and two show minor amounts. Of the three relatively undisturbed sites, one shows major amounts, the other two, minor (Garraffo et al. 1993a; Daly et al. 1996) Thus, neither the number of alkaloids nor the amounts of alkaloids appeared to correlate with the degree of disturbance at a location.

The present alkaloid analyses of *M. baroni* also do not support the conclusion that frogs from an undisturbed collection site will have a larger number or a greater diversity of alkaloids. However, it should be noted that sample sizes in the present study also were small and, therefore, any conclusion should be treated as tentative. For the sites of the five combined skin collections of *M. baroni*, Mangevo and Vohiparara in the Ranomafana National Park are considered relatively undisturbed sites, whereas Andriabe, Tsinjoarivo, and Vohindrazana are considered disturbed sites (see Table 7). The two relatively undisturbed sites have, respectively, a total of 17 alkaloids (of which ten are major/minor) and 19 alkaloids (of which four are major/minor). The total number of alkaloids for the disturbed sites, in the order listed above, is as follows: 24 alkaloids (of which 17 are major/minor), 22 alkaloids (of which 12 are major/minor); and 30 alkaloids (of which 12 are major/minor). A comparison of the number of alkaloids in *M. baroni* between these sites clearly demonstrates that there are not significantly more alkaloids in frogs from the undisturbed sites. Furthermore, in the present study, the 15 individual skins of *M. baroni* from Ranomafana (an undisturbed site) contained an average of 7.0 major/minor alkaloids per frog (average including trace alkaloids, 16), whereas the four individuals from Besariaka (a disturbed site) contained an average of 8.3 major/minor alkaloids (average including trace alkaloids, 13). Once again, there is no marked difference in the number of alkaloids (a measure of alkaloid diversity) between an undisturbed and a disturbed site for *M. baroni*. At the present time, the generalization by Clark et al. (2006) that a pristine site will yield frogs with either a greater diversity or amount of alkaloids cannot be supported with the available data.

The current study resulted in the detection of 46 alkaloids (including isomers) in 19 individuals of *M. baroni* from two different populations, 56 alkaloids (including isomers) in 11 individuals of *M. madagascariensis* from two different populations, and 48 alkaloids (including isomers) in 51 individuals of *M. bernhardi* from two different populations (Tables 1, 2, 3, 4, 5, and 6). Summaries of alkaloid composition of extracts of individuals from six populations (Table 7) and of extracts from combined skins (Table 11) indicate the marked dependence of alkaloid compositions on the geographic location of the collections.

A variety of factors undoubtedly affect the complex differences in alkaloid composition detected in mantellid frogs. The geographic location of mantellid species and associated differences in habitat between locations, as well as the availability of alkaloid-containing arthropod prey items within each habitat are likely the most important factors in explaining variation in alkaloid composition. Temporal differences in alkaloid composition are likely because of successional changes in habitat and the associated shifts in alkaloid-containing prey availability. In addition, difference in prey electivity and/or foraging

behavior, which may be correlated in some cases with certain species, sexes, age, and/or size of mantellids, may also play a role in mantellid alkaloid variation. Finally, it is possible that some of the variation in alkaloid composition is because of differences in the alkaloid uptake systems among different species, which are presumably involved in sequestration and retention of alkaloids. A simplistic analysis of the factors involved in explaining variation in alkaloid composition is clearly not possible, but the various confounding factors are amenable to further study.

There is currently no information on whether or not there is a preference for sequestration and/or storage of one alkaloid structural class over another in mantellid frogs. A series of controlled and extensive feeding experiments will be required to clarify these points. However, as yet, the resources, which include a large number of captive-raised frogs, have not been available. Several of the mantellid species are now at risk (Andreone et al. 2005; Vieites et al. 2006), which may preclude extensive studies of mantellids similar to the present study. The wide range of amounts of alkaloids present in individual frogs from a single collection site is perhaps the most important current observation and has required these extensive single skin analyses to verify. The rare complete absence of alkaloids or the presence of only a trace amount of alkaloids in individual frogs is somewhat puzzling. It is possible that such frogs have expelled most of their alkaloids just before capture; however, it is unlikely that all of the alkaloids are expelled as a defensive strategy. Therefore, a deficient or possibly absent uptake system for these rare individuals is the most likely explanation at the moment. Those frogs without alkaloids or with only trace amounts of alkaloids appear to occur randomly in every collection.

Further research is clearly needed to understand the complex trophic relationships between alkaloid-containing mites, ants, millipedes, and beetles and chemical defense in poison frogs. Crucial to such an understanding will be (1) the distribution and composition of alkaloids within oribatid mites and myrmicine ants, which appear likely to be the principal dietary sources of poison frog alkaloids, (2) the factors that affect abundance/availability of such prey, and (3) the electivity of frogs toward such alkaloid-containing prey. Multiple classes of alkaloids do occur together in mites, but can also occur alone (Saporito et al. 2007b). In ants, alkaloid composition differs not only with species, but also with caste and age (Deslippe and Guo 2000; Torres et al. 2001; Saporito et al. 2004). Studies of alkaloid composition in different species of Madagascan mites, the occurrence of such mites in stomach contents of mantellid frogs, and correlations between alkaloids of mites, ants, and frogs from the same site are required.

Analyses of alkaloid profiles in more than 80 individual frogs of the *M. milotympanum* group (Vences et al. 1999), namely, *M. aurantiaca*, *M. milotympanum*, and *M. crocea*, are in progress (Daly and Vences et al., unpublished data). In parallel studies, alkaloid profiles have been obtained for extracts of combined frogs of 13 species of *Mantella* from more than 40 different sites in Madagascar (N. R. Andriamaharavo, M. Andriantsiferana et al., unpublished data), and the profiles are being analyzed for further insights into the factors that determine alkaloid compositions in these poison frogs.

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References

ANDREONE, F., CADLE, J. E., COX, N., GLAW, F., NUSSBAUM, R. A., RAXWORTHY, C. J., STUART, S. N., VALLAN, D., and VENCES, M. 2005. Species review of amphibian extinction risks in Madagascar: conclusions from the global amphibian assessment. *Cons. Biol.* 19:1790–1802.

CHIARI, Y., VENCES, M., VIEITES, D. R., RABEMANANJARA, F., BORA, P., RAMILIJAONA RAVOAHANGIMALALA, O., and MEYER, A. 2004. New evidence for parallel evolution of colour patterns in Malagasy poison frogs (*Mantella*). *Mol. Ecol.* 13: 3763–3774.

CHIARI, Y., ANDREONE, F., VENCES, M., and MEYER, A. 2005. Genetic variation of an endangered Malagasy frog, *Mantella cowani*, and its phylogeographic relationship to the widespread *M. baroni*. *Cons. Genet*. 6:1041–1047.

CLARK, V. C., RAXWORTHY, C. J., RAKOTOMALALA, V., SIERWALD, P., and FISHER, B. L. 2005. Convergent evolution of chemical defense in poison frogs and arthropod prey between Madagascar and the Neotropics. *Proc. Natl. Acad. Sci. U S A* 102:11617–11622.

CLARK, V. C., RAKOTOMALALA, V., RAMILIJAONA, O., ABRELL, L., and FISHER, B. L. 2006. Individual variation in alkaloid content of poison frogs of Madagascar (*Mantella*; Mantellidae). *J. Chem. Ecol.* 32–2219–2233.

CLARKE, K. R. and WARWICK, R. M. 2001. Change in marine communities: an approach to statistical analysis and interpretation, 2nd edition. PRIMER-E: Plymouth.

DALOZE, D., BRAEKMAN, J. C., and PASTEELS, J. M. 1995. Ladybug defense alkaloids: structural, chemotaxonomic, and biosynthetic aspects. *Chemoecology* 5/6:173–183.

DALY, J. W. 1998. Thirty years of discovering arthropod alkaloids in amphibian skin. *J. Nat. Prod.* 61:162–172.

DALY, J. W., MYERS, C. W., and WHITTAKER, N. 1987. Further classification of skin alkaloids from Neotropical poison frogs (Dendrobatidae), with a general survey of toxic/noxious substances in the Amphibia. *Toxicon* 25:1023–1095.

DALY, J. W., GARRAFFO, H. M., PANNELL, L. K., and SPANDE, T. F. 1990. Alkaloids from Australian frogs (Myobatrachidae): Pseudophyrnamines and pumiliotoxins. *J. Nat. Prod*. 53:407–421.

DALY, J. W., SECUNDA, S. GARRAFFO, H. M., SPANDE, T. F., WISNIESKI, A. NISHIHIRA, C., and COVER, J. F., JR. 1992. Variability in alkaloid profiles in Neotropical poison frogs (Dendrobatidae): genetic versus environmental determinants. *Toxicon* 30:887–898.

DALY, J. W., GARRAFFO, H. M., SPANDE, T. F., JARAMILLO, C., and RAND, S. A. 1994a. Dietary source for skin alkaloids of poison frogs (Dendrobatidae)? *J. Chem. Ecol*. 20:943–955.

DALY, J. W., SECUNDA, S. I., GARRAFFO, H. M., SPANDE, T. F., WISNIESKI, A., and COVER, J. F., Jr. 1994b. An uptake system for dietary alkaloids in poison frogs (Dendrobatidae). *Toxicon* 32:657–663.

DALY, J. W., ANDRIAMAHARAVO, N. R., ANDRIANTSIFERANA, M., and MYERS, C. W. 1996. Madagascan poison frogs (*Mantella*) and their skin alkaloids. *Amer. Mus. Novitates* 3177:1–34.

DALY, J. W., GARRAFFO, H. M., HALL, G. S. E., and COVER, J. F., JR. 1997. Absence of skin alkaloids in captive-raised Madagascan mantelline frogs (*Mantella*) and sequestration of dietary alkaloids. *Toxicon* 35:1131–1135.

DALY, J. W., GARRAFFO, H. M., JAIN, P., SPANDE, T. F., SNELLING, R. R., JARAMILLO, C., and RAND, S. A. 2000. Arthropod-frog connection: decahydroquinoline and pyrrolizidine alkaloids common to microsympatric myrmicine ants and dendrobatid frogs. *J. Chem. Ecol.* 26:73–85.

DALY, J. W., KANEKO, T., WILHAM, J., GARRAFFO, H. M., SPANDE, T. F., ESPINOSA, A., and DONNELLY, M. A. 2002. Bioactive alkaloids of frog skin: combinatorial bioprospecting reveals that pumiliotoxins have an arthropod source. *Proc. Natl. Acad. Sci. U S A* 99:13996–14001.

DALY, J. W., SPANDE, T. F., and GARRAFFO, H. M. 2005. Alkaloids from amphibian skin: a tabulation of over eight-hundred alkaloids. *J. Nat. Prod.* 68:1556–1575.

DALY, J. W., WILHAM, J. M., SPANDE, T. F., GARRAFFO, H. M., GIL, R. R., SILVA, G. L., and VAIRA, M. 2007. Alkaloids in bufonid toads (*Melanophryniscus*): Temporal and geographic determinants for two Argentinian species. *J. Chem. Ecol.* 33:871–887.

DESLIPPE, R. J. and GUO Y. 2000. Venom alkaloids of fire ants in relation to worker size and age. *Toxicon* 38:223–232.

GARRAFFO, H. M., CACERES, J., DALY, J. W., and SPANDE, T. F. 1993a. Alkaloids in Madagascan frogs (*Mantella*): pumiliotoxins, indolizidines, quinolizidines, and pyrrolizidines. *J. Nat. Prod.* 56:1016–1038.

GARRAFFO, H. M., SPANDE, T. F., DALY, J. W., BALDESSARI, A., and GROS, E. G. 1993b. Alkaloids from bufonid toads (*Melanophryniscus*): decahydroquinolines, pumiliotoxins and homopumiliotoxins, indolizidines, pyrrolizidines, and quinolizidines. *J. Nat. Prod.* 56:357–373.

GLAW, F., and VENCES, M. 2006. Phylogeny and genus-level classification of mantellid frogs. *Org. Divers. Evol.* 6:236–253.

JONES, T. H., GORMAN, J. S. T., SNELLING, R. R., DELABIE, J. H. Q., BLUM, M. S., GARRAFFO, H. M., JAIN, P., DALY, J. W., and SPANDE, T. F. 1999. Further alkaloids common to ants and frogs: decahydroquinolines and a quinolizidine. *J. Chem. Ecol.* 25:1179–1193.

MEBS, D., POGODA, W., MANEYRO, R., and KWET, A. 2005. Studies on the poisonous skin secretions of individual red bellied toads, *Melanophryniscus montevidensis* (Anura, Bufonidae), from Uruguay. *Toxicon* 46:641–650.

MICHEL, P., RASSAT, A., DALY, J. W., and SPANDE, T. F. 2000. A stereospecific synthesis of (+/−)-5,8-disubstituted indolizidines and (+/−)-1,4-disubstituted quinolizidines found in poison frog skins. *J. Org. Chem.* 65:8908–8918.

MYERS, C. W., and DALY, J. W. 1976. Preliminary evaluation of skin toxins and vocalizations in taxonomic and evolutionary studies of poison-dart frogs (Dendrobatidae). *Bull. Am. Mus. Nat. Hist*. 157:175–262.

MYERS, C.W., DALY, J.W., GARRAFFO, H.M., WISNIESKI, A., and COVER, J., Jr 1995. Discovery of the Costa Rican frog *Dendrobates granuliferus* in sympatry with *Dendrobates pumilio*, and comments on taxonomic use of skin alkaloids. *Am. Mus. Novitates* 3144:1–21.

PINTAK, T. M., VENCES, M., GLAW, F., and BÖHME, W. 1998. Comparative chromosome morphology of Malagasy poison frogs (Amphibia: Ranidae: *Mantella*). *Folia Zool*. 47:197–204.

SAPORITO, R. A., DONNELLY, M. A., HOFFMAN, R. L., GARRAFFO, H. M., and DALY, J. W. 2003. A siphonotid millipede (*Rhinotus*) as the source of spiropyrrolizidine oximes of dendrobatid frogs. *J. Chem. Ecol*. 29:2781–2786.

SAPORITO, R. A., GARRAFFO, H. M., DONNELLY, M. A., EDWARDS, A. L., LONGINO, J. T., and DALY, J. W. 2004. Formicine ants: an arthropod source for the pumiliotoxin alkaloids of dendrobatid poison frogs. *Proc. Natl. Acad. Sci. USA* 101:8045–8050.

SAPORITO, R. A., DONNELLY, M. A., GARRAFFO, H. M., SPANDE, T. F., and DALY, J. W. 2006. Geographic and seasonal variation in alkaloid-based chemical defenses of *Dendrobates pumilio* from Bocas del Toro, Panama. *J. Chem. Ecol.* 32:795–814.

SAPORITO, R. A., DONNELLY, M. A., NORTON, R. A., GARRAFFO, H. M., SPANDE, T. F., and DALY, J. W. 2007a. Oribatid mites as a major dietary source for alkaloids in poison frogs. *Proc. Natl. Acad. Sci. U S A* 104:8885–8890.

SAPORITO, R. A., DONNELLY, M. A., JAIN, P., GARRAFFO, H. M., SPANDE, T. F., and DALY, J. W. 2007b. Spatial and temporal patterns of alkaloid variation in the poison frog *Oophaga pumilio* in Costa Rica and Panama over 30 years. *Toxicon* 50: 757–778.

SCHAEFER, H. C., VENCES, M., and VEITH, M. 2002. Molecular phylogeny of Malagasy poison frogs, genus *Mantella* (Anura: Mantellidae): homoplastic evolution of colour pattern in aposematic amphibians. *Org. Divers. Evol.* 2:97–105.

SMITH, B. P., TYLER, M. J., KANEKO, T., GARRAFFO, H. M., SPANDE, T. F., and DALY, J. W. 2002. Evidence of biosynthesis of pseudophrynamine alkaloids by an Australian myobatrachid frog (*Pseudophryne*) and for sequestration of dietary pumiliotoxins. *J. Nat. Prod.* 65:439–447.

TAKADA, W., SAKATA, T., SHIMANO, S., ENAMI, Y., MORI, N., NISHIDA, R., and KUWAHARA, Y. 2005. Scheloribatid mites as the source of pumiliotoxins in dendrobatid frogs. *J. Chem. Ecol.* 31:2403–2415.

TORRES, J.A., ZOTTIG, V. E., CO, J. E., JONES, T. H., and SNELLING, R. R. 2001. Caste specific alkaloid chemistry of *Solenopsis maboya* and *S. torresi* (Hymenoptera: Formicidae). *Sociobiology* 37:579–584.

VENCES, M., and KNIEL, C. 1998. Mikrophage und myrmecophage Ernährungsspezialisierung bei madagassischen Giftröschen der Gattung *Mantella*. *Salamandra* 34:245–254.

VENCES, M., GLAW, F., and BÖHME, W. 1998. Evolutionary correlates of microphagy in alkaloid-containing frogs (Amphibia: Anura). *Zool. Anz.* 236:217–230.

VENCES, M., GLAW, F., and BÖHME, W. 1999. A review of the genus *Mantella* (Anura, Ranidae, Mantellinae): taxonomy, distribution and conservation of Malagasy poison frogs. *Alytes* 17:3–72.

VENCES, M., CHIARI, Y., RAHARIVOLOLONIAINA, L., and MEYER, A. 2004. High mitochondrial diversity within and among populations of Malagasy poison frogs. *Mol. Phyl. Evol.* 30:295–307.

VIEITES, D. R., CHIARI, Y., VENCES, M., ANDREONE, F., RABEMANANJARA, F., BORA, P., NIETO-ROMÁN, S., and MEYER, A. 2006. Mitochondrial evidence for distinct phylogeographic units in the endangered Malagasy poison frog *Mantella bernhardi*. *Mol. Ecol.* 15:1617–1625.

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Prey and Non-prey Arthropods Sharing a Host Plant: Effects on Induced Volatile Emission and Predator Attraction

Jetske G. de Boer · Cornelis A. Hordijk · Maarten A. Posthumus · Marcel Dicke

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Abstract It is well established that plants infested with a single herbivore species can attract specific natural enemies through the emission of herbivore-induced volatiles. However, it is less clear what happens when plants are simultaneously attacked by more than one species. We analyzed volatile emissions of lima bean and cucumber plants upon multi-species herbivory by spider mites (*Tetranychus urticae*) and caterpillars (*Spodoptera exigua*) in comparison to single-species herbivory. Upon herbivory by single or multiple species, lima bean and cucumber plants emitted volatile blends that comprised mostly the same compounds. To detect additive, synergistic, or antagonistic effects, we compared the multi-species herbivory volatile blend with the sum of the volatile blends induced by each of the herbivore species feeding alone. In lima bean, the majority of compounds were more strongly induced by multi-species herbivory than expected based on the sum of volatile emissions by each of the herbivores separately, potentially caused by synergistic effects. In contrast, in cucumber, two compounds were suppressed by multi-species herbivory, suggesting the potential for antagonistic effects. We also studied the behavioral responses of the predatory mite *Phytoseiulus persimilis*, a specialized natural enemy of spider mites. Olfactometer experiments showed that *P. persimilis* preferred volatiles induced by multi-species herbivory to volatiles induced by *S. exigua* alone or by prey mites alone. We conclude that both lima bean and cucumber plants effectively attract predatory mites upon multi-species herbivory, but the underlying mechanisms appear different between these species.

Keywords Food webs · Indirect defense · Methyl salicylate · Terpenes · Tritrophic interactions · GC-MS · Signal transduction

J. G. de Boer (✉) · M. Dicke
Laboratory of Entomology, Wageningen University,
P.O. Box 8031, 6700 EH
Wageningen, The Netherlands
e-mail: j.g.de.boer@rug.nl
URL: www.insect-wur.nl

C. A. Hordijk
Netherlands Institute for Ecology, Centre for Limnology,
P.O. Box 1299, 3600 BG
Maarssen, The Netherlands

M. A. Posthumus
Laboratory of Organic Chemistry, Wageningen University,
P.O. Box 8026, 6700 EG
Wageningen, The Netherlands

Present address:
J. G. de Boer
Evolutionary Genetics, Centre for Ecological and Evolutionary Studies, University of Groningen,
P.O. Box 14, 9750 AA Haren, The Netherlands

Introduction

Plants can employ arthropod natural enemies and defend themselves against herbivorous insects and mites. Such arthropod natural enemies can have an impact on local herbivore populations and thereby also on plant fitness (Sabelis and van der Meer 1986; Dicke and Sabelis 1989; Pels and Sabelis 1999; van Loon et al. 2000; Fritzsche Hoballah and Turlings 2001; Kessler and Baldwin 2001). Plants can promote the effectiveness of natural enemies by providing alternative food, shelter, or volatiles (Price et al. 1980; Dicke and Sabelis 1988). The emission of herbivore-induced plant volatiles (HIPV) has been demonstrated in several families (e.g., Dicke et al. 1990a; Turlings et al.

1990; van den Boom et al. 2004; van Poecke and Dicke 2004). Many parasitoid wasps, predatory mites, and predatory insects use HIPV to locate their prey or hosts (reviewed in Dicke 1999; Dicke and Vet 1999; Sabelis et al. 1999). HIPV are thus thought to have an important influence on the interactions between plants, herbivores, and natural enemies in food webs (Turlings et al. 1995; Dicke and Vet 1999).

To date, studies on the production of HIPV by plants and the responses of natural enemies to these have been carried out mostly for systems with one species of plant, herbivore, and natural enemy. Yet, in the field, most plants are likely to be attacked by several herbivore species, or by herbivores and pathogens, at the same time. It is difficult to predict whether or not changes in HIPV blends upon multi-species herbivory affect attraction of natural enemies, and in what direction. Several studies have attempted to fill this gap by analyzing the chemical composition of volatile blends upon multi-species infestation of plants (Shiojiri et al. 2001; Cardoza et al. 2002; Rodriguez-Saona et al. 2003; Rostás et al. 2006; Moayeri et al. 2007; Rasmann and Turlings 2007; Soler et al. 2007). For instance, Shiojiri et al. (2000) showed that the parasitoid wasp *Cotesia plutellae* prefers the HIPV blend induced by its host *Plutella xylostella* to the blend induced by multi-species herbivory by its host and the non-host caterpillar *Pieris rapae* although volatile blends are similar (Shiojiri et al. 2001). *Cotesia glomerata*, on the other hand, prefers HIPV blends induced by its host *P. rapae* and non-host caterpillars to blends induced by one caterpillar species feeding alone (Shiojiri et al. 2000; Vos et al. 2001). *Cotesia marginiventris* and *Microplitis rufiventris* do not discriminate between the multi-species-induced HIPV blend of plants infested with their host (beet armyworm caterpillars) and a plant pathogen or plants infested with their host alone, although the pathogen significantly suppresses volatile induction (Rostás et al. 2006). Similarly, *C. glomerata* does not discriminate between mustard plants infested with a root herbivore and its host, or plants infested with its host alone (Soler et al. 2007). The parasitoid *C. marginiventris* and the entomopathogenic nematode *Heterorhabditis megidis* exhibited a reduced attraction to volatiles from maize plants that were infested by a non-host in addition to their host, although the non-host was feeding on different tissues from the host, i.e., leaves vs. roots (Rasmann and Turlings 2007).

In this study, we investigated the effects of multi-species herbivory by spider mites (*Tetranychus urticae*) and beet armyworm caterpillars (*Spodoptera exigua*) on volatile emissions by two different plant species: lima bean (*Phaseolus lunatus*) and cucumber (*Cucumis sativus*), and evaluated the responses of *Phytoseiulus persimilis*, a specialist natural enemy of spider mites. Both herbivore species are known to induce volatile emission in several plant species, and remarkable differences in HIPV blend composition have been found in lima bean infested by either of these herbivores (e.g., Ozawa et al. 2000; Schmelz et al. 2003; de Boer et al. 2004). We statistically compared volatile emission by multi-species-infested plants with the sum of volatile emissions of plants infested with either herbivore species alone to evaluate the presence of potentially antagonistic or synergistic effects among signal transduction pathways (e.g., Engelberth et al. 2001). Plant responses to multi-species herbivory may depend on feeding modes of the herbivore species involved (Walling 2000), but whether or not plant species differ in these responses is unknown. Therefore, we compared two plant species in their relative responses to multi-species vs. single-species herbivory by using the same two herbivore species to attack both plant species. We also studied the olfactory responses of predatory mites (*P. persimilis*). In nature, plants are commonly attacked by more than one herbivore. Knowledge on natural enemy responses to volatile blends induced by multi-species vs. single-species herbivory is essential to understand whether differences in volatile composition are relevant in an ecological context.

Methods and Materials

Plants, Herbivores, and Predators Lima bean plants (*Phaseolus lunatus* L. cv Sieva) and cucumber plants (*C. sativus* L. cv Lange Groene Giganten) were grown in a greenhouse at 20–30°C, 50–70% R.H. and 16 hr of light. Bean plants were used when their primary leaves had unfolded (and were about 8–12 cm wide), which was 10–15 d after sowing. Cucumber plants were used when their first two leaves were about 8–12 cm wide, which was 15–35 d after sowing.

Two-spotted spider mites, *Tetranychus urticae* Koch (Acari: Tetranychidae), were reared on lima bean plants in a greenhouse under the same conditions as uninfested plants. Eggs of the beet armyworm *Spodoptera exigua* Hübner (Lepidoptera: Noctuidae) were obtained from a colony reared on artificial diet in the laboratory of Virology, Wageningen University, The Netherlands (Smits et al. 1986). Two to three batches of eggs were placed in a Petri dish together with a lima bean or cucumber leaf in a growth chamber at 23±1°C. Larvae were used in experiments within 24 hr of hatching.

A colony of predatory mites, *Phytoseiulus persimilis* Athias-Henriot (Acari: Phytoseiidae), was kept on spider mite-infested lima bean leaves in a growth chamber at 23±1°C, 50–70% R.H. and continuous light. To obtain females of the same age for olfactometer experiments, gravid females were allowed to oviposit on a spider mite-infested bean leaf for 2 d. The female offspring were used

in experiments 9 d after initiation of the colonies, i.e., 1–2 d after their final molt. Before the experiments, females were kept individually in 1.5-ml microcentrifuge tubes with a small droplet of water, for 24±2 hr at 23±1°C.

Plant Treatments Treatments were applied to the two oldest leaves of lima bean or cucumber plants 3 d before an experiment. Plants were incubated in plastic cages in a climate-controlled chamber at 24±1°C, 50–70% R.H. and 16L:8D, each treatment in a separate cage. We used the following four treatments:

(1) *T. urticae*: Adult female spider mites were transferred to experimental plants. Twenty mites per leaf were used on lima bean, and 100 mites per leaf were used on cucumber because the same number of spider mites leads to lower attraction of *P. persimilis* to cucumber than to lima bean plants (Dicke et al. 1990b).
(2) *S. exigua*: Newly hatched *S. exigua* larvae were placed in clip cages to keep caterpillars on the leaves (clip cages were made of two plastic cylinders, 2.5 cm diam., and a hairpin; the upper side was covered with gauze and the underside with Parafilm). Two larvae in one clip cage were used per leaf. Clip cages were moved daily to a new position on the same leaf, and any missing larvae were replaced. The clip cages were supported by sticks to prevent the leaves from bending and incurring damage due to the weight of the cages.
(3) Multi-species herbivory: Two newly hatched *S. exigua* larvae in a clip cage and 20 (lima bean) or 100 (cucumber) *T. urticae* females were placed on each leaf.
(4) Uninfested: Plants without herbivores were treated in the same way as plants of the other treatments. This treatment was only used for volatile collections of cucumber. We did not analyze volatile emission from uninfested lima bean plants because it is documented that they emit low amounts of volatiles (e.g., de Boer et al. 2004).

When clip cages were used to keep caterpillars in place, *T. urticae*-infested or uninfested plants were treated in the same way with empty clip cages to avoid any cage effects.

Volatile Collections and Analyses Just before volatile collections, plants were cut above the soil line. Stems of individual plants were wrapped in moist cotton and aluminum foil to prevent them from wilting during volatile collection, which lasted at most 4 hr. All clip cages, caterpillars, and their products were removed. Five plants of one treatment were transferred to a 5-l glass vessel. A viton O-ring and a metal clamp were used to attach the glass lid to the vessel to make it airtight. Purified air (filtered through silica, a molecular sieve, activated charcoal, and 90 mg Tenax) was split into two air streams of about 100 ml/min each and led into the vessels through teflon tubing. Volatiles from two treatments were collected simultaneously in parallel. The system was purged for 30 min before attaching a collection tube with Tenax (90 mg for bean samples, 200 mg for cucumber samples) to the air outlet in the lid of the vessel. Bean volatiles were collected for 25 min, cucumber volatiles were collected for 3 hr. For lima bean, volatile collection was repeated four times for each treatment, except for uninfested from which volatiles were not sampled. For cucumber, volatile collections were replicated seven times for the uninfested, six times for *T. urticae*, and five times for *S. exigua* and multi-species herbivory each. One sample of the *T. urticae* and one of the *S. exigua* treatments of cucumber were lost during the analysis.

Volatiles from lima bean and cucumber were analyzed on two different gas chromatography-mass spectrometry (GC-MS) systems.

Lima bean Volatiles were released from Tenax traps with a thermodesorption cold trap setup (Chrompack, Middelburg, The Netherlands) by heating at 250°C for 10 min, with a He-flow of 12 ml/min. Desorbed compounds were collected in the cold trap at −90°C. Volatiles were injected in splitless mode into the DB5 column (60 m×0.25 mm ID, 0.25 μm film thickness) by heating the cold trap to 220°C. After an initial column temperature of 40°C for 4 min, the temperature was raised to 280°C at 4°C/min. The column was directly coupled to the ion source of a Finnigan MAT 95 mass spectrometer, which was operating in the 70-eV electron impact (EI) ionization mode with scanning from mass 24 to 300 at 0.5 scan/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 library and in the Wageningen Mass Spectral Database of Natural Products, and by checking the retention indices.

Cucumber Volatiles were released from Tenax traps with a thermodesorption cold trap setup (Markes, UK) by heating at 200°C for 10 min, with a He-flow of 30 ml/min. The desorbed volatiles were collected in the cold trap at −10°C. Volatiles were injected in splitless mode into the RTX-5Silms column (Restec, 30 m×0.32 mm ID, 0.33 μm film thickness) by heating of the coldtrap to 270°C. After an initial column temperature of 40°C for 2 min, the temperature was raised to 95°C at 3°C/min, then to 165°C at 2°C/min, and subsequently to 250°C at 15°C/min. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operated in the 70-eV EI ionization mode and scanned from mass 33 to 300 at three scans/sec. Compounds were identified by comparison of mass spectra with those in the NIST 98 and

Wiley 7th edition spectral libraries, and by checking the retention indices.

Analyses Not all compounds were detected in every replicate of every treatment. We therefore added one peak area unit to all measurements for lima bean and 10 units to all measurements for cucumber. These were the lowest recorded non-zero values for bean and cucumber, respectively. This enabled us to plot volatile emission on a log scale (Figs. 1a and 2a), and to determine ratios among treatments (see below).

For cucumber, we first determined which volatile compounds were induced by herbivory by using Kruskal–Wallis tests per compound. Variation in volatile emission levels of uninfested plants was low, indicating that other types of inducing factors (e.g., general stress) were absent or had only minor effects. Only herbivore-induced volatiles were used in subsequent analyses. We assumed that all

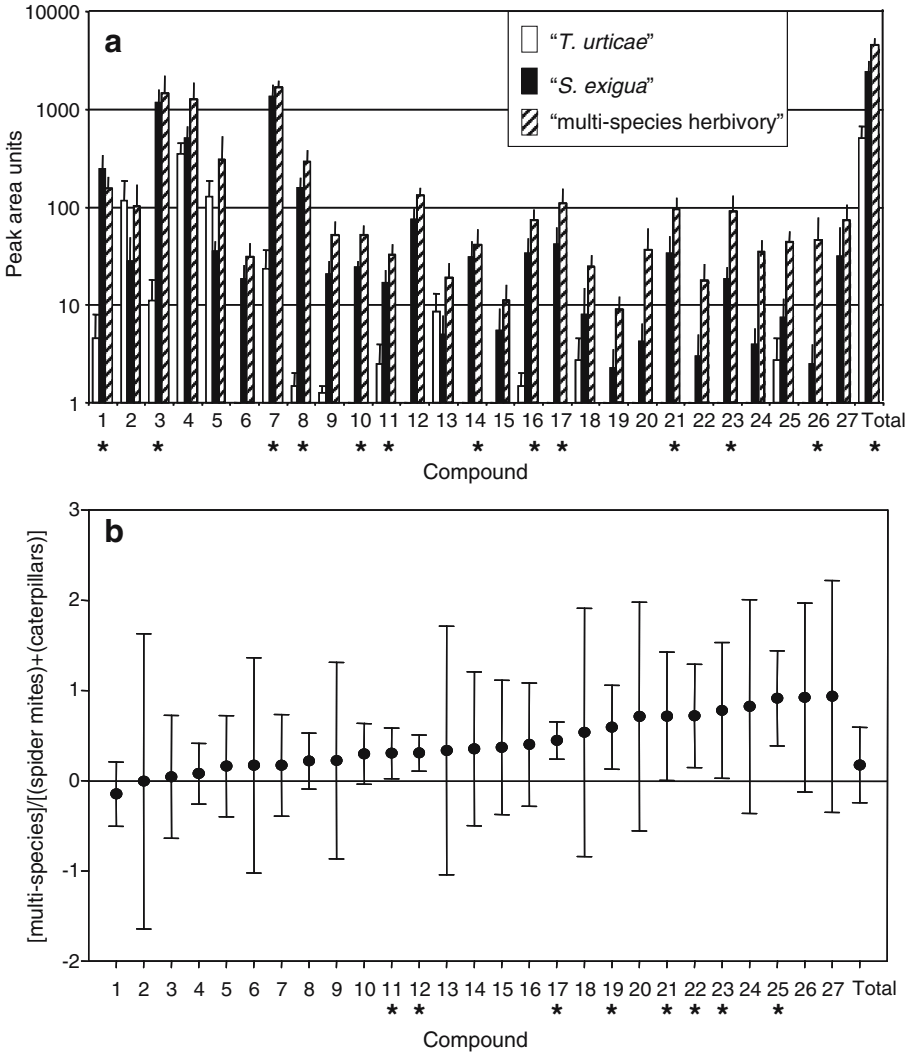

Fig. 1 Effect of multi-species herbivory on volatile emission by lima bean plants. (**a**) Volatile emission (mean + SE peak area units) upon single herbivory by *Tetranychus urticae* (20 mites per leaf) (open bars), single herbivory by *Spodoptera exigua* (two larvae per leaf) (filled bars), or multi-species herbivory (hatched bars). Asterisks indicate compounds that were significantly affected by herbivore treatment ($P<0.05$, Kruskal–Wallis tests). (**b**) Ratio of emission rates upon multi-species herbivory to the sum per volatile emitted by the two single herbivore treatments (see "Methods and Materials" for detailed explanation). Symbols indicate mean ratio, and error bars indicate upper and lower 95% confidence limits. Asterisks indicate compounds that were significantly more strongly induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (lower 95% confidence limit larger than 0). $N=4$ for all treatments. Compound numbers: (1) (*Z*)-3-hexen-1-ol, (2) methyl salicylate, (3) (*E*)-β-ocimene, (4) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (5) (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (6) *p*-mentha-1,3,8-triene, (7) (*Z*)-3-hexen-1-ol acetate, (8) 2-methylbutanal-*O*-methyl oxime, (9) 3-methylbutanal-*O*-methyl oxime, (10) linalool, (11) 1-octen-3-ol, (12) hexyl acetate, (13) limonene, (14) β-caryophyllene, (15) nonanal, (16) indole, (17) (*E*)-2-hexen-1-ol acetate, (18) 3-pentanone, (19) 3-octanone, (20) 2-methylbutanal nitrile, (21) (*Z*)-β-ocimene, (22) rose furan, (23) unknown 95B, 150, (24) 3-methylbutanal nitrile, (25) unknown 91B, 148, (26) unknown 41, 69B, 164, (27) 2-methylpropanal-*O*-methyl oxime

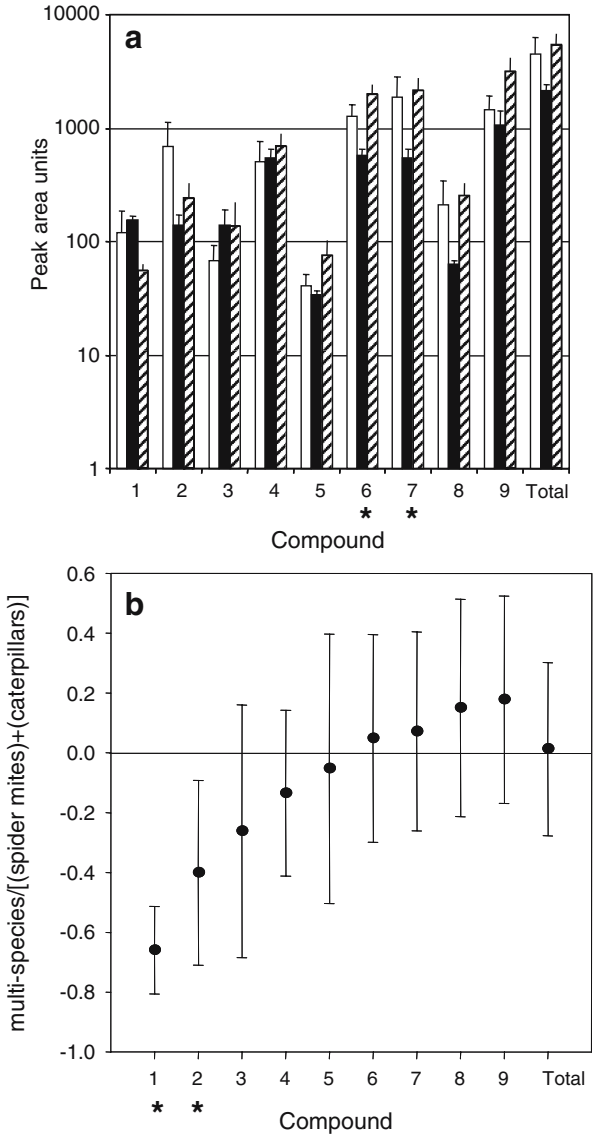

Fig. 2 Effect of multi-species herbivory on volatile emission by cucumber plants. (**a**) Volatile emission (mean ± SE peak area units) upon single herbivory by *Tetranychus urticae* (100 mites per leaf, N=5, open bars), single herbivory by *Spodoptera exigua* (2 larvae per leaf, N=4, filled bars), or multi-species herbivory (N=4, hatched bars). Asterisks indicate compounds that are significantly affected by herbivore treatment (P<0.05, Kruskal–Wallis tests). (**b**) Ratio of emission rates upon multi-species herbivory to the sum per volatile emitted by the two single herbivore treatments (see "Methods and Materials" and for detailed explanation). Symbols indicate mean ratio, and error bars indicate upper and lower 95% confidence limits. Asterisks indicate compounds that were significantly less induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (upper 95% confidence limit smaller than 0) (N=4) Compound numbers: (1) (*E*)-2-hexenal + (*Z*)-3-hexanal, (2) (*Z*)-3-hexen-1-ol acetate, (3) indole, (4) (*E,E*)-α-farnesene, (5) butyl aldoxime, (6) 3-methylbutanal-*O*-methyl oxime, (7) (*E*)-4,8-dimethyl-1,3,7-nonatriene, (8) (*3E,7E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (9) (*E*)-β-ocimene. Note that peak area units in Fig. 2a cannot be compared with Fig. 1a because measurements were done on a different GC-MS system

compounds emitted by infested lima bean plants were induced by herbivores based on previous measurements of volatile emissions by uninfested lima bean plants (e.g., de Boer et al. 2004). Per plant species, we then used Kruskal–Wallis tests to analyze which compounds were differentially induced by the three herbivore treatments (*T. urticae*, *S. exigua*, or multi-species herbivory) (α=0.05). Note that we did not statistically compare absolute levels of volatile emission across plant species because volatiles were collected for different periods of time, and analyses were run on two different GC-MS systems.

We also tested whether the effect on HIPV of multi-species herbivory was the same as the combined effect of the two herbivores feeding alone, i.e., *T. urticae* + *S. exigua*. Per replicate, the amount of each volatile compound of the multi-species herbivory treatment was divided by the sum of the amounts emitted by *T. urticae* and *S. exigua*. The ratios were log-transformed before taking their averages. An average ratio of 0 means the effect of multi-species herbivory and the sum of *T. urticae* and *S. exigua* is equal. We concluded that a compound was significantly more strongly induced by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* if the lower 95% confidence limit was larger than 0. Similarly, we concluded a significantly weaker effect of multi-species herbivory if the upper 95% confidence limit was smaller than 0 (see Figs. 1 and 2). We also used a χ^2 test to test whether the number of compounds with a ratio >0 and a ratio <0 differed from a 50:50 distribution. A 50:50 distribution would be expected if the effect of multi-species herbivory and the sum of *T. urticae* and *S. exigua* is equal. One replicate of the *T. urticae* treatment in cucumber was excluded from this analysis because we had no corresponding replicates of the *S. exigua* and multi-species herbivory treatments. Note that these tests reflect volatile responses to multi-species herbivory relative to responses to single-species herbivory. This allows us to compare these data between lima bean and cucumber because the data no longer depend on the absolute levels of volatiles detected by the different GC-MS systems.

Y-tube Olfactometer Experiments A closed system Y-tube olfactometer setup was used to test the responses of predatory mites to volatiles induced by multi-species herbivory (Takabayashi and Dicke 1992; de Boer et al. 2004). Odor sources consisted of four leaves of the same treatment that were cut from the plant just before an experiment; the petioles were wrapped in wet cotton wool and aluminum foil. Individual female predators were observed for a maximum of 5 min. A choice was recorded when the finish line, halfway one of the olfactometer arms, was reached within this period. Otherwise, it was recorded as "no-choice". Sixteen to 23 predators were tested per

experimental day, and each experiment was repeated on 4 different days. Per replicate, new groups of predatory mites and new sets of leaves were used. We tested the responses of predatory mites to volatiles from bean or cucumber plants of the multi-species herbivory treatment vs. volatiles from plants of the *T. urticae* or *S. exigua* treatments.

The choices of predatory mites between two odor sources in the Y-tube olfactometer were analyzed with two-sided binomial tests to investigate whether the distribution of the predators differed from 50:50. Predators that did not make a choice were excluded from statistical analyses.

Results

Volatile Emission by Lima Bean Plants The main compounds emitted by *T. urticae*-infested bean plants were methyl salicylate (MeSA; compound number 2 in Fig. 1a), (3*E*)-4,8-dimethyl-1,3,7-nonatriene [(*E*)-DMNT] (4), and (3*E*,7*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene [(*E,E*)-TMTT] (5). Herbivory by *S. exigua* alone resulted in the emission of similar amounts of (*E*)-DMNT (4), but amounts of MeSA (2) and (*E,E*)-TMTT (5) were small. Instead, *S. exigua*-infested bean leaves emitted large amounts of (*E*)-β-ocimene (3) and (*Z*)-3-hexen-1-ol acetate (7). Multi-species herbivory on bean plants induced the same volatile compounds as *S. exigua* feeding alone. Multi-species-infested plants emitted larger amounts of all volatile compounds than plants infested with a single herbivore species, except for (*Z*)-3-hexen-1-ol (1) that was emitted in larger amounts by *S. exigua*-infested plants, and MeSA (2) that was emitted in somewhat larger absolute amounts by *T. urticae*-infested plants.

In lima bean, herbivore treatment had a significant effect on the emission of (*Z*)-3-hexen-1-ol (1), (*E*)-β-ocimene (3), (*Z*)-3-hexen-1-ol acetate (7), 2-methylbutanal-*O*-methyl oxime (8), linalool (10), 1-octen-3-ol (11), hexyl acetate (12), β-caryophyllene (14), indole (16), (*E*)-2-hexen-1-ol acetate (17), (*Z*)-β-ocimene (21), and two unknown compounds (23 and 26) (all $P<0.05$, Kruskal–Wallis tests, Fig. 1a). These compounds were all induced in larger amounts by *S. exigua* and/or multi-species herbivory than by *T. urticae*. Total volatile emission was also significantly affected by treatment ($P<0.006$, Kruskal–Wallis test, Fig. 1a).

We compared the ratio of volatiles emitted in the multi-species herbivory treatment to the sum of volatiles emitted by the *T. urticae* and *S. exigua* treatments to determine whether infestation by two herbivore species simultaneously had additive, synergistic or antagonistic effects. Figure 1b shows the average ratio of multi-species herbivory to the sum of *T. urticae* and *S. exigua* for lima bean. Eight of 27 compounds were significantly more strongly induced by multi-species herbivory than by the sum of the two herbivore species feeding alone, suggesting synergistic effects (Fig. 1b). These compounds included members of various groups of chemicals, for example, (*E*)-2-hexen-1-ol acetate (17) and (*Z*)-β-ocimene (21). In addition, more compounds had a ratio >0 than expected ($\chi^2=9.80$, $P=0.002$). None of the compounds was significantly less induced by multi-species herbivory, suggesting the absence of antagonistic effects of *T. urticae* and *S. exigua* feeding simultaneously on lima bean.

Volatile Emission by Cucumber Plants Nine compounds were induced by herbivory on cucumber (all $P<0.10$, Kruskal–Wallis tests; data not shown). The main compounds emitted by *T. urticae*-infested cucumber plants were (*Z*)-3-hexen-1-ol acetate (compound number 2 in Fig. 2a), (*E,E*)-α-farnesene (4), 3-methylbutanal-*O*-methyl oxime (6), (*E*)-DMNT (7), and (*E*)-β-ocimene (9) (Fig. 2a). Similar compounds were emitted in the largest amounts by *S. exigua*-infested cucumber plants. Multi-species herbivory resulted in a volatile blend that consisted of the same compounds as the blends induced upon herbivory by each of the herbivore species alone. The multi-species herbivory treatment resulted in the strongest induction of 3-methylbutanal-*O*-methyloxime (6) and (*E*)-β-ocimene (9), while *T. urticae*-infested plants emitted the largest amounts of (*Z*)-3-hexen-1-ol acetate (2). Herbivory by *T. urticae* alone and multi-species herbivory induced larger amounts of (*E,E*)-TMTT (8) than single herbivory by *S. exigua* alone. No MeSA was detected in any of the cucumber samples.

In cucumber, herbivore treatment had an effect on the emission of 3-methylbutanal-*O*-methyloxime (6) and (*E*)-DMNT (9) ($P<0.05$, Kruskal–Wallis tests, Fig. 2a). Both compounds were induced in larger amounts by *T. urticae* or multi-species herbivory than by *S. exigua*. Total volatile emission was not affected by herbivore treatment in cucumber ($P>0.10$, Kruskal–Wallis test, Fig. 2a).

Two volatile compounds [(*E*)-2-hexanal/(*Z*)-3-hexenal (1) and (*Z*)-3-hexen-1-ol acetate (2)] were induced significantly less by multi-species herbivory than by the sum of *T. urticae* and *S. exigua* (Fig. 2b), suggesting the presence of antagonistic effects of the two herbivore species feeding on cucumber. The seven other compounds were equally induced by multispecies herbivory and the sum of the two herbivores feeding alone, indicating an additive effect of *T. urticae* and *S. exigua*. The distribution of compounds with a ratio >0 or <0 did not differ from 50:50 ($\chi^2=0.06$, $P=0.81$).

Responses of P. persimilis We tested the preferences of the predatory mite *P. persimilis* for the volatile blends analyzed above in a Y-tube olfactometer. On bean and cucumber plants, predatory mites preferred HIPV induced by multi-

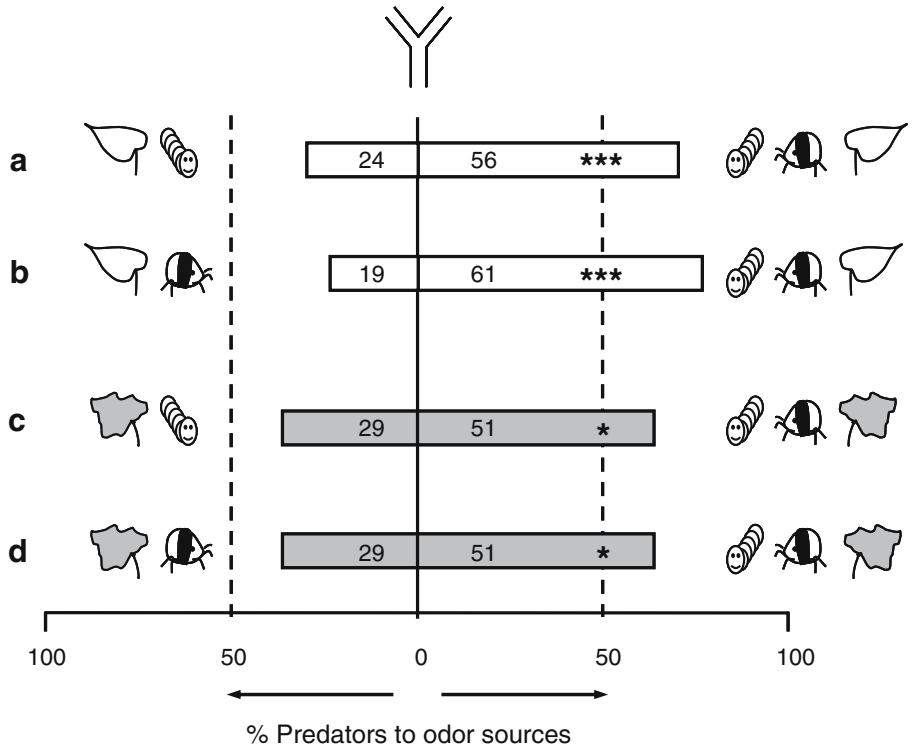

Fig. 3 Responses of *P. persimilis* to volatile blends in the Y-tube olfactometer. Odor sources consisted of four leaves from lima bean (**a** and **b**) or cucumber plant (**c** and **d**) simultaneously infested by *Tetranychus urticae* and *Spodoptera exigua* or infested by one of the herbivore species alone (*S. exigua*: **a** and **c**, *T. urticae*: **b** and **d**). Bars present the overall percentages of predators choosing for each odor source. Numbers in bars are the total numbers of predators responding to each odor source. Choices between odor sources were analyzed with a two-sided binomial test (*$P<0.05$; ***$P<0.001$)

species herbivory to blends induced by either *T. urticae* or *S. exigua* alone (Fig. 3, $P \leq 0.02$, binomial tests).

Discussion

We investigated volatile emissions by lima bean and cucumber plants upon single and multi-species herbivory by *T. urticae* and *S. exigua*. In lima bean, we found that most compounds were more strongly induced by multi-species herbivory than by the sum of each of the herbivores feeding separately (Fig. 1b). This suggests that the two herbivores feeding together on the same plant have more than an additive effect on volatile emission, potentially indicating that biosynthetic pathways may act synergistically. It is also possible that one or both herbivore species inflicted more damage on multi-species-infested plants than on plants with conspecifics only. However, it is unlikely that spider mite feeding was affected by the presence of *S. exigua* because in a preliminary experiment oviposition rates of mites, which are correlated with food intake rates, were similar on leaf discs with or without *S. exigua* damage (de Boer et al., unpublished). Some studies have observed that feeding by *S. exigua* is enhanced by the presence of aphids or a fungal pathogen (Rodriguez-Saona et al. 2005; Cardoza and Tumlinson 2006), but other studies have found no effect of the presence of whiteflies or a fungus (Rodriguez-Saona et al. 2003; Rostás et al. 2006).

In contrast to bean, we found that in cucumber plants, two volatile compounds were induced less by multi-species herbivory than by the sum of the two herbivores feeding separately, suggesting that their induction was suppressed by simultaneous feeding of *T. urticae* and *S. exigua*. Whether this reduction is caused by a negative interaction between the signal transduction pathways induced by spider mites and beet armyworms, or to reduced feeding of one or both herbivore species as a result of feeding on the same plant, remains to be investigated. From our data, it appears that plant species is an important determinant in the way in which herbivore-induced defense pathways interact upon multispecies herbivory.

The plant hormones salicylic acid (SA) and jasmonic acid (JA) play important roles in plant defense pathways and the production of HIPV (e.g., Kessler and Baldwin 2002; van Poecke and Dicke 2004). Interaction between these pathways may occur, for example, when SA inhibits JA-dependent defense pathways (e.g., Doares et al. 1995; Niki et al. 1998; Engelberth et al. 2001). To determine whether

synergistic (lima bean) and antagonistic (cucumber) interactions between biosynthetic defense pathways caused the changes in HIPV blends that we observed, endogenous levels of JA and SA should be determined in future studies. Arimura et al. (2002) showed that both JA and SA levels are significantly elevated in lima bean after 3 d of spider mite feeding, whereas several *Spodoptera* species are known to increase JA levels in corn and broad bean (Blechert et al. 1995; Schmelz et al. 2003). We are not aware of analyses of SA levels in response to *Spodoptera* feeding. No studies of JA and SA levels in plants upon multi-species herbivory are known to us either.

Another important question that needs to be addressed is the effect of herbivore sequence on volatile emission patterns, since in nature two herbivore species may infest an individual plant sequentially. In fact, some herbivore species are known to be attracted to HIPV themselves (e.g., Bolter et al. 1997; Kalberer et al. 2001), but spider mites are not attracted to *S. exigua*-infested lima bean plants (Horiuchi et al. 2003).

To determine whether the differences in volatile profiles upon multi-species herbivory and single-species herbivory are relevant in an ecological context, we investigated the behavior of the predatory mite *P. persimilis*, a specialist natural enemy of spider mites. Our results show that *P. persimilis* prefers volatiles induced by multi-species herbivory by spider mites and the non-prey caterpillar *S. exigua* to volatiles induced by one of the herbivore species feeding alone (Fig. 3). The strong preference of *P. persimilis* for the volatiles induced by multi-species herbivory may be explained by the relatively large amount of some specific compounds. Of the three major compounds emitted by spider mite-infested lima bean, two, namely (*E,E*)-TMTT and especially (*E*)-DMNT, were induced more strongly by multi-species herbivory, whereas induction of the third, MeSA, was somewhat reduced (Fig. 1a). Of the five major compounds emitted by spider mite-infested cucumber, four were induced more strongly by multi-species herbivory: (*E*)-DMNT, (*E*)-β-ocimene, (*E,E*)-α-farnesene, and 3-methylbutanal-*O*-methyl oxime, while (*Z*)-3-hexen-1-ol acetate emission was reduced (Fig. 2a). (*E*)-DMNT, (*E,E*)-TMTT, and (*E*)-β-ocimene are known attractants for *P. persimilis* (Dicke et al. 1990a, de Boer et al. 2004), and (*E,E*)-TMTT can be used by *P. persimilis* to discriminate between complex blends of HIPV (de Boer et al. 2004). Similarly, when *S. exigua*-induced volatiles are compared to the blend induced by multi-species herbivory, all of these compounds are produced in larger amounts in the latter. This suggests that a greater emission of (the sum of) attractive compounds may be the main determinant in attracting the predatory mite *P. persimilis.*

Previous studies on the responses of parasitoid wasps to HIPV also demonstrated a preference for HIPV induced by multi-species herbivory compared to single-species herbivory (Shiojiri et al. 2000; Vos et al. 2001; Cardoza et al. 2003; Moayeri et al. 2007). Rodriguez-Saona et al. (2005) even showed that naïve *Cotesia marginiventris* females were only attracted to tomato plants infested with their host *S. exigua* when the same plant was also infested with aphids. The presence of aphids thus made the host-infested plants more detectable. The opposite, i.e., multi-species herbivory-induced volatiles being less attractive than volatiles induced by only the host has been found in two cases: *C. plutellae* wasps preferred volatiles induced by their host, *P. xylostella*, to volatiles induced by their host and the non-host caterpillar *P. rapae* (Shiojiri et al. 2000), and *C. marginiventris* as well as the entomopathogenic nematode *H. megidis* were less attracted to volatiles from maize plants that were infested by a non-host in addition to their host (Rasmann and Turlings 2007). In such cases, the presence of non-host or non-prey herbivores may decrease the detectability or reliability of HIPV cues for natural enemies. For example, feeding by a non-prey or non-host herbivore may suppress the induction of volatile compounds used by natural enemies. Multi-species herbivory thus adds a new dimension to the trade-off between reliability and detectability of searching cues used by natural enemies of herbivorous arthropods (Vet and Dicke 1992).

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References

ARIMURA, G., OZAWA, R., NISHIOKA, T., BOLAND, W., KOCH, T., KÜHNEMANN, F., and TAKABAYASHI, J. 2002. Herbivore-induced volatiles induce the emission of ethylene in neighboring Lima bean plants. *Plant J.* 29:87–98.

BLECHERT, S., BRODSCHELM, W., HOLDER, S., KANNERER, L., KUTCHAM, T., MUELLER, M. J., XIA, Z. Q., and ZENK, M. H. 1995. The octadecanoic pathway: signal molecules for the regulation of secondary patways. *Proc. Natl. Acad. Sci. U S A* 92:4099–4105.

BOLTER, C. J., DICKE, M., VAN LOON, J. J. A., VISSER, J. H., and POSTHUMUS, M. A. 1997. Attraction of Colorado potato beetle to herbivore damaged plants during herbivory and after its termination. *J. Chem. Ecol.* 23:1003–1023.

CARDOZA, Y. J. and TUMLINSON, J. H. 2006. Compatible and incompatible *Xanthomonas* infections differentially affect herbi-

vore-induced volatile emission by pepper plants. *J. Chem. Ecol*. 32:1755–1768.

CARDOZA, Y. J., ALBORN, H. T., and TUMLINSON, J. H. 2002. In vivo volatile emissions from peanut plants induced by simultaneous fungal infection and insect damage. *J. Chem. Ecol*. 28:161–174.

CARDOZA, Y. J., TEAL, P. E. A., and TUMLINSON, J. H. 2003. Effect of peanut plant fungal infection on oviposition preference by *Spodoptera exigua* and on host-searching behavior by *Cotesia marginiventris*. *Environ. Entomol*. 32:970–976.

DE BOER, J. G., POSTHUMUS, M. A., and DICKE, M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or non-prey herbivores by a predatory mite. *J. Chem. Ecol*. 30:2215–2230.

DICKE, M. 1999. Evolution of induced indirect defense of plants, pp. 62–88, in R. Tollrian and C. J. Harvell (eds.). The Ecology and Evolution of Inducible Defenses. Princeton University Press, Princeton, New Jersey.

DICKE M. and SABELIS M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M. and SABELIS, M. W. 1989. Does it pay plants to advertise for bodyguards? Towards a cost–benefit analysis of induced synomone production, pp. 341–358, in H. Lambers, M. L. Cambridge, H. Konings, and T. L. Pons (eds.). Causes and Consequences of Variation in Growth Rate and Productivity of Higher Plants. SPB Publishing, The Hague, The Netherlands.

DICKE, M., and VET, L. E. M. 1999. Plant–carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore, pp. 483–520, in H. Olff, V. K. Brown, and R. H. Drent (eds.). Herbivores: Between Plants and Predators. Blackwell Science, Oxford, U.K.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990a. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKE, M., VAN DER MAAS, K. J., TAKABAYASHI, J., and VET, L. E. M. 1990b. Learning affects response to volatile allelochemicals by predatory mites. *Proc. Exp. Appl. Entomol.* 1:31–36.

DOARES, S. H., NARVÁEZ-VÁSQUEZ, J., CONCONI, A., and RYAN, C. A. 1995. Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. *Plant Physiol.* 108:1741–1746.

ENGELBERTH, J., KOCH, T., SCHÜLER, G., BACHMANN, N., RECHTENBACH, J., and BOLAND, W. 2001. Ion channel-forming alamecithin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between the jasmonate and salicylate signaling in Lima bean. *Plant Physiol.* 125:369–377.

FRITZSCHE HOBALLAH, M. E. and TURLINGS, T. C. J. 2001. Experimental evidence that plants under caterpillar attack may benefit from attracting parasitoids. *Evol. Ecol. Res.* 3:553–565.

HORIUCHI, J., ARIMURA, G., OZAWA, R., SHIMODA, T., TAKABAYASHI, J., and NISHIOKA, T. 2003. A comparison of the responses of *Tetranychus urticae* (Acari: Tetranychidae) and *Phytoseiulus persimilis* (Acari: Phytoseiidae) to volatiles emitted from lima bean leaves with different levels of damage made by *T. urticae* or *Spodoptera exigua* (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* 38:109–116.

KALBERER, N. M., TURLINGS, T. C. J., and RAHIER, M. 2001. Attraction of a leaf beetle (*Oreina cacaliae*) to damaged host plants. *J. Chem. Ecol.* 27:647–661.

KESSLER, A., and BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141–2144.

KESSLER, A., and BALDWIN, I. T. 2002. Plant responses to insect herbivory: The emerging molecular analysis. *Annu. Rev. Plant Biol.* 53:299–328.

MOAYERI, H. R. S., ASHOURI, A., POLL, L., and ENKEGAARD, A. 2007. Olfactory responses of a predatory mirid to herbivore induced plant volatiles: Multiple herbivory vs. single herbivory. *J. Appl. Entomol.* 131:326–332.

NIKI, T., MITSUHARA, I., SEO, S., OHTSUBO, N., and OHASHI, Y. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* 39:500–507.

OZAWA, R., ARIMURA, G., TAKABAYASHI, J., SHIMODA, T., and NISHIOKA, T. 2000. Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41:391–398.

PELS, B., and SABELIS, M. W. 1999. Local dynamics, overexploitation and predator dispersal in an acarine predator-prey system. *Oikos* 86:573–583.

PRICE, P. W., BOUTON, C. E., GROSS, P., MCPHERON, B. A., THOMPSON, J. N., and WEISS, A. E. 1980. Interactions among three trophic levels: Influence of plants on interactions between insect herbivores and natural enemies. *Annu. Rev. Ecol. Syst.* 11:41–65.

RASMANN, S., and TURLINGS, T. C. J. 2007. Simultaneous feeding by aboveground and belowground herbivores attenuates plant-mediated attraction of their respective natural enemies. *Ecol. Lett.* 10:926–936.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDNER, S. J., and CAÑAS, L. A. 2003. Volatile emissions triggered by multiple herbivore damage: Beet armyworm and whitefly feeding on cotton plants. *J. Chem. Ecol.* 29:2539–2550.

RODRIGUEZ-SAONA, C., CHALMERS, J. A., RAJ, S., and THALER, J. S. 2005. Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. *Oecologia* 143:566–577.

ROSTÁS, M., TON, J., MAUCH-MANI, B., and TURLINGS, T. C. J. 2006. Fungal infection reduces herbivore-induced plant volatiles of maize but does not affect naïve parasitoids. *J. Chem. Ecol.* 32:1897–1909.

SABELIS, M. W., and VAN DER MEER, J. 1986. Local dynamics of the interaction between predatory mites and two-spotted spider mites, pp. 322–344, in J. A. J. Metz and O. Diekmann (eds.). Dynamics of physiologically structured populations. Lecture notes in biomathematics 68. Springer-Verlag, Berlin, Germany.

SABELIS, M. W., JANSSEN, A., PALLINI, A., VENZON, M., BRUIN, J., DRUKKER, B., and SCUTAREANU, P. 1999. Behavioral responses of predatory and herbivorous arthropods to induced plant volatiles: From evolutionary ecology to agricultural applications, pp. 269–296, in A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses Against Pathogens and Herbivores. Biochemistry, Ecology and Agriculture. APS Press, St. Paul, Minnesota.

SCHMELZ, E. A., ALBORN, H. T., BANCHIO, E., and TUMLINSON, J. H. 2003. Quantitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory. *Planta* 216:665–673.

SHIOJIRI, K., TAKABAYASHI, J., YANO, S., and TAKAFUJI, A. 2000. Flight response of parasitoids toward plant–herbivore complexes: A comparative study of two parasitoid-herbivore systems on cabbage plants. *Appl. Entomol. Zool.* 35:87–92.

SHIOJIRI, K., TAKABAYASHI, J., YANO, S., and TAKAFUJI, A. 2001. Infochemically mediated tritrophic interaction webs on cabbage plants. *Popul. Ecol.* 43:23–29.

SMITS, P. H., VAN DE VRIE, M., and VLAK, J. M. 1986. Oviposition of beet armyworm (Lepidoptera: Noctuidae) on greenhouse crops. *Environ. Entomol.* 15:1189–1191.

SOLER R., HARVEY, J. A., KAMP, A. F. D., VET, L. E. M., VAN DER PUTTEN, W. H., VAN DAM, N. M., STUEFER, J. F., GOLS, R., HORDIJK, C. A., and BEZEMER, T. M. 2007. Parasitoids of leaf herbivores repelled by root herbivores. *Oikos* 116:367–376.

TAKABAYASHI, J. and DICKE, M. 1992. Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. Exp. Appl.* 64:187–193.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 30:1251–1253.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL P. J., RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U S A* 92:4169–4174.

VAN DEN BOOM, C. E. M., VAN BEEK, T. A., POSTHUMUS, M. A., DE GROOT, A., and DICKE, M. 2004. Qualitative and quantitative variation among volatile profiles induced by Tetranychus urticae feeding on plants from various families. *J. Chem. Ecol.* 30: 69–89.

VAN LOON, J. J. A., DE BOER, J. G., and DICKE, M. 2000. Parasitoid-plant mutualism: Parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl.* 97:219–227.

VAN POECKE, R. M. P. and DICKE, M. 2004. Indirect defence of plants against herbivores: Using *Arabidopsis thaliana* as a model plant. *Plant Biol.* 6:387–401.

VET, L. E. M. and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VOS, M., BERROCAL, S. M., KARAMAOUNA, F., HEMERIK, L., and VET, L. E. M. 2001. Plant-mediated indirect effects and the persistence of parasitoid-herbivore communities. *Ecol. Lett.* 4:38–45.

WALLING, L. L. 2000. The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19:195–216.

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The Attraction of *Spodoptera frugiperda* Neonates to Cowpea Seedlings is Mediated by Volatiles Induced by Conspecific Herbivory and the Elicitor Inceptin

Mark J. Carroll · Eric A. Schmelz · Peter E. A. Teal

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Abstract Neonate fall armyworms [FAW; *Spodoptera frugiperda* (Smith)] often encounter conspecific herbivore damage as they disperse from an egg mass to an initial feeding site. We investigated the orientation responses of dispersing neonates to herbivore damage in cowpea seedlings, specifically examining whether neonate behaviors were affected by inceptin, the primary elicitor of FAW-induced defenses in cowpea leaves. We focused on responses to damage caused by conspecific first instars, as might occur during the dispersal of siblings from an egg mass. Inceptin contents of damaging first instar FAW were controlled through their diets, with leaf-fed FAW producing inceptins in their oral secretions, and root-fed or starved FAW lacking these elicitors. In a bioassay designed to evaluate neonate dispersal off a host plant, a higher percentage of neonates remained on herbivore-induced or inceptin-treated plants than on undamaged plants, mechanically damaged plants, freshly damaged plants, or on plants damaged by FAW lacking inceptins. Further investigations of neonate responses to plant odors with a four-arm olfactometer demonstrated that neonate attraction to odors from 4-h old FAW damage was strongly dependent on previous diet of the damaging larvae. Neonates were attracted to odors from 4-h old FAW damage over odors from undamaged plants or fresh FAW damage, provided that the damaging larvae had previously ingested leaf material. In a direct comparison of odors from induced plants, plants damaged by leaf-fed FAW were as attractive as plants treated with synthetic inceptin. GC-MS analysis confirmed that (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was the major volatile induced by FAW herbivory. While both DMNT and undamaged plant odors were more attractive than air, neonates preferred DMNT-supplemented plant odors. These results suggest that neonate FAW exploit herbivore-induced plant volatiles as host plant location and recognition cues.

Keywords Olfaction · Dispersal · Fall armyworm · Plant-herbivore interactions · Induction · (*E*)-4,8-dimethyl-1,3,7-nonatriene · Four-arm olfactometer · Lepidoptera · Noctuidae

Introduction

Plants respond to herbivore feeding through increased biosynthesis and emission of volatile compounds from the damaged tissues (Paré and Tumlinson 1997, 1999). While these volatiles often benefit the plant through enhanced attraction of natural enemies (i.e., parasitoids and predators; Dicke and Sabelis 1988; Kessler and Baldwin 2001; Turlings et al. 1990, 1995), the same volatiles can potentially serve as olfactory cues to the herbivores themselves by making the damaged tissues more apparent (Bolter et al. 1997; Dicke and Vet 1999; Carroll et al. 2006). Such enhanced detectability of host plant tissue may be particularly useful to herbivores with limited search capacities (Ward 1987; Stamps and Krishnan 2005), such as neonate caterpillars (Zalucki et al. 2002). Despite their

M. J. Carroll (✉) · E. A. Schmelz · P. E. A. Teal
Center for Medical, Agricultural, and Veterinary Entomology,
U.S. Department of Agriculture, Agricultural Research Service,
Chemistry Research Unit, 1700 SW 23rd Dr.,
Gainesville, FL 32608, USA
e-mail: Mark.Carroll@ars.usda.gov

greatly restricted mobility, neonates must locate and establish feeding sites on appropriate host tissues within a limited amount of time, or they will starve to death due to minimal energy reserves (Zalucki et al. 2002). In many species, location of an initial neonate feeding site is accomplished largely by female oviposition choice (Thompson 1988). However, the oviposition site may be inappropriate or insufficient for neonate development for a variety of reasons. Females may oviposit off the plant or on tissues that are not consumed by the neonate (Harris et al. 1999; Zalucki et al. 2002). Ovipositing females may be phenologically displaced from the tissues that their offspring will consume, particularly in rapidly developing tissues, and therefore, they are often unable to assess host quality or to lay eggs in an appropriate location (Zangerl and Berenbaum 1992). Females may also lay more offspring on the natal plant than can be supported by the available resources (Chapman et al. 1999; Prokopy and Roitberg 2001). In such cases, neonates may compensate for suboptimal conditions at the oviposition site by dispersing to other host tissues via ballooning or crawling (Roitberg and Mangel 1993; Harris et al. 1999; Doak 2000; Zalucki et al. 2002). Since location and recognition of appropriate host plant tissue by neonates involves chemosensory and tactile inputs (del Campo et al. 2001; Schoonhoven and van Loon 2002), herbivore damage may affect dispersal behavior and eventual recruitment of neonates to damaged tissues.

The fall armyworm (FAW) *Spodoptera frugiperda* (Smith) is a highly mobile lepidopteran that disperses as a neonate from its oviposition site (Yang et al. 1993), thus escaping from the frequent and negative effects of overcrowding (Chapman et al. 1999). Larval overcrowding can be limited through the use of larval frass as an oviposition deterrent, as in the related noctuid *S. littoralis* (Hilker and Klein 1989); however, an ovipositing FAW female lays up to 200 eggs in a single egg mass on a host plant, often exceeding the resources available (Sparks 1979; Pitre et al. 1983; Chapman et al. 1999). Larvae that remain in proximity to conspecifics face not only depletion of host plant material, but the threat of cannibalism (Chapman et al. 1999). To compensate for unfavorable conditions at the oviposition site, neonates disperse *en masse* several hours after hatching from the egg mass before initiating feeding on the natal host plant and on neighboring ones (Sparks 1979).

During dispersal from the egg mass, neonates often encounter various phases of herbivore damage caused by feeding conspecifics, from fresh damage inflicted at new feeding sites to older damage at abandoned feeding sites, to a combination of fresh and older damage at feeding sites established by actively feeding (often older) conspecifics. Eventually, the neonate will experience the whole phenological sequence of damage volatiles as it establishes its own feeding site. The volatile compositions emitted during these various damage phases change considerably over time as individual volatile components are induced at different rates (Turlings et al. 1998). In many plants, fresh damage volatiles that consist of lipoxygenase products (i.e., green leaf volatiles) rapidly released by mechanical damage differ from older damage volatiles consisting of terpenoids and other compounds slowly synthesized by the attacked plant (Hoballah and Turlings 2005). As such, the damage-induced volatiles encountered by dispersing neonates may convey information not only about host tissue location, but also the presence of other herbivores (Landolt 1993; Prokopy and Roitberg 2001; Carroll et al. 2006), host tissue quality *vis-à-vis* damage phenology (Bernasconi et al. 1998), and eventually the location of the larva's own feeding site.

We examined the responses of dispersing neonate FAW to herbivore damage in cowpea [*Vigna unguiculata* (L.) Walp.], an occasional, yet marginal, host of the fall armyworm (Meagher et al. 2004). We compared neonate responses to both fresh damage and older damage separately. We also evaluated neonate responses to damage-induced volatiles alone. While host location, recognition, and eventual acceptance of host tissues involves several sensory inputs, the initial opportunity for interactions with damaged host tissues are likely to occur through long distance modalities such as olfaction and vision (Harris et al. 1999; Schoonhoven and van Loon 2002).

We also asked whether neonate responses to herbivore damage could be mediated by elicitor-induced defensive responses in cowpea. Inceptin, a peptide formed by the partial proteolysis of chloroplastic ATPase, is the primary elicitor of induced volatile responses to sixth instar FAW in cowpea leaves (Schmelz et al. 2006). We examined whether neonate responses to older damage were dependent on full induction of herbivore-induced responses (*sensu* Dicke et al. 1999 and Turlings et al. 2000), both through the application of synthetic inceptin to wounded plant tissues and the use of damaging FAW with different predicted inceptin contents. Since only FAW larvae that have previously ingested chloroplast-containing tissues produce inceptin in their oral secretions, the inceptin contents of damaging larvae, and therefore the extent of herbivore-induced responses in cowpea leaves, can be readily manipulated through the larval diet (Schmelz et al. 2006). We also examined whether neonate responses to whole plant odors could be altered by a single induced volatile. The terpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) is a major constituent of induced volatiles in many plants, not only in cowpea but also in preferred graminaceous hosts such as maize (Turlings et al. 1998; D'Alessandro and Turlings 2005; Carroll et al. 2006). Given the prominence

of DMNT in the induced volatile profile of such unrelated host plants, we investigated the orientation responses of dispersing neonates to DMNT volatiles.

Methods and Materials

Plants Seeds of cowpea [*Vigna unguiculata* (L.) Walp. var. Cal 5] were planted in professional growers mix potting soil (Metro Mix 300, SunGro Horticulture, Vancouver, British Columbia) in 4 in. square pots and initially maintained for the first 7 days under 350 μmol m^{-2} s^{-1} photosynthetic active radiation under a 12:12 h (L/D) photoperiod. On the eighth day, plants were transferred and maintained at 25 °C under ambient greenhouse light supplemented with high-pressure sodium lamps under a 12:12 h (L/D) photoperiod. Supplemental lighting was used from 6:00 A.M. to 6:00 P.M. Seedlings (14–17 days old) with two fully expanded V2 leaves were used in all experiments.

Insects Fall armyworm eggs and neonates used in the behavioral bioassays were obtained from a colony maintained on an agar-based artificial diet at CMAVE, USDA-ARS (Gainesville, FL, USA). All insects were reared at 25° C under a 14:10 h (L/D) photoperiod.

Damage Treatments of Cowpea Seedlings Herbivore damage was inflicted by first instar FAW that either contained or lacked inceptins in their oral secretions. Because inceptins are formed by the partial proteolysis of chloroplastic ATP synthase, only larvae that have previously ingested and partially digested chloroplast-containing plant material produce inceptins (Schmelz et al. 2006). To obtain damaging first instars with inceptins, neonates were prefed on cowpea leaves (LF-FAW) for 10 h before their use in the damage treatments. Larvae without inceptins in their oral secretions were acquired by prefeeding neonates on cowpea roots [which lack chloroplasts (RF-FAW)] for 10 h or starving the larvae (unfed; UF-FAW) before their use in the damage treatments (Schmelz et al. 2006). We also specifically included a treatment that stimulates fresh damage by unfed [UF-FAW (fresh)] first instars, as this type of herbivore damage is commonly encountered by dispersing neonates near the initial feeding sites of their neonate siblings. Herbivore damage to leaves was initiated by transferring 120 first instar FAW to the V2 leaves of each plant. To minimize the production of inceptin-related peptides from recently ingested foliage during the damage treatments, we restricted the time period that damaging larvae were allowed to feed on the plants to 60 min or less. In cowpea leaves, emissions of damage volatiles induced by FAW herbivory or inceptin treatment increase substantially 4 h after the initial attack (Schmelz et al. 2006). To obtain cowpea seedlings that emitted only fresh damage or older damage odor phenologies, bioassays or volatile collections were conducted on plants either 1 h (fresh) or 4 h (old) after feeding damage had been inflicted.

Artificial damage treatments were initiated by superficially scratching three small wound patches (about 2 cm^2 each) with repeated shallow swipes of a razor blade on the adaxial side of a newly expanded V2 trifoliate. Each trifoliate was treated by applying either inceptin [2.5 ng synthetic inceptin in 10 μl 50 mM sodium phosphate pH 8.0 buffer, inceptin-treated (INC)] or a buffer solution [10 μl buffer, mechanically-damaged (MD)] across its wounds. To obtain cowpea seedlings that emitted older damage odors, all artificial damage treatments were conducted 4 h before use in bioassays or volatile collections. All damage treatments were inflicted under normal light conditions (previously described) to obtain maximal induction of photosynthesis-dependent volatiles (Turlings et al. 1995).

Plant Volatile Collections Collections of headspace volatiles emitted by cowpea plants were performed in glass chambers with a push–pull system (Analytical Research Systems, Gainesville, FL, USA) modified after Heath and Manukian (1994). An excess of pre-humidified, carbon-filtered air was added to the top of the chamber at 3 l/min to ensure that outside air did not enter the chamber. Volatiles were sampled by pulling air over the plants and through a Super Q absorbent (80–100 mesh, Alltech Associates, Deerfield, IL, USA) filter at 600 ml/min for 1 h. Adsorbed volatiles were eluted from the filter with 200 μl dichloromethane that contained 400 ng nonyl acetate as an internal standard.

Identification of Plant Volatiles Volatile analysis was performed by positive ion electron impact gas chromatography-mass spectrometry (EI GS-MS) on an HP 6890 gas chromatograph coupled to an HP 5973 MS detector. 1 μl of the sample was injected (240°C) onto an Agilent HP-5MS dimethylpolysiloxane column (30 m×250 μm (i.d.)× 0.25 μm, Agilent Technologies, Palo Alto, CA, USA) and separated by temperatures programmed from 35°C (1.0 min hold) to 230°C at 10°C/min. Helium was used as a carrier gas at 1.2 ml/min. Volatiles were identified by comparison of mass spectra (a) with mass spectra libraries (NIST and Department of Chemical Ecology, Göteborg University, Sweden) and (b) with mass spectra and retention times of authentic standards.

Chemicals Inceptin (ICDINGVCVDA) was synthesized and purified at the Protein Core Chemistry Facility (University of Florida, Gainesville, FL) as previously

described by Schmelz et al. (2006). Synthetic stocks of 4,8-dimethyl-1,3,7-nonatriene were acquired as a gift from Robert J. Bartlett (Crop Bioprotection Research Unit, USDA-ARS, Peoria, IL, USA). To enrich the content of the naturally emitted (*E*) isomer over the (*Z*) isomer, the mixture was separated three times sequentially by normal phase HPLC on a $AgNO_3$-coated (25%) silica gel column with benzene as an eluent (Heath and Sonnet 1980). Although we were unable to completely separate the isomers, the ratio of (*E*):(*Z*) DMNT was improved from 1:2 to approximately 20:1. The benzene was carefully removed under gentle N_2 flow.

Neonate Dispersal Bioassay To assess the effects of plant damage on neonate dispersal, we recorded the number of first instars remaining on a plant 24 h after transfer to leaves as dispersing neonates. We compared first instar retention on plants induced by inceptin [LF-FAW (old) and INC (old)] with retention on plants that were not induced by inceptin [CON (undamaged control), MD (old), UF-FAW (fresh), RF-FAW (old)]. The bioassay was conducted in a walk-in incubator (2.4×2.4×2.4 m, Aminco, Silver Springs, MD) maintained at 30°C and 80% RH under a 12:12 h (L/D) photoperiod. The ambient vibration and airflow within this incubator was minimal, thereby reducing the chance of disturbance or successful ballooning off the plant in a strong airflow. A group of 20 newly dispersing neonates were transferred by a camel hair brush to the adaxial side of V2 leaflets on each plant at 8:00 P.M. (2 h after the onset of scotophase). Retention of larvae was tested on three plants per treatment. One replicate set of plants containing one plant from each treatment (six plants total) was placed in the walk-in incubator at a time. Three replicate sets total were performed during separate overnight periods. To limit the exposure of neonates to volatiles from adjacent plants, the plants were placed in a single, staggered line that ran perpendicular to the reduced air flow. The order of plants in the linear array was randomly assigned by treatment in each replicate. Each plant was isolated from other plants by at least 50 cm and placed on a water-filled plastic saucer (25 cm diameter) to ensure that neonates that left the plant could not return. Larvae observed attempting to silk off the plant were unable to become aloft and simply lowered to the ground or returned to the plant above. The number of first instars left alive on each plant was recorded 24 h after introduction. No dead larvae were observed on any of the plants at the end of the bioassay.

Four-Arm Olfactometer Bioassays Orientation responses of neonate FAW to plant and synthetic volatile odors were evaluated with a four-arm olfactometer (Suazo et al. 2003). The olfactometer consisted of a quadrilateral-shaped choice arena with an arm at each corner and a 3 cm diameter base for air outflow at the center. Air flow from distal odors sources through the olfactometer was maintained with a push–pull system. Volatiles were obtained in distal volatile collection systems (previously described, Heath and Manukian 1994) by passing 3 l/min of pre-humidified, carbon-filtered air over the odor source. A portion of these volatiles were relayed from the distal odor source to an arm by 1/4" OD corrugated Teflon tubing. Air was then pulled through the olfactometer and out of the bottom at 2.4 l/min (600 ml/min per arm) by a vacuum line attached to the central base. Neonates were prevented from entering the vacuum line by a fine nylon mesh screen and the odor source lines by a glass trap placed at the distal end of each arm.

We evaluated neonate responses to odors in the olfactometer by using groups of neonates rather than individuals, since neonate FAW disperse from egg masses containing many siblings. A group of 120–200 neonates was transferred by a camel hairbrush to a 10 ml glass scintillation vial. Each group of neonates was placed in the center of the olfactometer by rapidly inverting the vial directly over the base and gently tapping the larvae off the glass vial. To minimize phenological changes in odors from damaged plants, the neonates were given a limited amount of time (up to 1 h) to move toward an odor source in the arena. A choice preference for an odor source was scored only if a neonate entered an arm. Larvae that did not enter an arm (up to 65%) within the allotted time were scored as neutrals and excluded from the statistical analysis.

Whole plant odors were sampled by the methods previously described for volatile collection. Synthetic DMNT was introduced into the odor source airstream by capillary release, a method that allows for the predictable and stable release of volatile compounds from a capillary tube based on their physiochemical characteristics (Weatherston et al. 1985). We based our protocol on the modifications of D'Alessandro et al. (2006), who greatly improved handling ease and conservation of volatile compounds by creating a volatile-saturated atmosphere in an auxillary container rather than the capillary tube itself. A DMNT-saturated atmosphere was created within a 4 ml glass vial by placing 5 µl DMNT inside a 250 µl glass GC vial insert and sealing the vial with a GC septum. A defined capillary release of DMNT (80 ng/h) was achieved by inserting one end of a 50 µl capillary tube (Drummond Scientific Company, Broomall, PA, USA) through the vial septum and projecting the other end into the odor source airstream. The released DMNT was thoroughly mixed with the airstream through the use of multiple nylon mesh baffles.

The capillary-release rate of DMNT in odor source airstreams was estimated by analysis of SuperQ filter-trapped volatiles following the methods of Schmelz et al.

(2001). Quantification of DMNT was performed on an Agilent 6890 gas chromatograph coupled to a flame ionization detector (250°C). One microliter of the sample was injected (splitless, 220°C) onto an Agilent DB-1 dimethylpolysiloxane column (15 m×250 μm (i.d.)× 0.25 μm, Agilent Technologies, Palo Alto, CA, USA) and separated with temperatures programmed from 40°C (0.50 min hold) to 180°C at 12°C/min followed by a 220° C post run (2 min). Helium was used as a carrier gas at 1.2 ml/min.

Statistics In both the neonate dispersal bioassay and the olfactometer bioassays, the proportion of neonates remaining on the plant or selecting the source were arcsin-transformed and compared either across treatments or odor sources by a one-way ANOVA (JMP 4.0.4, SAS Institute, Cary, NC, USA). In all bioassays, proportion means were compared by Tukey-Kramer HSD tests (JMP 4.0.4, SAS Institute, Cary, NC, USA).

Results

Retention of Dispersing Neonates on Herbivore-Induced Plants The percentage of dispersing neonates remaining on induced plants (plants damaged by leaf-fed first instar FAW or inceptin-treatment) was significantly higher than on undamaged plants or plants that experienced other forms of damage (Fig. 1). The retention of first instars on these plants could not be attributed to feeding damage alone, as plants that experienced fresh damage or damage by root-fed first instar FAW retained first instars in proportions similar to undamaged or mechanically damaged plants. These results are consistent with the hypothesis that neonate retention on damaged cowpea plants is largely mediated by inceptin-induced plant responses. Of all the damaging larvae, only leaf-fed first instar FAW would be expected to produce inceptins from previously ingested chloroplast-containing plant material. Curiously, we observed that the majority of first instars retained on the damaged plants established feeding sites in pre-existing wound sites, despite the fact that these tissues comprised less than 5% of the total leaf area.

Neonate Attraction to Herbivore-Induced Odors in Olfactometer Bioassays In the olfactometry bioassays, dispersing neonates were strongly attracted to odors emitted by herbivore-induced plants (plants damaged by leaf-fed FAW), but not to odors from undamaged plants, plants with fresh damage, or plants damaged by larvae lacking inceptins (starved or root-fed FAW; Figs. 2 and 3). These results support the hypothesis that neonate attraction to cowpea volatiles induced by plant damage, like neonate retention, is strongly mediated by inceptin-induced plant responses. A direct comparison of the larval response to volatiles released from plants with old feeding damage and to odors from inceptin-treated plants revealed that neonates do not distinguish between these odor sources (Fig. 4).

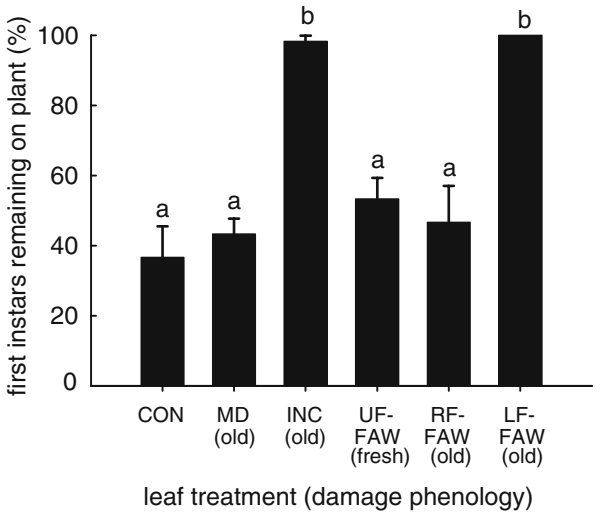

Fig. 1 Retention of *Spodoptera frugiperda* neonates on cowpea plants subjected to different leaf damage treatments: undamaged (*CON*), mechanically damaged (*MD*), inceptin-treated (*INC*), or FAW-damaged [unfed (*UF-FAW*), root-fed (*RF-FAW*), or leaf-fed (*LF-FAW*) first instars] plants. Mean percentage(±SE) of first instars represents the number of FAW remaining (out of 20 dispersing neonates introduced) on a plant 24 h after placement on the upper foliage (N=3 plants per treatment). To obtain only fresh or older damage odor phenologies, FAW-damaged plants were attacked by damaging larvae either 1 h (fresh) or 4 h (old) before use in the bioassay. Treatments not sharing the same letter are significantly different by *Tukey-Kramer HSD* ($P<0.05$; *one-way ANOVA*; $P<0.001$, $F=32.55$, $df=5$)

Herbivore-Induced Plants Emit DMNT In a comparison of volatiles from FAW-damaged, inceptin-treated, and undamaged cowpea seedlings during the early afternoon, the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was the major induced volatile produced by cowpea foliage (Fig. 5). DMNT emissions were strongly induced by inceptin-treatment or feeding damage by leaf-fed first instar FAW after 4 h, but only moderately by mechanical damage, feeding damage from unfed FAW, or fresh feeding damage. The greater induction of DMNT in inceptin-treated plants was likely due to the large amount of inceptin (2.2 pmol/trifoliate) applied, which equates to 4 μl of oral secretions by a 6th instar, a quantity that probably exceeds the amount contributed by leaf-fed first instars (Schmelz et al. 2006). Other volatiles commonly encountered in damaged leaf tissues, including lipoxygenase products (green leaf volatiles) from freshly damaged plants, were not detected by the methods used.

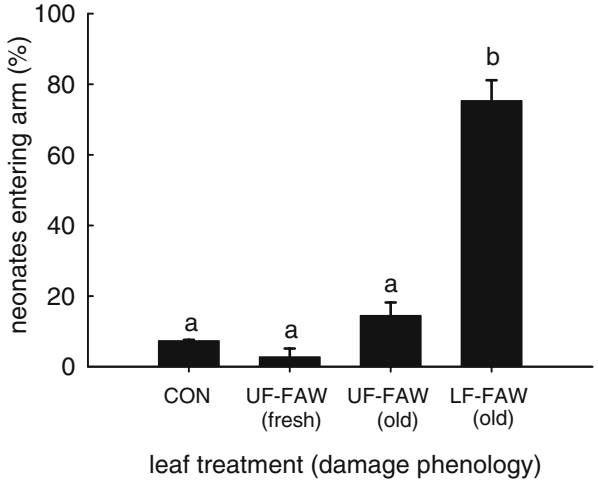

Fig. 2 Response of *Spodoptera frugiperda* neonates to different odor sources offered simultaneously in a four-arm olfactometer: undamaged cowpea plants (*CON*), or cowpea plants attacked by FAW [unfed (*UF-FAW*) or leaf-fed (*LF-FAW*) first instars]. Mean percentage (±SE) of neonates is the proportion of neonates entering a respective olfactometer arm out of the total number that responded positively (entered arms; $N=2$ replicates, a total of 93 and 179 larvae responded in each replicate). To obtain only fresh or older damage odor phenologies, FAW-damaged plants were attacked by damaging larvae either 1 h (fresh) or 4 h (old) before use in the bioassay. Treatments not sharing the same letter are significantly different by *Tukey-Kramer HSD* ($P<0.05$; *one-way ANOVA*; $P<0.001$, $F=27.60$, $df=3$)

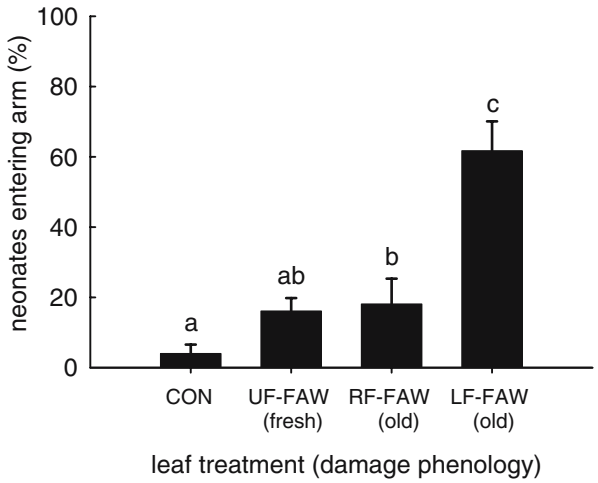

Fig. 3 Response of *Spodoptera frugiperda* neonates to different odor sources offered simultaneously in a four-arm olfactometer: undamaged cowpea plants (*CON*), or cowpea plants attacked by FAW [unfed (*UF-FAW*), root-fed (*RF-FAW*), or leaf-fed (*LF-FAW*) first instars]. Mean percentage (±SE) of neonates is the proportion of neonates entering a respective olfactometer arm out of the total number that responded positively (entered arms; $N=3$ replicates, a total of 95, 109, and 149 larvae responded in each replicate). To obtain only fresh or older damage odor phenologies, FAW-damaged plants were attacked by damaging larvae either 1 h (fresh) or 4 h (old) before use in the bioassay. Treatments not sharing the same letter are significantly different by *Tukey-Kramer HSD* ($P<0.05$; *one-way ANOVA*; $P=0.003$, $F=34.81$, $df=3$)

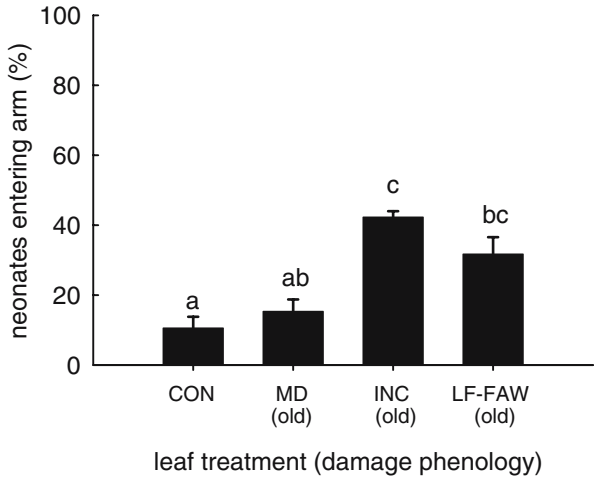

Fig. 4 Response of *Spodoptera frugiperda* neonates to different odor sources offered simultaneously in a four-arm olfactometer: undamaged cowpea plants (*CON*), mechanically damaged cowpea plants (*MD*), inceptin-treated cowpea plants (*INC*), or cowpea plants damaged by leaf-fed first instar FAW (*LF-FAW*). Mean percentage (±SE) of neonates is the proportion of neonates entering a respective olfactometer arm out of the total number that responded positively (entered arms; $N=2$ replicates, a total of 104 and 112 larvae responded in each replicate). To obtain only older damage odor phenologies, FAW-damaged plants were attacked by damaging larvae 4 h before use in the bioassay. Treatments not sharing the same letter are significantly different by *Tukey-Kramer HSD* ($P<0.05$; *one-way ANOVA*; $P=0.014$, $F=14.14$, $df=3$)

Attraction of Neonates to DMNT DMNT was an attractant to dispersing neonates in the four-arm olfactometer, both by itself and as a supplement to undamaged cowpea plant odors (Fig. 6). The combination of supplementary DMNT and undamaged plant odors was significantly more attractive than either odor source alone. This observation, combined with the equivalent response of neonates to DMNT or undamaged plant odors (which contain much lower amounts of DMNT than damaged plants), indicates that undamaged leaves may emit volatiles other than DMNT that are also attractive to dispersing neonates.

Discussion

Neonate FAW are attracted to volatiles released from herbivore-damaged cowpea leaves. In cowpea leaves, inceptin induces both volatile and nonvolatile anti-herbivore defenses against FAW larvae, as implicated by phenylpropanoid markers and protease inhibitor transcripts (Schmelz et al. 2006). Sixth instar FAW fed on inceptin-induced cowpea leaves endure a significant reduction in growth rate (17%) compared to larvae given undamaged leaves (Schmelz et al. 2006). Furthermore, herbivore-induced volatiles are well-known to serve as signals to

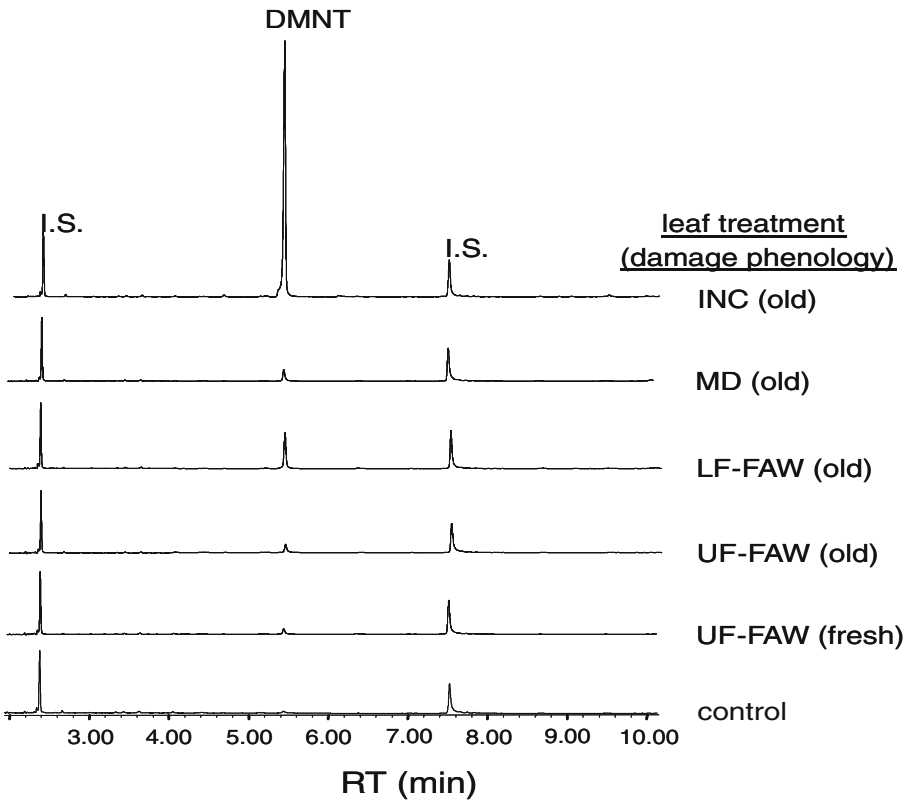

Fig. 5 Representative GC-FID chromatographic profiles of headspace volatiles collected in the early afternoon from cowpea plants subjected to different leaf damage treatments: inceptin-treated (*INC*), mechanically damaged (*MD*), FAW-damaged [unfed (*UF-FAW*) or leaf-fed FAW (*LF-FAW*)], or undamaged (*control*) plants. The homoterpene [*E*]-4,8-dimethyl-1,3,7-nonatriene (DMNT, RT 5.22 min) is the most abundant volatile emitted by both the induced and uninduced cowpea plants used in the bioassays. Samples represent 1/5× the total headspace emissions collected over 60 min. Octane (280 ng, RT 2.38 min) and nonyl acetate (400 ng, RT 7.53 min) were added separately as internal standards (I.S.). To obtain only fresh damage or older damage odor phenologies, FAW-damaged plants were attacked by damaging larvae either 1 h (fresh) or 4 h (old) before the volatile collections

natural enemies that forage for plants infested with herbivores (Kessler and Baldwin 2001).

Despite the role of herbivore-induced plant volatiles as cues for natural enemies, some lepidopteran neonates orient to herbivore-damaged hosts during the search for an initial feeding site. Many neonates are not positioned on preferred host tissues during oviposition, but move within the plant to an appropriate feeding site after hatching (Zalucki et al. 2002). Movement of codling moth (*Cydia pomonella*; Lepidoptera: Tortricidae) neonates from oviposition sites on foliage to feeding sites on apple fruit is enhanced by conspecific damage and induction of the fruit volatile [*E, E*]-α-farnesene (Landolt et al. 2000), despite the fact that herbivore damage attracts the larval parasitoid *Hyssopus pallidus* (Hymenoptera: Eulophidae; Rott et al. 2005). In this case, the utility of induced volatiles as olfactory attractants to appropriate host tissue may outweigh secondary consequences of induced defenses or enhanced recruitment of natural enemies.

Noticeably, dispersing FAW neonates are more attracted to odors from plants with old (4 h) neonate feeding damage than to odors from plants with fresh (1 h) neonate feeding damage. These olfactory preferences occur despite an absence of prior feeding experience. Unlike older instars, neonates have not yet been exposed to plant odors during feeding. Experience-based olfactory preferences are thought to be an important component in the development of host preferences in related polyphagous noctuids, such as *Spodoptera littoralis* (Anderson et al. 1995; Carlsson et al. 1999). However, an innate lack of attraction to fresh damage volatiles would enhance dispersal of FAW neonates away from the egg mass and limit orientation to new feeding sites established by neonate siblings. Neonate attraction might also be targeted toward host plant volatiles induced by oviposition itself. Recent studies indicate that some plants respond to insect oviposition by emitting volatiles that attract egg parasitoids to the oviposition site (Meiners and Hilker 1997, 2000; Colazza et al. 2004). Although induction of volatiles by oviposition has not been examined in either cowpea or the grasses preferred by FAW, the large size of the egg mass and the female's preference for readily inducible leaf tissue (leaf vein creases) as a preferred oviposition site (Sparks 1979) are factors that merit further investigation.

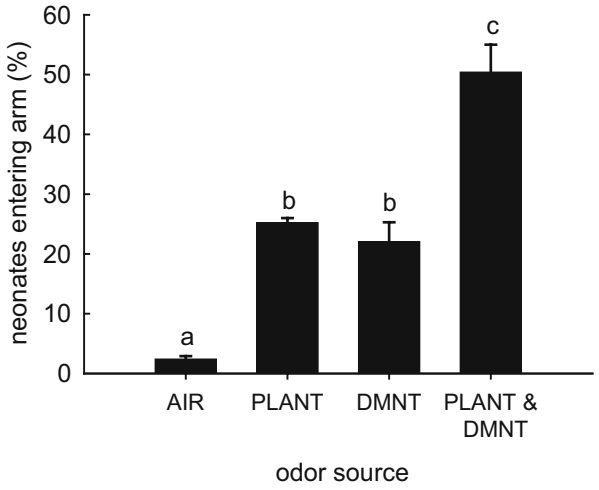

Fig. 6 Response of *Spodoptera frugiperda* neonates to different odor sources offered simultaneously in a four-arm olfactometer: filtered air (AIR), undamaged cowpea plant (PLANT), DMNT-supplemented filtered air (DMNT), or a DMNT-supplemented air stream from an undamaged cowpea plant (PLANT & DMNT). Mean percentage (±SE) of neonates is the proportion of neonates entering a respective olfactometer arm out of the total number that responded positively (entered arms; $N=3$ replicates, a total of 97, 107, and 123 larvae responded in each replicate). For the DMNT supplemented odor sources, 80 ng/h of synthetic DMNT was introduced into the air stream by capillary release. Treatments not sharing the same letter are significantly different by *Tukey-Kramer HSD* ($P<0.05$; *one-way ANOVA*; $P<0.001$, $F=27.60$, $df=3$)

The use of herbivore-induced plant volatiles as neonate orientation cues may facilitate and reinforce a broad host range in polyphagous herbivores such as FAW. Although FAW has been reported from over 80 plant species, the preferred oviposition hosts are overwhelmingly grasses (Luginbill 1928; Sparks 1979; Meagher et al. 2004). On grasses, FAW neonates feed preferentially and performed better on younger tissues, especially leaf whorls (Yang et al. 1993; Davis et al. 1999), developing tissues that usually emit higher levels of constitutive and induced volatiles than comparable mature tissues (Hoballah et al. 2004). While unrelated hosts such as cowpea and maize differ considerably in plant architecture and overall secondary chemistry, these plants share some similarities in damage odors derived from a relatively restricted pool of volatiles (Gouinguené et al. 2003; D'Alessandro and Turlings 2005). DMNT is one of the few major induced volatiles emitted by both cowpea and maize (Carroll et al. 2006). FAW attraction to the major induced volatile emitted by a marginal host (cowpea) may reflect its co-occurrence in the preferred tissues of a major host (maize). A polyphagous caterpillar therefore may exploit induced volatile odors, not only because of their high emission rates, but also because qualitatively these compounds are fairly ubiquitous components of diverse host volatile profiles. The use of induced volatiles as cues may be further promoted by their familiar presence around actively feeding FAW at their own feeding sites, as larvae routinely encounter induced volatiles emitted by host tissues damaged by themselves.

Compared with other hosts of FAW, herbivore-damaged cowpea releases relatively few major volatile components, yet is readily detected by both feeding herbivores and parasitoids (Hoballah et al. 2002). It is unclear if herbivores and parasitoids are attracted to exactly the same induced volatiles. FAW neonates orient toward herbivore-induced volatiles that may be poorly perceived by some parasitoids (Gouinguené et al. 2005) but used as cues by others (Pickett et al. 2006). For example, DMNT, the major volatile induced in cowpea leaves, is readily detected (as measured by GC-EAG) in *Spodoptera littoralis*-damaged cowpea leaves by the parasitoid *Campoletis sonerensis* (Hymenoptera: Ichneumonidae) but not *Cotesia marginiventris* (Hymenoptera: Braconidae) or *Microplitis rufiventris* (Hymenoptera: Braconidae; Gouinguené et al. 2005). Other induced volatile components from cowpea leaves may be used as olfactory cues by both herbivores and parasitoids. For example, the monoterpene linalool, which has been detected as a minor component of herbivore-damaged cowpea leaves by several authors (Hoballah et al. 2002; D'Alessandro and Turlings 2005) but not in one of our previous studies (Schmelz et al. 2006), is highly attractive to both sixth instar FAW (Carroll et al. 2006) and several noctuid parasitoids (Hoballah et al. 2002; Gouinguené et al. 2005). Neonates and parasitoids have likely converged on a limited subset of induced plant volatiles as olfactory cues based on their functionality (Stamps and Krishnan 2005)—as ubiquitous and reliable markers of herbivore damage, induced volatiles are among the most apparent indicators of host plant location available to a forager. The extent of overlap and exclusion between herbivores and parasitoids in their use of particular volatiles as cues remains to be determined.

While much research has focused on how plants respond to herbivory and how natural enemies react to induced plant volatiles (Kessler and Baldwin 2002; Dicke et al. 2003; Paré et al. 2005), little is known about responses of feeding herbivores themselves. The few studies that have been performed on generalist responses to induced volatiles have focused primarily on ovipositing females for the simple reason that oviposition is often a critical determinant of host plant selection (De Moraes et al. 2001; Kessler and Baldwin 2001). However, we should not ignore the fact that it is neonate establishment of an initial feeding site on a host, rather than oviposition alone, that is the *sine qua non* of host acceptance by herbivores. Paradoxically, it is often the limited and unspectacular search capabilities and acceptance behaviors of neonates that determine whether host acceptance or rejection occurs after oviposition (del Campo et al. 2001; Renwick 2001). Despite their underlying simplicity (Bernays et al. 2004), caterpillar feeding

behaviors can be readily influenced by host plant volatiles (Shiojiri et al. 2006a).

Given that the induced volatile profiles of herbivore-damaged plants often differ considerably from undamaged plants and change rapidly over time, it is necessary to consider both the role of volatile induction in herbivore host plant acceptance as well as the reciprocal impact of herbivore behavioral responses on the plant. Recent studies with parasitoids demonstrate a surprising complexity in the functions attributed to individual components of an attractive herbivore-induced plant odor, with some compounds being attractive, others repellent, some potentially masking the effects of others, and some inaffectably neutral (D'Alessandro et al. 2006). Comparable studies need to be conducted to determine to what extent these volatiles are directed against herbivores. In particular, the potential of induced volatile components to mask or alter the attractive qualities of a host plant to an herbivore should be further explored. Future attempts to improve plant resistance through alteration of induced volatiles must take into account the different roles of individual components, not only for natural enemies but also for direct mediation of herbivore behavior (Zangerl 2003; Shiojiri et al. 2006a, b).

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References

ANDERSON, P., HILKER, M., and LÖFQVIST, J. 1995. Larval diet influence on oviposition behavior in *Spodoptera littoralis*. *Entomol. Exp. Appl* 74:71–82.

BERNASCONI, M. L., TURLINGS, T. C. J., AMBROSETTI, L., BASSETTI, P., and DORN, S. 1998. Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis*. *Entomol. Exp. Appl* 87:133–142.

BERNAYS, E. A., SINGER, M. S., and ODRIGUES, D. 2004. Foraging in nature: foraging efficiency and attentiveness in caterpillars with different diet breadths. *Ecol. Entomol* 29:389–397.

BOLTER, C. J., DICKE, M., VANLOON, J. J. A., VISSER, J. H., and POSTHUMUS, M. A. 1997. Attraction of Colorado potato beetle to herbivore-damaged plants during herbivory and after its termination. *J. Chem. Ecol* 23:1003–1023.

CARROLL, M. J., SCHMELZ, E. A., MEAGHER, R. L., and TEAL, P. E. A. 2006. Attraction of *Spodoptera frugiperda* larvae to volatiles from herbivore-damaged maize seedlings. *J. Chem. Ecol* 32:1911–1924.

CARLSSON, M. A., ANDERSON, P., HARTLIEB, E., and HANSSON, B. S. 1999. Experience-dependent modification of orientational response to olfactory cues in larvae of *Spodoptera littoralis*. *J. Chem. Ecol* 25:2445–2454.

CHAPMAN, J. W., WILLIAMS, T., ESCRIBANO, A., CABALLERO, P., CAVE, R. D., and GOULSON, D. 1999. Fitness consequences of cannibalism in the fall armyworm, *Spodoptera frugiperda*. *Behav. Ecol* 10:298–303.

COLAZZA, S., FUCARINO, A., PERI, E., SALERNO, G., CONTI, E., and BIN, F. 2004. Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *J. Exp. Biol* 207:47–53.

D'ALESSANDRO, M., HELD, M., TRIPONEC, Y., and TURLINGS, T. C. J. 2006. The role of indole and other shikimic acid derived maize volatiles in the attraction of two parasitic wasps. *J. Chem. Ecol* 32:2733–2748.

D'ALESSANDRO, M., and TURLINGS, T. C. J. 2005. *In situ* modification of herbivore-induced plant odors: A novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem. Senses* 30:739–753.

DAVIS, F. M., WILLIAMS, W. P., CHANG, Y. M., BAKER, G. T., and HEDIN, P. A. 1999. Differential growth of fall armyworm larvae (Lepidoptera: Noctuidae) reared on three phenotypic regions of whorl leaves from a resistant and a susceptible maize hybrid. *Fla. Entomol* 82:248–254.

DE MORAES, C. M., MESCHER, M. C., and TUMLINSON, J. H. 2001. Caterpillar-induced nocturnal plant volatiles repel nonspecific females. *Nature* 410:577–580.

DEL CAMPO, M. L., MILES, C. I., SCHROEDER, F. C., MUELLER, C., BOOKER, R., and ENWICK, J. A. 2001. Host recognition by the tobacco hornworm is mediated by a host plant compound. *Nature* 411:186–189.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol* 25:1907–1922.

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool* 38:148–165.

DICKE, M., VAN POECKE, R. M. P., and DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Bas. Appl. Ecol* 4:27–42.

DICKE, M., and VET, L. E. M. 1999. Plant-carnivore interactions: evolutionary and ecological consequences for plant, herbivore and carnivore, pp. 483–520, in H. Olff, V. K. Brown, and R. H. Drent (eds.). Herbivores: Between Plants and PredatorsOxford, Blackwell Science Oxford.

DOAK, P. 2000. Population consequences of restricted dispersal for an insect herbivore in a subdivided habitat. *Ecology* 81:1828–1841.

GOUINGUENÉ, S., ALBORN, H., and TURLINGS, T. C. J. 2003. Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. *J. Chem. Ecol* 29:145–162.

GOUINGUENÉ, S., PICKETT, J. A., WADHAMS, L. J., BIRKETT, M. A., and TURLINGS, T. C. J. 2005. Antennal electrophysiological responses of three parasitic wasps to caterpillar-induced volatiles from maize (*Zea mays mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*). *J. Chem. Ecol* 31:1023–1038.

HARRIS, M. O., SANDANAYAKE, M., and FOSTER, S. P. 1999. Chemical stimuli from apple influence the behavior of neonate caterpillars of the generalist herbivore, *Epiphyas postvittana*. *J. Chem. Ecol* 25:1717–1738.

HEATH, R. R., and MANUKIAN, A. 1994. An automated-system for use in collecting volatile chemicals released from plants. *J. Chem. Ecol* 20:593–608.

HEATH, R. R., and SONNET, P. E. 1980. Technique for *in situ* coating of Ag^{+} onto silica gel in HPLC columns for the separation of geometrical isomers. *J. Liquid Chromatogr* 3:1129–1135.

HILKER, M., and KLEIN, B. 1989. Investigation of oviposition deterrent in larval frass of *Spodoptera littoralis* (Boisd.). *J. Chem. Ecol* 15:929–938.

HOBALLAH, M. E., KOLLNER, T. G., DEGENHARDT, J., and TURLINGS, T. C. J. 2004. Costs of induced volatile production in maize. *Oikos* 105:168–180.

HOBALLAH, M. E., and TURLINGS, T. C. J. 2005. The role of fresh versus old leaf damage in the attraction of parasitic wasps to herbivore-induced maize volatiles. *J. Chem. Ecol* 31:2003–2018.

HOBALLAH, M. E. F., TAMO, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: Is quality or quantity important? *J. Chem. Ecol* 28:951–968.

KESSLER, A., and BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141–2144.

KESSLER, A., and BALDWIN, I. T. 2002. Plant responses to insect herbivory: The emerging molecular analysis. *Annu. Rev. Plant Biol* 53:299–328.

LANDOLT, P. J. 1993. Effects of host plant leaf damage on cabbage moth attraction and oviposition. *Entomol. Exp. Appl* 67:79–85.

LANDOLT, P. J., BRUMLEY, J. A., SMITHHISLER, C. L., BIDDICK, L. L., and HOFSTETTER, R. W. 2000. Apple fruit infested with codling moth are more attractive to neonate codling moth larvae and possess increased amounts of (*E,E*)-alpha-farnesene. *J. Chem. Ecol* 26:1685–1699.

LUGINBILL, P. 1928. The fall armyworm. *U.S. Department of Agriculture Technical Bulletin* 34:1–91.

MEAGHER, R. L., NAGOSHI, R. N., STUHL, C., M, and E. R. 2004. Larval development of fall armyworm (Lepidoptera: Noctuidae) on different cover crop plants. *Fla. Entomol* 87:454–460.

MEINERS, T., and HILKER, M. 1997. Host location in *Oomyzus gallerucae* (Hymenoptera: Eulophidae), an egg parasitoid of the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera, Chrysomelidae). *Oecologia* 112:87–93.

MEINERS, T., and HILKER, M. 2000. Induction of plant synomones by oviposition of a phytophagous insect. *J. Chem. Ecol* 26:221–232.

PARÉ, P. W., FARAG, M. A., KRISHNAMACHARI, V., ZHANG, H. M., YU, C. M., and KLOEPPER, J. W. 2005. Elicitors and priming agents initiate plant defense responses. *Photosynth. Res* 85:149–159.

PARÉ, P. W., and TUMLINSON, J. H. 1997. Induced synthesis of plant volatiles. *Nature* 385:30–31.

PARÉ, P. W., and TUMLINSON, J. H. 1999. Plant volatiles as a defense against insect herbivores. *Plant Physiol* 121:325–331.

PICKETT, J. A., BRUCE, T. J. A., CHAMBERLAIN, K., HASSANNALI, A., KHAN, Z. R., MATTHES, M. C., NAPIER, J. A., SMART, L. E., WADHAMS, L. J., and WOODCOCK, C. M. 2006. Plant volatiles yielding new ways to exploit plant defence, pp. 161–173, in M. Dicke, and W. Takken (eds.). Chemical Ecology: from Gene to EcosystemSpringer, Wageningen, The Netherlands.

PITRE, H. N., MULROONEY, J. E., and HOGG, D. B. 1983. Fall armyworm (Lepidoptera: Noctuidae) oviposition: crop preferences and egg distribution on plants. *J. Econ. Entomol* 76:463–466.

PROKOPY, R. J., and ROITBERG, B. D. 2001. Joining and avoidance behavior in nonsocial insects. *Annu. Rev. Entomol* 46:631–665.

RENWICK, J. A. A. 2001. Variable diets and changing taste in plant-insect relationships. *J. Chem. Ecol* 27:1063–1076.

ROITBERG, B. D., and MANGEL, M. 1993. Parent-offspring conflict and life history consequences in herbivorous insects. *Am. Nat* 1442:443–456.

ROTT, A. S., HACKERMANN, J., BRAND, N., VALLAT, A., and DORN, S. 2005. Parasitoid exploitation of the seasonal variation in host plant volatile emission for herbivore location. *Entomol. Exp. Appl* 115:199–205.

SCHMELZ, E. A., ALBORN, H. T., and TUMLINSON, J. H. 2001. The influence of intact-plant and excised-leaf bioassay designs on volicitin- and jasmonic acid-induced sesquiterpene volatile release in *Zea mays*. *Planta* 214:171–179.

SCHMELZ, E. A., CARROLL, M. J., LECLERE, S., PHIPPS, S. M., MEREDITH, J., CHOUREY, P. S., ALBORN, H. T., and TEAL, P. E. A. 2006. Fragments of ATP synthase mediate plant perception of insect attack. *Proc. Natl. Acad. Sci. U.S.A* 103:8894–8899.

SCHOONHOVEN, L. M., and VAN LOON, J. J. A. 2002. An inventory of taste in caterpillars: Each species its own key. *Acta Zool. Acad. Sci. H* 48:215–263.

SHIOJIRI, K., OZAWA, R., and TAKABAYASHI, J. 2006a. Plant volatiles, rather than light, determine the nocturnal behavior of a caterpillar. *Plos Biology* 4:1044–1047.

SHIOJIRI, K., KISHIMOTO, K., OZAWA, R., KUGIMIYA, S., URASHIMO, S., ARIMURA, G., HORIUCHI, J., NISHIOKA, T., MATSUI, K., and TAKABAYASHI, J. 2006b. Changing green leaf volatile biosynthesis in plants: An approach for improving plant resistance against both herbivores and pathogens. *Proc. Natl. Acad. Sci. U.S.A* 103:16672–16676.

SPARKS, A. N. 1979. A review of the biology of the fall armyworm. *Fla. Entomol* 62:82–86.

STAMPS, J., and KRISHNAN, V. V. 2005. Nonintuitive cue use in habitat selection. *Ecology* 86:2860–2867.

SUAZO, A., TORTO, B., TEAL, P. E. A., and TUMLINSON, J. H. 2003. Response of the small hive beetle (*Aethina tumida*) to honey bee (*Apis mellifera*) and beehive-produced volatiles. *Apidologie* 34:525–533.

THOMPSON, J. N. 1988. Evolutionary ecology of the relationship between oviposition preference and performance of offspring in phytophagous insects. *Entomol. Exp. Appl* 47:3–14.

TURLINGS, T. C. J., ALBORN, H. T., LOUGHRIN, J. H., and TUMLINSON, J. H. 2000. Volicitin, an elicitor of maize volatiles in oral secretion of *Spodoptera exigua*: Isolation and bioactivity. *J. Chem. Ecol* 26:189–202.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* 207:146–152.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., OSE, U. S., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. U.S.A* 92:4169–4174.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking wasps. *Science* 250:1251–1253.

WARD, S. A. 1987. Optimal habitat selection in time-limited dispersers. *Am. Nat* 129:568–579.

WEATHERSTON, I., MILLER, D., and DOHSE, L. 1985. Capillaries as controlled-release devices for insect pheromones and other volatile substances - a reevaluation. Part I. Kinetics and development of predictive model for glass capillaries. *J. Chem. Ecol* 11:953–965.

YANG, G., WISEMAN, B. R., and ESPELIE, K. E. 1993. Movement of neonate fall armyworm (Lepidoptera: Noctuidae) larvae on resistant and susceptible genotypes of corn. *Environ. Entomol* 22:547–553.

ZALUCKI, M. P., CLARKE, A. R., and MALCOLM, S. B. 2002. Ecology and behavior of first instar larval Lepidoptera. *Annu. Rev. Entomol* 47:361–393.

ZANGERL, A. R. 2003. Evolution of induced plant responses to herbivores. *Bas. Appl. Ecol* 4:91–103.

ZANGERL, A. R., and BERENBAUM, M. R. 1992. Oviposition patterns and hostplant suitability - parsnip webworms and wild parsnip. *Am. Midl. Nat* 128:292–298.

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Aphid and Plant Volatiles Induce Oviposition in an Aphidophagous Hoverfly

Francois J. Verheggen · Ludovic Arnaud · Stefan Bartram · Marie Gohy · Eric Haubruge

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Abstract *Episyrphus balteatus* DeGeer (Diptera, Syrphidae) is an abundant and efficient aphid-specific predator. We tested the electroantennographic (EAG) response of this syrphid fly to the common aphid alarm pheromone, (E)-β-farnesene (EβF), and to several plant volatiles, including terpenoids (mono- and sesquiterpenes) and green leaf volatiles (C6 and C9 alcohols and aldehydes). Monoterpenes evoked significant EAG responses, whereas sesquiterpenes were inactive, except for the aphid alarm pheromone (EβF). The most pronounced antennal responses were elicited by six and nine carbon green leaf alcohols and aldehydes [i.e., (*Z*)-3-hexenol, (*E*)-2-hexenol, (*E*)-2-hexenal, and hexanal]. To investigate the behavioral activity of some of these EAG-active compounds, *E. balteatus* females were exposed to *R*-(+)-limonene (monoterpene), (*Z*)-3-hexenol (green leaf alcohol), and EβF (sesquiterpene, common aphid alarm pheromone). A single *E. balteatus* gravid female was exposed for 10 min to an aphid-free *Vicia faba* plant that was co-located with a semiochemical dispenser. Without additional semiochemical, hoverfly females were not attracted to this plant, and no oviposition was observed. The monoterpene *R*-(+)-limonene did not affect the females' foraging behavior, whereas (*Z*)-3-hexenol and EβF increased the time of flight and acceptance of the host plant. Moreover, these two chemicals induced oviposition on aphid-free plants, suggesting that selection of the oviposition site by predatory hoverflies relies on the perception of a volatile blend composed of prey pheromone and typical plant green leaf volatiles.

Keywords *Episyrphus balteatus* · Predator · Plant-insect interaction · Oviposition induction · Green leaf volatiles · Terpenoids · *(E)*-β-Farnesene · Electroantennography · EAG

F. J. Verheggen (✉) · L. Arnaud · E. Haubruge
Department of Functional and Evolutionary Entomology,
Gembloux Agricultural University,
Gembloux, Belgium
e-mail: entomologie@fsagx.ac.be

S. Bartram
Department of Bioorganic chemistry,
Max Planck Institute for Chemical Ecology,
Jena, Germany

M. Gohy
Unité Technique COVs Qualité de l'air,
Institut scientifique de service public,
Liege, Belgium

Introduction

Episyrphus balteatus DeGeer (Diptera: Syrphidae) is the most frequently encountered syrphid species at aphid-infested sites in temperate regions (Schneider 1969), and one of the most efficient aphid-specific predators (Entwistle and Dixon 1989; Tenhumberg and Poehling 1991). Because syrphid larvae have limited dispersal abilities (Chandler 1969), oviposition site selection has an important impact on offspring performance. Several studies have addressed a wide range of parameters that influence the foraging and oviposition behavior of aphid natural enemies. Those studied so far are (1) aphid species and their associated chemicals (Budenberg and Powell 1992; Bargen et al. 1998; Sadeghi and Gilbert 2000a, b; Zhu et al. 2005; Almohamad et al. 2007; Verheggen et al. 2007a), (2) physical and chemical characteristics of plants associated with aphid species (Chandler 1968; Sanders 1983; Vanhaelen et al. 2001, 2002; Tumlinson et al. 1992; Zhu et al. 2005; Videla et al. 2006; Almohamad et al. 2007; Harmel et al. 2007), (3) aphid colony size and density (Bargen et al. 1998; Scholz and

Poehling 2000; Sutherland et al. 2001), (4) age of the hoverfly female (Sadeghi and Gilbert 2000c; Frechette et al. 2004) and (5) floral characters (Sutherland et al. 1999).

Location of herbivorous prey by carnivorous arthropods is known to be mediated by many semiochemicals emitted by the prey or its host plants (Dicke and Sabelis 1988; Vet and Dicke 1992; Harmel et al. 2007). Many studies on tritrophic interactions between plants, herbivorous insects, and natural enemies have demonstrated that attack-induced plant volatiles (synomones) may attract carnivorous species (Nordlund and Lewis 1976; Turlings et al. 1990; Vet and Dicke 1992; Tumlinson et al. 1992; Turlings and Tumlinson 1992; De Moraes et al. 2001). Plants infested by herbivores can qualitatively and/or quantitatively change their volatile emissions. These emissions usually consist of terpenoids (monoterpenes and sesquiterpenes) and green leaf volatiles (GLVs; alcohols, aldehydes, or esters), the latter being specifically released just upon tissue damage (Paré and Tumlinson 1997; Farag and Paré 2002; Tholl et al. 2006).

Compared to the large body of information on parasitoids, less information is available on those chemical cues that guide predators during location and acceptance of oviposition sites (Steidle and van Loon 2002). Hoverflies are subjected to various chemical blends when searching for an oviposition site. These blends consist of plant and insect semiochemicals such as (*E*)-β-farnesene (EβF), the main component of the alarm pheromone of most aphid species (Nault et al. 1973; Francis et al. 2005a). This sesquiterpene has been found to act as a kairomone for several aphid predators, including *E. balteatus* larvae, *Harmonia axyridis* adults, and *Adalia bipunctata* larvae and adults (Francis et al. 2004, 2005b; Verheggen et al. 2007a). More than 20 additional chemicals, including α- and β-pinene, cymene, α-phellandrene, or limonene, were found by Francis et al. (2005a) to be released by some aphid species. These volatiles are also commonly found in the headspace of many plant families, such as the Solanaceae, Fabaceae, or Brassicaceae (Agelopoulos et al. 1999; Farag and Paré 2002; Verheggen et al. 2005; Harmel et al. 2007). Terpenoids and GLVs are potential semiochemicals that are used by aphid predators, such as syrphids, lady beetles, or lacewings, to locate their prey (Zhu et al. 1999; Steidle and van Loon 2002; Harmel et al. 2007).

In this study, we investigated the olfactory perception and behavioral activity of various plant volatiles and the common aphid alarm pheromone (EβF) to highlight those that may act on the prey-seeking behavior of *E. balteatus*.

Methods and Materials

Chemicals All chemicals, except EβF, were purchased from Sigma-Aldrich (Chemie Gmbh, Steinheim, Germany) and had chemical purity >97% [determined by gas chromatography (GC)]. EβF was synthesized from farnesol (Tanaka et al. 1975) and had a chemical purity of 98% (also determined by GC).

Biological Material All plants, aphids, and hoverflies were reared in climate-controlled rooms (16 hrs light photoperiod; 70% RH; 20±2°C). Broad beans (*Vicia faba* L.) were grown in plastic pots (9×8 cm) filled with a mixture of vermiculite and perlite (1/1) and were used as host plants for the pea aphid, *Acyrthosiphon pisum* Harris. Adult *E. balteatus* were reared in cages (75×60×90 cm) and fed with pollen, sugar, and water *ad libitum*. Hoverfly oviposition was induced by placing broad beans in the cage for 3 hrs. *E. balteatus* larvae were fed with *A. pisum* and pupae were placed in aerated plastic boxes (14×11×4 cm) until hatching. Experiments were carried out with 2- to 4-wk-old adults.

Electroantennography The hoverfly was immobilized by covering its abdomen and thorax with modeling clay. This setup enabled the recording of electroantennograms for a longer time period than if the antenna was excised (Verheggen et al. 2007b). Two glass Ag-AgCl electrodes (Harvard Apparatus; 1.5 mm OD×1.17 mm ID) filled with saline solution (NaCl, 7.5 g/l; $CaCl_2$, 0.21 g/l; KCl, 0.35 g/l; $NaHCO_3$, 0.2 g/l) and in contact with a silver wire were placed on the insect antennae. The ground glass electrode entirely covered one antenna, whereas the recording electrode that was linked to an amplifier (IDAC-4, Syntech®, Hilversum, The Netherlands) with a ×100 amplification was placed on the bottom of the last segment of the other antenna. A 0.5-cm^2 piece of filter paper that was impregnated with 10 µl of the chemical under examination was placed in a Pasteur pipette. This was then used to puff an air sample in a constant 1.5 l/min airstream. Paraffin oil was used to make chemical solutions with seven concentrations ranging from 10^{-1} to 10^5 ng/µl (by 10× increments). Electroantennograms were collected by using Autospike 3.0 (Syntech, Hilversum, The Netherlands). Stimulation with paraffin oil was used as a negative control before and after the stimulations with the seven concentrations. Time between stimulations was 30 sec. Preliminary results indicated that this length of time was adequate to allow the antenna to recover and regain its full reactivity to stimuli. Five insects from both sexes were tested with each chemical.

Behavioral Observations A single female hoverfly was placed in a cage (30×30×60 cm) with a *V. faba* plant (height, 20 cm). As a positive control, the hoverflies were offered a *V. faba* plant that was infested with 1 g of the pea aphid *A. pisum* 24 hrs before the experiment started. A non-

infested *V. faba* plant was offered to the hoverfly as negative control. A rubber septum was used as dispenser to test the behavioral activity of *(R)*-(+)-limonene (monoterpene), *(Z)*-3-hexenol (GLV), and EβF (sesquiterpene). The dispenser was placed on the first pair of true leaves of a non-infested plant and contained a 100-µl paraffin oil solution (400 ng/µl) of the test chemical. The solution was changed after each replication. Paraffin oil was chosen because of its chemical inertness and ability to continuously release chemicals that are diluted within it. The foraging behavior was recorded for 10 min by using the software The Observer5.0® (Noldus information Technology, version 5.0, Wageningen, The Netherlands). This allows hoverfly behavior to be easily observed, subdivided, and recorded (Harmel et al. 2007). Descriptions of the four observed behavioral subdivisions are presented in Table 1. The number of eggs laid by each female was counted at the end of each observation. Experiments were conducted in a climate-controlled room at 22±1°C. The *E. balteatus* females were 15- to 30-d old, and no aphid-infested plant was offered for 24 hrs before the experiment. Ten replications were performed for each tested chemical.

Statistical Analyses One-way analysis of variance (ANOVA) followed by Tukey's test (pairwise comparisons) was used to evaluate the EAG results. Two sample *t* tests were used to compare EAG responses by males and females. A one-way ANOVA followed by Dunnett's test (comparison with a control) was used to compare the behavioral data observed for the four treatments compared to the control. The one sample *t* test was applied to compare the mean number of eggs laid in behavioral assays to the "0" value observed with the control. All statistical tests were conducted by using Minitab v.14 for Windows®.

Table 1 Description of the behavioral sequences recorded for aphidophagous hoverfly *Episyrphus balteatus* exposed to *Vicia faba*

Observed Behavioral Sequences	Descriptions
Immobility	Predator immobilized on the cage
Searching	
Fly/cage	Predator flies in the cage
Fly/plant	Predator flies near the plant
Acceptance	
Immobile/plant	Predator lands on the plant
Walking/plant	Predator moves on the plant
Proboscis/plant	Predator extends its proboscis and identifies the stimulatory substrate to accept the host
Oviposition	
Immobile abdomen/plant	Predator exhibits an abdominal protraction
Walking abdomen/plant	
Egg laying	Oviposition

Results

Electroantennography Antennal responses increased significantly in both sexes with the concentration of the tested compound (that ranged from 0.1 ng/µl to 0.1 mg/µl). No saturation of the antenna was observed for any tested chemical. The three lowest concentrations (0.1, 1, and 10 ng/µl) did not elicit antennal response, regardless of the tested compound. Because we aimed to compare EAG data to results previously obtained when similar chemicals were tested on other aphid predators, we did not correct our EAG responses by taking into account their differences of volatility, as stated by Brockerhoff and Grant (1999).

EAG responses were significantly different among the five tested chemical families ($F_{4,152}$=117.82, P<0.001), namely, monoterpenes, monoterpenes with an alcohol function, sesquiterpenes, and C6 and C9 green leaf chemicals (Fig. 1). The eight tested monoterpenes elicited electrical depolarization that ranged from −400 to −800 µV, and they were all equally perceived by both sexes. Linalool induced an average depolarization of −1,300 µV. This monoterpene was similarly perceived by males and females (t_{obs}=0.95, P=0.372). The sesquiterpene EβF was the only chemical to be perceived differently by males and females (t_{obs}=2.62, P=0.031). The two other sesquiterpenes (α-humulene and β-caryophyllene) did not elicit electrical depolarization in either sex. The green leaf alcohols and aldehydes tested elicited high EAG responses, statistically equal in both males and females, ranging from −1,750 to −2,250 µV for the six-carbon chain GLV and from −600 to −1,400 µV for the nine-carbons chain GLV.

Behavioral Observations Hoverfly gravid females were not activated by a non-infested *V. faba* and stayed immobile during 80% of the observation time (Fig. 2). However, when an aphid-infested plant was presented, the duration of immobility was significantly reduced, and the time spent on the plant (acceptance) increased. Moreover, the number of eggs laid increased compared to non-infested plants (7.4 eggs/female; t_{obs}=7.38, P<0.001; Fig. 3). *(R)*-(+)-Limonene did not significantly attract the predatory hoverflies to the non-infested plant and did not increase the number of eggs laid, when compared with a non-infested semiochemical-free plant (0.2 egg/female; t_{obs}= 1.50, P=0.084). *(Z)*-3-Hexenol presented on a non-infested plant significantly increased the mobility of females and plant acceptance. Furthermore, this GLV induced oviposition on the non-infested plant compared to oviposition on

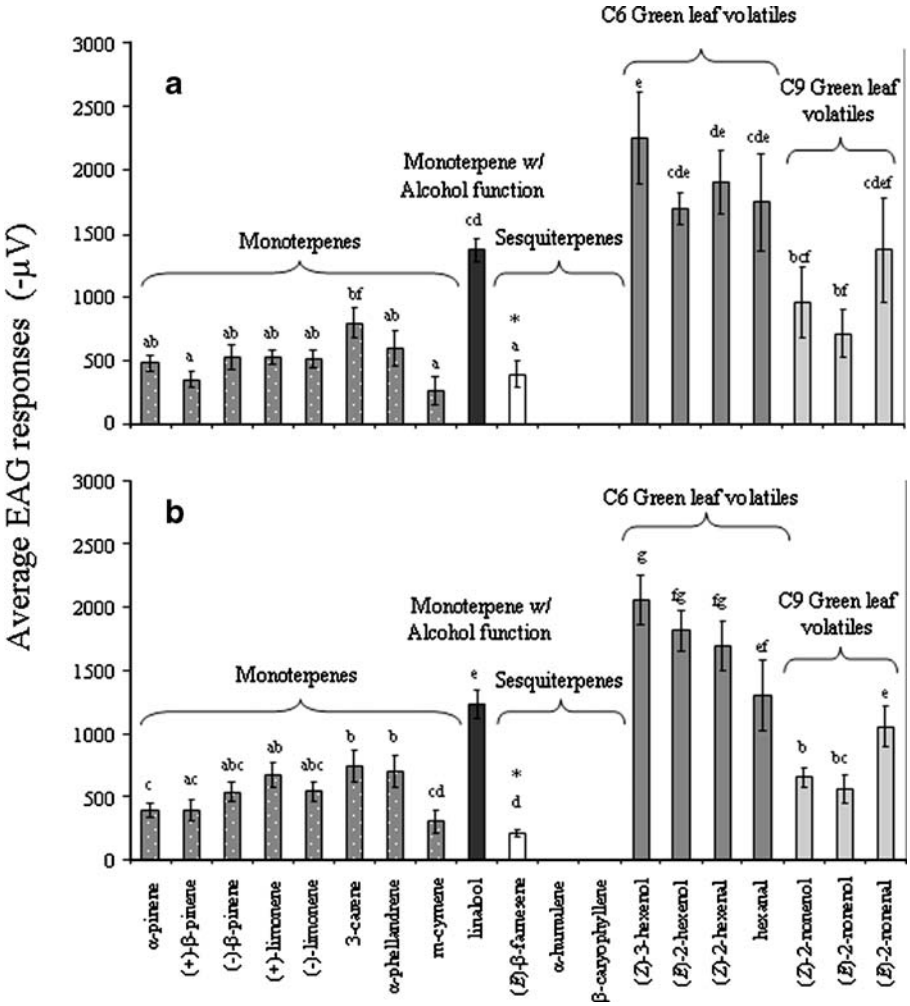

Fig. 1 EAG activity of female (**a**) and male (**b**) *Episyrphus balteatus* antennae to aphid and plant volatiles (100 μg/μl). Means (±SE) with no letter in common are significantly different (ANOVA followed by Tukey's test, $P<0.05$). An *asterisk* indicates significant difference in EAG activity between male and female antennae (two-sample Student's *t* test, $P<0.05$). $N=5$ for both sexes and each chemical

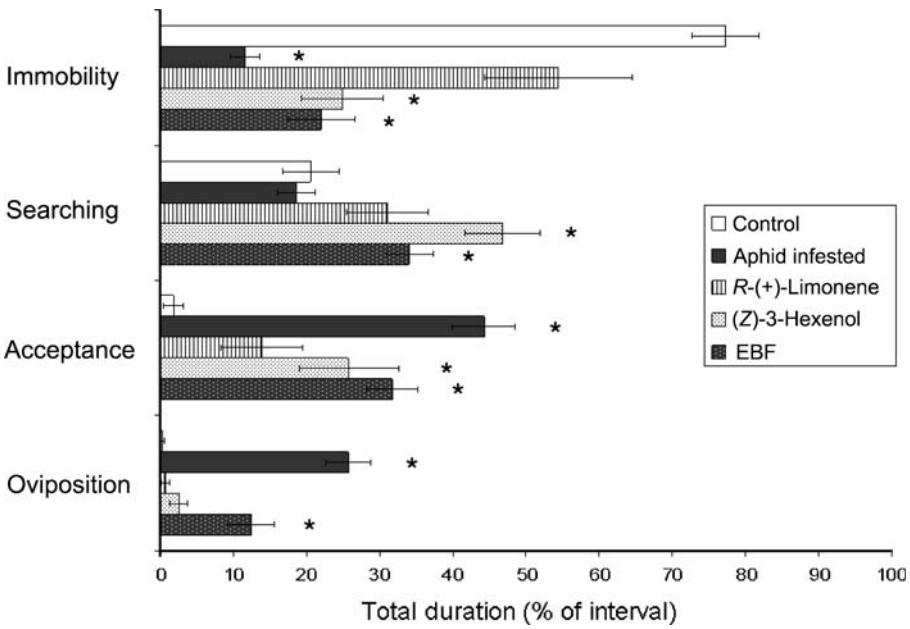

Fig. 2 Effect of plant and aphid volatiles on different sequences of the foraging behavior of *Episyrphus balteatus* females (mean duration in percent of interval, ±SE). Volatiles [*R*-(+)-limonene, (*Z*)-3-hexenol, and (*E*)-β-farnesene (EBF)] were offered on a non-infested plant. *Stars* indicate significant differences from the non-infested semiochemical-free plant (=Control) for the respective behavioral sequence (ANOVA followed by Dunnett's test, $P<0.05$). $N=10$ for each treatment

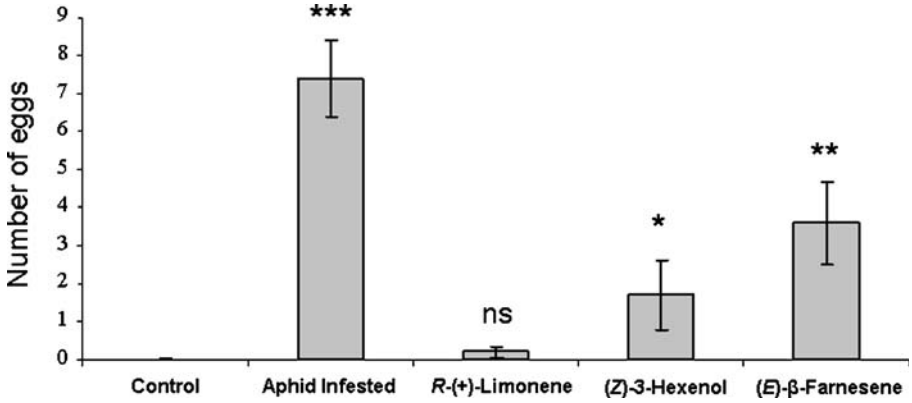

Fig. 3 Effect of plant and aphid semiochemicals on oviposition of *Episyrphus balteatus* females (mean number of eggs ±SE). *ns* No significant differences from control; **P*<0.05, ***P*<0.01, and ****P*<0.001 significant differences from control (one-sample Student's *t* test). *N*=10 for each treatment

semiochemical-free, non-infested plants (1.7 eggs/female; t_{obs}=1.85, *P*=0.049). The main compound of the aphid alarm pheromone (EβF) significantly increased hoverfly mobility on non-infested plants, searching duration, acceptance of the host plant, and oviposition. The mean number of eggs laid per female was 3.6. This was significantly higher than the control (t_{obs}=3.31, *P*=0.005) and lower than the number of eggs laid on an aphid-infested *V. faba* plant ($F_{1,18}$=6.60, *P*=0.019).

Discussion

Tritrophic interactions among infested plants, herbivorous arthropods, and their natural enemies are complex because of the many semiochemicals typically involved. In addition to the semiochemicals emitted by herbivorous insects, most plant species respond to insect infestation by synthesizing and releasing complex blends of volatiles. These can be used by predators and parasitoids as foraging cues, thereby enhancing the plants' defense ability (Dicke et al. 1990; Dicke 1994; Turlings et al. 1995). There are several previous electrophysiological studies of antennal responses by aphid natural enemies to prey and host plant semiochemicals that include lady beetles (Coleoptera, Coccinellidae) (Zhu et al. 1999; Al Abassi et al. 2000; Verheggen et al. 2007a) and lacewings (Nevroptera, Chrysopidae) (Zhu et al. 1999; 2005). Some chemicals attract predators, but no information is available about their impact on predator foraging behavior and oviposition (Zhu et al. 1999, 2005). However, several species respond with oviposition to aphid-produced honeydew alone, whereas in others, the aphid prey itself is needed for oviposition (Steidle and van Loon 2002).

Syrphid larvae do not use semiochemicals to locate aphids, or exclusively at short distances (Bargen et al. 1998; Francis et al. 2005b). Because of their limited dispersal abilities (Chandler 1969), the choice of the oviposition site by adult females has an impact on offspring performance. Volatile organic compounds are therefore presumed to guide their foraging behavior. To the best of our knowledge, this is the first published report of successful EAG recordings from *E. balteatus* antennae, and we found that adult hoverflies are able to sense their environment by odors. Generalists may need to invest less time in searching particular host and prey species than specialists. Therefore, the use of infochemicals to reduce searching time should be less important (Vet and Dicke 1992). However, previous studies have indicated that the use of infochemicals for foraging is an adaptive strategy regardless of dietary specialization and that physiological constraints on sensory processing in generalists might be less severe than previously supposed (Steidle and van Loon 2003). We demonstrated in this paper that some EAG-active compounds play a key role in foraging behavior of the generalist *E. balteatus*. Plant and prey volatiles activate this predatory species and induce oviposition, even in absence of aphids. Additionally, we have confirmed that *E. balteatus* females do not lay eggs on a non-infested plant (Scholz and Poehling 2000).

The electrophysiologically tested terpenoids α-pinene, β-pinene, α-phellandrene, and limonene are not only common plant volatiles (Farag and Paré 2002; Tholl et al. 2006) but are also emitted by some aphid species such as *Megoura viciae* Buckton or *Drepanosiphum platanoides* Schrank (Francis et al. 2005a). It is not surprising that they, like other monoterpenes, elicited EAG responses in both male and female *E. balteatus*. *(R)*-(+)-Limonene did not attract the hoverflies and that the number of eggs laid by females exposed to *(R)*-(+)-limonene on a non-infested plant was not significantly different from a non-infested plant. This monoterpene is commonly found in the headspace of various plant species (Agelopoulos et al. 1999; Farag and Paré 2002; Verheggen et al. 2005) and does not provide information about prey presence on a stressed plant. This might explain why *(R)*-(+)-limonene did not provoke a behavioral effect on a gravid hoverfly female.

Whereas the tested monoterpenes elicited small EAG responses, the GLVs showed EAG responses that were three to four times higher for the six-carbon chain GLVs, and two to three times higher for the nine-carbon chain GLVs. These responses by *E. balteatus* differ from those by other aphid predators (Zhu et al. 1999). In addition, our results show that *(Z)*-3-hexenol increased the female's mobility, plant acceptance, and oviposition activity even in the absence of prey.

EβF, the common aphid alarm pheromone (Francis et al. 2005a), which acts as a kairomonal substance for several aphid predators (Francis et al. 2004, 2005b; Verheggen et al. 2007a), was detected by both male and female *E. balteatus* adults. In contrast to lacewings and lady beetles, *E. balteatus* showed a sex-specific response to EβF (Zhu et al. 1999; Verheggen et al. 2007a). This difference in antennal activity between sexes accentuates the importance of EβF in hoverfly foraging behavior, as females are looking for a suitable oviposition site. In contrast to the strong response to EβF, hoverflies showed no response to the two other tested sesquiterpenes, α-humulene and β-caryophyllene. Whereas β-caryophyllene induced antennal activity in both lady beetles and lacewings, α-humulene was not tested on these two aphidophagous predators (Zhu et al. 1999; Verheggen et al. 2007a). The lack of electrical response by *E. balteatus* to the two sesquiterpenes and the lower responses observed to the C9-GLV compared to the C6-GLV may also be caused by their lower volatility. Brockerhoff and Grant (1999) stated that EAG responses should be corrected by taking into account the volatility of the tested chemicals. However, EβF is as volatile as β-caryophyllene and α-humulene. EβF significantly increased hoverfly mobility, acceptance of the host plant, and the number of eggs laid (3.6 eggs/females). This aphid alarm pheromone is thus a key compound in prey-seeking behavior in aphidophagous hoverflies. Previous results have demonstrated a kairomonal role for *E. balteatus* larvae, which were attracted in a four-arm olfactometer (Francis et al. 2005b). In this study, we confirmed that female hoverflies are able to perceive this sesquiterpene and use it to select an oviposition site. Behavioral results obtained with an EβF-treated aphid-free plant and an aphid-infested plant were different. Therefore, the data suggest that predatory hoverfly oviposition site selection is influenced by a blend rather than a single chemical. This includes not only EβF but also secondary metabolites related to plant damage, such as GLVs.

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References

AGELOPOULOS, N. G., HOOPER, A. M., MANIAR, S. P., PICKETT, J. A., and WADHAMS, L. J. 1999. A novel approach for isolation of volatile chemicals released by individual leaves of a plant in situ. *J. Chem. Ecol*. 25:1411–1425.

AL ABASSI, S. A., BIRKETT, M. A., PETTERSSON, J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2000. Response of the seven-spot ladybird to an alarm pheromone and an alarm pheromone inhibitor is mediated by paired olfactory cells. *J. Chem. Ecol.* 26:1765–1771.

ALMOHAMAD, R., VERHEGGEN, F. J., FRANCIS, F., and HAUBRUGE, E. 2007. Predatory hoverflies select their oviposition site according to aphid host plant and aphid species. *Entomol. Exp. Appl.* 125:13–21.

BARGEN, H., SAUDHOF, K., and POEHLING, H. M. 1998. Prey finding by larvae and adult females of *Episyrphus balteatus*. *Entomol. Exp. Appl.* 87:245–254.

BROCKERHOFF, E. G., and GRANT, G. G. 1999. Correction for differences in volatility among olfactory stimuli and effect on EAG responses of *Dioryctria abietivorella* to plant volatiles. *J. Chem. Ecol.* 25:1353–1367.

BUDENBERG, W. J., and POWELL, B. 1992. The role of honeydew as an oviposition stimulant for two species of syrphids. *Entomol. Exp. Appl.* 64:57–61.

CHANDLER, A. E. F. 1968. Some host-plant factors affecting oviposition by aphidophagous (Diptera: Syrphidae). *Ann. Appl. Biol.* 61:415–423.

CHANDLER, A. E. F. 1969. Locomotive behavior of first instar larvae of aphidophagous Syrphidae (Diptera) after contact with aphids. *An. Behav.* 17:673–678.

DE MORAES, C. M., MESCHER, M. C., and TUMLINSON, J. H. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410:577–580.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids: their role in plant-carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M., and SABELIS, M. W. 1988. Infochemical terminology: based cost-benefit analysis rather than origin of compounds. *Funct. Ecol.* 2:131–139.

DICKE, M., BEEK, T. A. V., POSTHUMUS, M. A., DOM, N. B., BOKHOVEN, H. V., and GROOT, A. D. 1990. Isolation and identification of volatiles kairomones that affects acarine predator-prey interactions. Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

ENTWISTLE, J. C., and DIXON, A. F. G. 1989. The effect of augmenting grain aphid (*Sitobium avenae*) numbers in a field of winter wheat in spring on the aphids abundance in summer and its relevance to the forecasting of outbreaks. *Ann. Appl. Biol.* 114:397–408.

FARAG, M. A., and PARÉ, P. W. 2002. C6-Green leaf volatiles trigger local and systemic VOC emissions in tomato. *Phytochemistry* 61:545–554.

FRANCIS, F., LOGNAY, G., and HAUBRUGE, E. 2004. Olfactory responses to aphid and host plant volatile releases: E-β-Farnesene, an effective kairomone for the predator *Adalia bipunctata*. *J. Chem. Ecol.* 30:741–755.

FRANCIS, F., VANDERMOTEN, S., VERHEGGEN, F. J., LOGNAY, G., and HAUBRUGE, E. 2005a. Is (E)-β-farnesene the only volatile terpenoid in aphids. *J. Appl. Entomol.* 129:6–11.

FRANCIS, F., MARTIN, T., LOGNAY, G., and HAUBRUGE, E. 2005b. Role of (E)-β-farnesene in systematic aphid prey location by *Episyrphus balteatus* larvae (Diptera: Syrphidae). *Eur. J. Entomol.* 102:431–436.

FRECHETTE, B., DIXON, A. F. G., ALAUZET, C., and HEMPTINNE, J.-L. 2004. Age and experience influence patch assessment for oviposition by an insect predator. *Ecol. Entomol.* 29:578–583.

HARMEL, N., ALMOHAMAD, R., FAUCONNIER, M.-L., DU JARDIN, P., VERHEGGEN, F., MARLIER, M., HAUBRUGE, E., and FRANCIS, F. 2007. Role of terpenes from aphid-infested potato on searching and oviposition behavior of the hoverfly predator *Episyrphus balteatus*. *Insect Science* 14:57–6.

NAULT, L. R., EDWARDS, L. J., and STYER, W. E. 1973. Aphid alarm pheromones: Secretion and reception. *Environ. Entomol.* 2:101–105.

NORDLUND, D. A., and LEWIS, W. J. 1976. Terminology of chemical releasing stimuli in intraspecific and interspecific interactions. *J. Chem. Ecol.* 2:211–220.

PARÉ, P. W., and TUMLINSON, J. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114:1161–1167.

SADEGHI, H., and GILBERT, F. 2000a. Oviposition preferences of aphidophagous hoverflies. *Ecol. Entomol.* 25:91–100.

SADEGHI, H., and GILBERT, F. 2000b. Aphid suitability and its relationship to oviposition preference in predatory hoverflies. *J. Anim. Ecol.* 69:771–784.

SADEGHI, H., and GILBERT, F. 2000c. The effect of egg load and host deprivation on oviposition behaviour in aphidophagous hoverflies. *Ecol. Entomol.* 25:101–108.

SANDERS, W. 1983. The searching behaviour of gravide *Syrphus corollae* Fabr. (Diptera: Syrphidae) and its depending on the optical cues. *Z. Angew. Zool.* 70:235–247.

SCHNEIDER, F. 1969. Bionomics and physiology of aphidophagous Syrphidae. *Annu. Rev. Entomol.* 14:103–121.

SCHOLZ, D., and POEHLING, H. M. 2000. Oviposition site selection of *Episyrphus balteatus*. *Entomol. Exp. Appl.* 94:149–158.

STEIDLE, J. L. M., and VAN LOON, J. J. A. 2002. Chemoecology of parasitoid and predator oviposition behaviour, pp. 291–317, in M. Hilker, and T. Meiners (eds.). Chemoecology of Insect Eggs and Egg DepositionBlackwell, Berlin.

STEIDLE, J. L. M., and VAN LOON, J. J. A. 2003. Dietary specialization and infochemical use in carnivorous arthropods: testing a concept. *Entomol. Exp. Appl.* 108:133–148.

SUTHERLAND, J. P., SULLIVAN, M. S., and POPPY, G. M. 1999. The influence of floral character on the foraging behaviour of the hoverfly, *Episyrphus balteatus*. *Entomol. Exp. Appl.* 93:157–164.

SUTHERLAND, J. P., SULLIVAN, M. S., and POPPY, G. M. 2001. Oviposition behaviour and host colony size discrimination in *Episyrphus balteatus* (Diptera: Syrphidae). *Bull. Entomol. Res.* 91:411–417.

TANAKA, S., YASUDA, A., YAMAMOTO, H., and NOZAKI, H. 1975. A general method for the synthesis of 1,3-dienes. Simple syntheses of β- and trans-α-farnesene from farnesol. *J. Am. Chem. Soc.* 97:3252–3254.

TENHUMBERG, B., and POEHLING, H. M. 1991. Studies on the efficiency of syrphid larvae, as predators of aphids on winter wheat, pp. 281–288, in L. Polgar, R. J. Chambers, A. F. G. Dixon, and I. Hodek (eds.). Behaviour and Impact of AphidophagaSPB Academic Publishing BV, The Hague, The Netherlands.

THOLL, D., BOLAND, W., HANSEL, A., LORETO, F., ÖSE, U. S. R., and SCHNITZLER, J.-P. 2006. Practical approaches to plant volatile analysis. *Plant J.* 45:540–560.

TUMLINSON, J. H., TURLINGS, T. C. J., and LEWIS, W. J. 1992. The semiochemical complexes that mediate insect parasitoid foraging. *Agr. Zool. Reviews* 5:221–252.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odor by host-seeking parasitic wasps. *Science* 250:1251–1253.

TURLINGS, T. C. J., and TUMLINSON, J. H. 1992. Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. USA* 89:8399–8402.

TURLINGS, T. C. J., LOUGHRIN, J. H., MC CALL, P. J., OSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parasitic wasps. *Proc. Natl. Acad. Sci. USA* 92:4169–4174.

VANHAELEN, N., HAUBRUGE, E., GASPAR, C., and FRANCIS, F. 2001. Oviposition preferences of *Episyrphus balteatus*. *Med. Fac. Landbouw. Univ.Gent*. 66/2a.

VANHAELEN, N., GASPAR, C., F, and F. 2002. Influence of prey host plant on a generalist aphidophagous predator: *Episyrphus balteatus* (Diptera: Syrphidae). *Eur. J. Entomol.* 99:561–564.

VERHEGGEN, F. J., ARNAUD, L., CAPELLA, Q., FRANCIS, F., and HAUBRUGE, E. 2005. Perception of aphid infested tomato plant volatiles by the predator *Episyrphus balteatus*. *Comp. Biochem. Phys* 3:141A.

VERHEGGEN, F. J., FAGEL, Q., HEUSKIN, S., LOGNAY, G., FRANCIS, F., and HAUBRUGE, E. 2007a. Electrophysiological and behavioral responses of the multicolored Asian lady beetle, *Harmonia axyridis* Pallas, to sesquiterpene semiochemicals. *J. Chem. Ecol.* 33:2148–2155.

VERHEGGEN, F. J., RYNE, C., OLSSON, C., ARNAUD, L., LOGNAY, G., PERSSON, D., HAUBRUGE, E., and LÖFSTEDT, C. 2007b. Electrophysiological and behavioural study of some secondary metabolites in the confused flour beetle, *Tribolium confusum*. *J. Chem. Ecol.* 33:525–539.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VIDELA, M., VALLADARES, G., and SALVO, A. 2006. A tritrophic analysis of host preference and performance in a polyphagous leafminer. *Entomol. Exp. Appl.* 121:105–114.

ZHU, J., COSSÉ, A. A., OBRYCKI, J. J., BOO, K. S., and BAKER, T. C. 1999. Olfactory reactions of the twelve-spotted lady beetle, *Coleomegilla maculata* and the green lacewing, *Chrysoperla* to semiochemicals released from their prey and host plant: Electro-antennogram and behavioral responses. *J. Chem. Ecol.* 5:1163–1177.

ZHU, J., OBRYCKI, J. J., OCHIENG, S., BAKER, T. C., and PICKETT, J. 2005. Attraction of two lacewing species to volatiles produced by host plants and aphid prey. *Naturwissenschaften* 92:277–281.

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Identification of Human-Derived Volatile Chemicals that Interfere with Attraction of *Aedes aegypti* Mosquitoes

James G. Logan · Michael A. Birkett · Suzanne J. Clark · Stephen Powers · Nicola J. Seal · Lester J. Wadhams · A. Jennifer Mordue (Luntz) · John A. Pickett

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Abstract It is known that human individuals show different levels of attractiveness to mosquitoes. In this study, we investigated the chemical basis for low attractiveness. We recorded behaviors of *Aedes aegypti* toward the hands of human volunteers and toward the volatile chemicals produced by their bodies. Some individuals, and their corresponding volatiles, elicited low upwind flight, relative attraction, and probing activity. Analyzing the components by gas chromatography coupled to electrophysiological recordings from the antennae of *Aedes aegypti*, enabled the location of 33 physiologically relevant compounds. The results indicated that higher levels of specific compounds may be responsible for decreased "attractiveness." In behavioral experiments, five of the compounds caused a significant reduction in upwind flight of *Aedes aegypti* to attractive human hands. Thus, unattractiveness of individuals may result from a repellent, or attractant "masking," mechanism.

Keywords Mosquito · Differential attraction · Semiochemical · Attractant "masking" effect

J. G. Logan · M. A. Birkett · S. J. Clark · S. Powers · L. J. Wadhams · J. A. Pickett (✉)
Rothamsted Research,
Harpenden,
Hertfordshire AL5 2JQ, UK
e-mail: John.Pickett@bbsrc.ac.uk

N. J. Seal · A. J. Mordue (Luntz)
School of Biological Sciences, University of Aberdeen,
Tillydrone Avenue,
Aberdeen AB24 2TZ, UK

Introduction

Mosquitoes (Diptera: Culicidae) have a sophisticated olfactory system that allows location of potential hosts and demonstrates interspecific preferences for certain vertebrate species. For example, *Anopheles* spp., which include vectors of malarial parasites, feed on higher vertebrates such as human beings and members of the bovidae; *Culex* spp., which include vectors of West Nile virus, are mostly ornithophilic while most *Aedes* spp., which include the yellow fever mosquito, *Aedes aegypti*, and some *Anopheles* spp. are highly anthropophilic (Takken 1991). These mosquitoes and other flies that bite vertebrates to obtain blood (sandflies, Diptera: Psychodidae and some blackfly species, Diptera: Simuliidae) also show intraspecific preferences for certain individuals (Acree et al. 1968; Hamilton and Ramsoondar 1994; Schofield and Sutcliffe 1996; Takken and Knols 1999; Dekker et al. 2001; Besansky et al. 2004; Jensen et al. 2004; Qiu et al. 2004). However, although this behavioral preference for certain individuals has been demonstrated, the basis for the phenomenon is unknown (Brouwer 1960; Khan and Maibach 1966; Mayer and James 1969; Burkot 1988; Schreck et al. 1990; Lindsay et al. 1993; Brady et al. 1997; Costantini et al. 1997; Qiu et al. 2004).

During host location, female mosquitoes use volatile chemicals released by their vertebrate hosts as long-range host location cues (Allan et al. 1987; Takken 1991; Takken and Knols 1999). Physical cues such as heat, moisture, and vision play a role during orientation and landing at close range (Khan et al. 1968; Eiras and Jepson 1994). The known olfactory stimuli from long-range orientation include CO_2, 1-octen-3-ol, acetone, and ammonia (Allan et al. 1987; Costantini et al. 1998; Takken and Knols 1999; Bernier et al. 2000, 2002; Bhasin et al. 2000, 2001; Jensen

et al. 2004). Different levels of attraction can be determined by volatile olfactory cues from breath and skin with its associated microorganisms (Allan et al. 1987; Steib et al. 2001; Bernier et al. 2002; Jensen et al. 2004). *Aedes aegypti*, *Anopheles stephensi*, *Anopheles gambiae*, and *Anopheles quadrimaculatus* show variation in attraction to hand odors from different individuals (Brouwer 1960; Khan and Maibach 1966; Mayer and James 1969; Schreck et al. 1990). Olfactometer studies have shown that human hands can be used as a "standard stimulus" typical of the whole body (Freyvogel 1961; Takken 1991; Eiras and Jepson 1994; Geier et al. 1996). Olfactometers have also shown that mosquitoes show positive behavioral responses to natural host volatiles that have been collected and then presented as an extract (Schreck et al. 1981; Takken 1991; Geier et al. 1996; Qiu et al. 2004). However, few studies have attempted to identify the chemicals within the extracts that are actually responsible for the attraction or lack of attraction of different individuals (Braks and Takken 1999; Braks et al. 2001; Healy et al. 2002; Qiu et al. 2004). One study tentatively identified 346 chemicals from human hand odors deposited onto glass beads (Bernier et al. 2000) that include members of diverse chemical groups: aldehydes, carboxylic acids, alcohols, aliphatics/aromatics, amides, amines, esters, halides, heterocyclics, ketones, sulfides, and thioesters. Of the 346 chemicals, 277 were suggested to be candidate attractants for *Aedes aegypti* (Bernier et al. 2000). Most investigations have focused on components of human odor that are described as attractants to mosquitoes, i.e., those that increase upwind flight activity in olfactometers or increase trap catches in field traps. These commonly include lactic acid, carbon dioxide (CO_2), ammonia, and acetone, with each showing varying abilities to elicit behavioral responses (Acree et al. 1968; Kline et al. 1990; Eiras and Jepson 1994; Geier et al. 1996; Bernier et al. 2003). It might be assumed that a lack of or reduction in such chemicals would contribute to low human attractiveness. It has been shown that adding lactic acid to the skin of formerly unattractive individuals increases their attractiveness (Steib et al. 2001) and that the removal of lactic acid from an attractive extract gives a loss of attraction (Geier et al. 1996). However, it is unlikely that compounds such as lactic acid and CO_2, which are produced ubiquitously by vertebrates as products of primary metabolism, are associated with differential attraction. It seems more likely that secondary volatile metabolites produced by the body or bacteria on the skin are involved. Three such human-specific compounds, 7-octenoic acid, (*E*) and (*Z*)-3-methyl-2-hexenoic acid, reduce catches of *Anopheles gambiae* at some concentrations in odor baited entry traps (Costantini et al. 2001).

An alternative basis for differential attractiveness is that some individuals produce additional compounds that interfere with their location by insects. This has been demonstrated in the attraction of the horn fly, *Haematobia irritans* (Diptera: Muscidae) to cattle (Steelman et al. 1993; Birkett et al. 2004; Jensen et al. 2004) where a volatile compound, 6-methyl-5-hepten-2-one, produced by unattractive cattle prevents fly attack. Furthermore, when this compound was applied in a slow-release sachet to cattle during a field study, fly-loads were significantly reduced (Birkett et al. 2004). Because many compounds, when applied at concentrations higher than they would occur naturally, can act as repellents (Pickett et al. 1998; Gikonyo et al. 2002, 2003), the mechanism for the reduction in fly-loads was suggested to be repellency or 'masking'.

In the present study, we examined the chemical basis of differential attractiveness of human subjects to *Aedes aegypti*. A newly developed volatile collection technique, along with coupled gas chromatography-electroantennography (GC-EAG), allowed the examination of complex extracts of human odors, at naturally occurring levels, and the identification of compounds that are physiologically relevant to the insects.

Methods and Materials

Insects A culture of *Aedes aegypti* was set up from the 'refm' strain obtained from the London School of Hygiene and Tropical Medicine. The culture was maintained at 27± 2°C, with a relative humidity of 55–60% and a L/D 12:12 hr photoperiod. Larvae were reared in trays and were fed daily with Tetramin® Tropical Fish Flakes. Adults were kept in plastic culture cages with a constant supply of 15% sucrose solution, and females were given access to sheep blood once a week via an artificial feeding system. For behavioral assays, female mosquitoes that had not been previously blood-fed were chosen at random from a population aged between 5 and 12 d. Mosquitoes were held in culture cages in the bioassay room (with access to 15% sucrose) for 24 hr and transferred to plastic pre-release chambers (without sucrose) 2 hr before use in the bioassay. Each replicate had 20–30 female mosquitoes.

Behavioral Assays Behavior of mosquitoes to human odors was assessed by using a Y-tube olfactometer as described by Geier and Boeckh (1999) in which adult insects (see above) could "choose" between a test sample or air alone. Two fluorescent light tubes (70 W; Luminux) were positioned overhead, approximately 1 m from the Y-tube, and the temperature and relative humidity were maintained at 27°C and 60%, respectively. Air was pushed through a glass bell jar (5 l) that contained distilled water (1 l) and an activated charcoal filter before being split between the arms

of the Y-tube (10 l min^{-1}). Two flowmeters controlled the airflow, to a mean speed of 0.13 m sec^{-1} in the arms and 0.11 m sec^{-1} in the stem of the olfactometer.

Three behaviors were recorded: (1) upwind flight activity (the proportion of mosquitoes that flew upwind beyond 30 cm in the stem of the Y-tube); (2) relative attraction (the proportion of mosquitoes in the arm of the Y-tube that contained the odor stimulus); (3) probing activity (the proportion of mosquitoes that probed their proboscis through the mesh in the treated arm of the Y-tube).

To test the effect of compounds that emanated from hands, 'test hands' were compared with a 'standard hand' (Y01) previously shown to be attractive, (i.e. causing approximately 70% of mosquitoes to fly upwind and with 80% relative attraction). Each test hand was placed in one side of the Y-tube with nothing in the other. This was repeated 10–12 times, and the average responses were calculated to determine levels of 'attractiveness'. Nine people were tested in this way. All were white European men and women, ranging from 21 to 60 years of age. The study was approved by the Bedfordshire and Hertfordshire Strategic Health Authority Local Research Ethics Committee, UK (Reference number: EC03652). Responses toward the 'standard hand' were shown to be consistent on each testing day by comparing the mean proportion for flight activity and relative attraction in response to the 'standard hand' and the control treatment using a χ^2 test for heterogeneity (Zar 1984).

To test the effect of compounds that emanated from whole bodies, air entrainment samples were applied to filter paper (10 µl) in one side of the Y-tube with a solvent control (redistilled diethyl ether on filter paper, 10 µl) in the other. CO_2 (0.1%) was added to the main air stream to activate mosquito flight (Dekker et al. 2005). This was not necessary for the hand experiments because hands themselves emit carbon dioxide (G. Lees, personal communication, University of Aberdeen).

For testing hands supplemented with other chemicals, the test compounds were applied to sterile filter paper contained in an external glass vessel, and air, controlled by flowmeters, was pumped (500 ml min^{-1}) from the glass vessel directly into the main air stream of the Y-tube, in front of the hand, via polytetrafluoroethylene tubing.

Individual chemical compounds identified from hands or extracts were tested by comparing control (clean air) vs control (clean air), the 'standard hand' vs control (clean air), and the 'standard hand' plus 5-decadic serial dilutions of a test compound ranging from 1×10^{-4} to 1×10^{-8} g in hexane (10 µl) vs 10 µl hexane control. The test compounds used were 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone. CO_2 was not added. Each odor stimulus was tested in a complete randomized block design, i.e. a control, a standard, and each dose of the same chemical was tested once within a block, and blocks were performed over several days, with 10 replicates for each.

Collection of Human-Derived Volatile Chemicals For 24 hr before the collection of volatiles, volunteers avoided spicy food, garlic, and alcohol, and used non-perfumed soap. Whole-body volatiles were collected by using a new system whereby volunteers were placed in individual aluminumized plastic bags (Lifesystems ™), with only their heads outside. Charcoal-filtered air was introduced into the top of the bag (flow rate, 2.2 l min^{-1}) and extracted from the bottom into two glass tubes that contained pre-conditioned 50 mg Porapak Q (Porapak tubes), while a positive pressure was maintained inside the bag. Air entrainments of individuals were run for 2 hr and were performed on the same day as the behavioral assays with hands. Moisture that had gathered in the Porapak tubes was removed by passing purified nitrogen through the tubes for 2 min after the entrainment. Porapak tubes were then eluted with freshly re-distilled diethyl ether (750 µl) to provide extracts.

Coupled Gas Chromatography-Electrophysiology GC-EAG was used to locate peaks within mixtures that could be detected by the antennae of mosquitoes. Test insects were unfed and mated 5- to 12-d-old, *Aedes aegypti* females, 'cooled' on ice for 30 sec before removing the head and the tips of both antennae. The indifferent electrode was inserted into the back of the head, and the distal ends of both antennae were inserted into the recording electrode. Recordings were made with Ag/AgCl electrodes inserted into glass pipettes filled with a saline solution (insect ringer—7.55 g NaCl, 0.64 g KCl, 0.22 g $CaCl_2$, 1.73 g $MgCl_2$, 0.86 g Na_2HCO_3, and 0.61 g Na_3PO_4 l^{-1} water). Electrodes were connected to an Autospike interface box and an AC/DC amplifier UN-06 (Syntech, The Netherlands). Preparations were held in a continuous, humidified, and charcoal-filtered air stream (1 l min^{-1}, Syntech Stimulus Controller CS-02, Syntech) that came from a glass tube outlet that was positioned 0.5 cm from the preparation. An HP 6890 gas chromatograph was used to separate the components of mixtures on a polydimethylsiloxane (HP1) column 30 m× 0.32 mm (film thickness, 0.52 µm). The oven temperature was maintained at 40°C for 2 min and then programmed at 5°C min^{-1} to 100°C and then at 10°C min^{-1} to 250°C. The carrier gas was hydrogen. Consistent antennal responses to individual components of the mixtures were determined by replicating the GC-EAG experiments (N=6–10), and EAG activity was confirmed by testing authentic chemicals obtained commercially.

Gas Chromatography and Coupled Gas Chromatography–Mass Spectrometry To identify compounds within air

entrainment extracts that elicited EAG responses, samples were injected into an HP 5890 GC fitted with a non-polar HP1 cross-linked polydimethylsiloxane capillary column (50 m, 0.32 mm ID, 0.52 μm film thickness), a cool on-column injector, and a deactivated HP1 pre-column (0.53 mm ID). The carrier gas was helium. The GC oven temperature was maintained at 30°C for 5 min and programmed at 5°C min^{-1} to 250°C. The GC was coupled to a VG Autospec double-focusing magnetic sector mass spectrometer (MS) and an integrated data system (Fisons Instruments, Manchester, UK). Ionization was by electron impact at 70 eV, 250°C (source temperature). All compounds of interest were tentatively identified by comparison of peaks on the GC with MS databases [NIST Standard Reference Database (Version 3.0.1.), 2002] and were confirmed by coinjection of authentic compounds on an HP1 and a DB-WAX column.

To analyze the air entrainment extracts, a Hewlett Packard (HP) 6890 gas chromatograph (GC) was used. This was fitted with a non-polar polydimethylsiloxane (HP1) cross-linked capillary column (50 m×0.32 mm ID, film thickness: 0.52 μm) and a polar DB-WAX column (30 m×0.32 mm ID, film thickness 0.82 μm) with hydrogen as the carrier gas. A cool on-column injector and a flame ionization detector (FID) were used. The GC oven temperature was held at 30°C for 1 min and programmed to increase at 5°C min^{-1} to 150°C, then 10°C min^{-1} to 240°C. To quantify the compounds within the air entrainment extracts, a multiple point external method was used.

Statistical Analyses For behavioral studies, pairwise comparisons were made between the 'standard hand' and the 'test hand', by using a generalized linear model (GLM) with binomial error and logit link (logistic regression)*r*. A logistic GLM was fitted (as above) to the data from extracts and standard hands plus chemicals (testing chemicals alongside a human hand). The mean proportions of mosquitoes that responded to all treatments were compared. Confidence intervals (95%) for each mean were calculated from the standard errors on the logit scale and $t_{0.05}$. These means, and 95% confidence intervals, were back-transformed to the proportion scale for presentation.

Volunteers were classified as 'attractive' or 'less attractive' based on significant behavioral differences in responses of mosquitoes to hands (see Fig. 1). Following analysis of the volunteers' extracts by GC, mean absolute amounts of each compound for the attractive and less attractive volunteer groups were compared by using a one-way analysis of variance (ANOVA). For each compound, the mean relative amount (percentage, p) was calculated (i.e., mean absolute amount divided by the mean total amount of all EAG-active compounds) and transformed by using the logit transformation [$\text{logit}(p)=\ln(p/(1-p))$]. Mean relative amounts were compared between the two groups by using a one-way ANOVA.

For the GC data from two groups of individuals, Group 1 (attractive to mosquitoes) and Group 2 (unattractive to mosquitoes), canonical variate analysis (CVA) was applied where $N=6$ for Group 1 and $N=4$ for Group 2. In this case, the method produces a one-dimensional representation of the data (because there are only two groups but many chemicals), which emphasizes group differences. Therefore, the new axis (canonical variate) is the linear combination of the original variates (chemicals), which maximizes the 'between-group' variability relative to the 'within-group' variability. Subsequent axes account for further variation not accounted for by previous canonical variates (CVs). For CVA, the loadings on the variates (chemicals) are scaled so that the average within-group variability in each canonical variate dimension is 1. Thus, the within-group variation is equally represented in each dimension. Loadings corresponding to roots less than 1 are for dimensions in the canonical variate space that exhibit more within-group variation than between-group variation.

Results

To test the responses of mosquitoes toward human odors directly from hands or whole body air-entrainment extracts, behavioral experiments were used to determine upwind flight activity, relative attraction, and probing activity.

Behavioral Assays—Hand Odors To ensure that valid comparisons of mosquito responses to hand odors from different individuals could be made on different days, each hand was compared to the standard hand of an 'attractive' individual on the day of testing. This showed that responses to the standard stimulus were consistent among testing days with the mean upwind flight activity for the standard stimulus being 71% (data not shown).

The responses of mosquitoes to odors from the hands of different individuals varied significantly (Fig. 1). For flight activity (Fig. 1a), test hands X09 ($P=0.001$), X07 ($P=0.012$), and X04 ($P=0.016$) induced greater proportions of *Aedes aegypti* mosquitoes to respond than did the standard hand. Hands X08 and X05 were not significantly different from the standard hand ($P=0.395$ and $P=0.935$, respectively), although they were different from clean air ($P=0.001$; data not shown). Significantly fewer mosquitoes performed upwind flight activity in response to four hands X06, Y04, Y05, and Y07 ($P<0.001$ for all volunteers) compared to the standard hand. However, all volunteers were also different from the control ($P=0.001$).

For relative attraction (Fig. 1b), hands X04 or X08 showed greater effects than did the standard hand ($P=0.002$

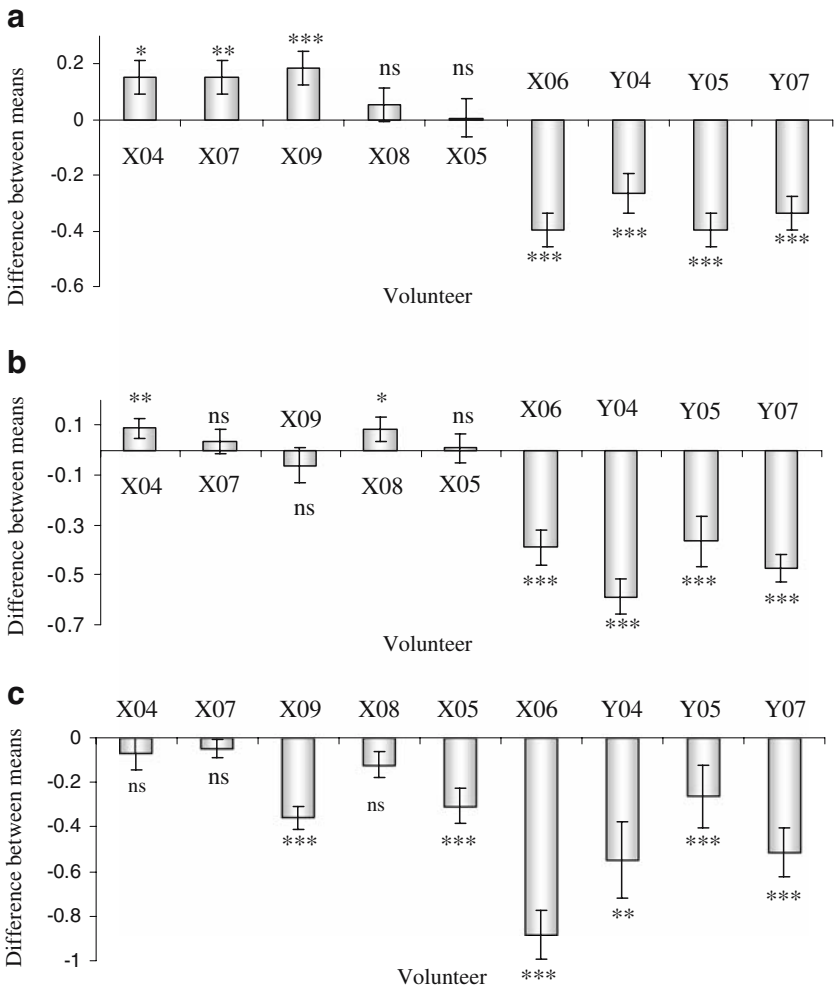

Fig. 1 Behavioral responses of *Aedes aegypti* female mosquitoes to nine test hands expressed as a difference from the standard hand. **a** Flight activity—the proportion of mosquitoes recorded upwind in the Y-tube; **b** relative attraction—the proportion of mosquitoes upwind recorded in the treated arm of the Y-tube; **c** probing activity—the proportion of mosquitoes upwind recorded probing in the treated arm of the Y-tube. Data are presented as differences between the response to the test hand and the response to the standard hand. Back transformed means+SED are shown. Asterisks indicate statistically significant differences determined by using a GLM (see text) (GLM, *$P<0.05$, **$P<0.01$, ***$P<0.001$; $n=10$–12 tests per volunteer; total mosquitoes per treatment, 287–485; total mosquitoes, 2,870–4,850)

and $P=0.019$, respectively), with the relative attraction of X07 and X09 being the same. However, relative attraction of hands X06, Y04, Y05, and Y07 was lower than that of the standard hand ($P<0.001$). These are the same four hands that induced low-flight activity.

For probing activity (Fig. 1c), hands X04 ($P=0.354$), X07 ($P=0.532$), and X08 ($P=0.082$) were not different form the standard, but all other hands showed lower responses to the standard stimulus (X09, $P<0.001$; X05, $P<0.001$; X06, $P=0.001$; Y04, $P=0.01$; Y05, $P=0.01$; Y07, $P<0.001$). Probing activity was not seen in response to the clean air control.

Behavioral Assays—Air Entrainment Extracts When air entrainment extracts were tested (Fig. 2a), the greatest flight activity was recorded for the extract from individual X07, followed by the extract from the standard (Y01), and extracts X09 and X04, all of which were greater than the clean air control ($P<0.001$ for all extracts; Fig. 2a). In good correlation with the hand experiments, extracts from individuals X06, Y04, Y05, and Y07 induced significantly less flight activity than the standard extract (Y01), and the response to these four extracts was not different from that of the clean air control ($P=0.54$, $P=0.71$, $P=0.69$, $P=0.226$, respectively; Fig. 2a). Extracts collected from the standard (Y01), X04, and X09 induced a significantly greater relative attraction than the control ($P<0.001$ for all extracts; Fig. 2b). However, again in correspondence with the hand experiments, relative attraction toward the extracts from X06, Y04, Y05, and Y07 was not significantly different from the solvent control ($P=0.562$, $P=0.296$, $P=0.889$, $P=0.767$, respectively; Fig. 2b). Probing activity did not occur in these experiments.

Electrophysiology, Gas Chromatography, and Gas Chromatography–Mass Spectrometry Four of the extracts shown in Fig. 2, i.e., those from the standard hand (Y01), the equally attractive test hand X04, and two much less attractive hands Y04 and Y05, were chosen for analysis by coupled GC-EAG. This revealed 33 peaks associated with EAG activity (an example is given in Fig. 3, which is labeled to show the eight EAG-active peaks found in

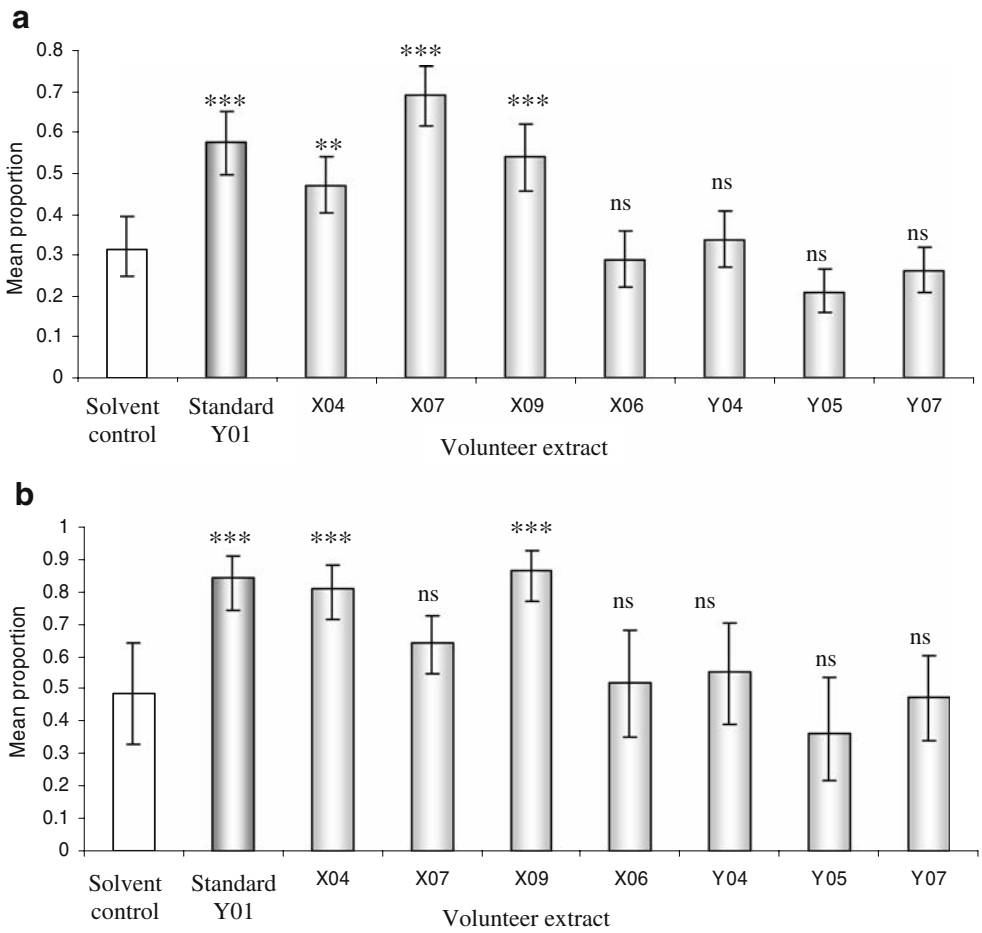

Fig. 2 Behavioral responses of *Aedes aegypti* female mosquitoes to air entrainment extracts (plus 0.1% CO_2) collected from seven people and the standard volunteer. **a** Flight activity—the proportion of mosquitoes recorded upwind in the Y-tube; **b** relative attraction—the proportion of mosquitoes upwind recorded in the treated arm of the Y-tube. Back-transformed means with 95% confidence intervals are shown. Asterisks indicate statistically significant differences from the control (solvent+CO_2) determined using a GLM (see text) (GLM, *P<0.05, **P<0.01, ***P<0.001; n=10–12 tests per extract; total mosquitoes per treatment, 151–232; total mosquitoes, 1449–2088)

greater amounts in the less-attractive group extracts). The compounds that gave rise to 23 of these peaks were tentatively identified by gas chromatography–mass spectrometry (GC-MS), and their identity was confirmed by peak enhancement on GC using two columns of different polarity by coinjection with authentic compounds (Table 1). The 23 peaks were thus identified and were examined by EAG using authentic samples, and the activity was confirmed. Ten EAG-active peaks, retention indices: 735, 792, 857, 829, 875, 907, 1030, 1130, 1345, and 2006 remain unidentified.

Thus, the coupled GC-EAG allowed location of physiologically relevant components within extracts. A comparison of mean absolute amounts of EAG-active compounds between the attractive and less-attractive subjects revealed a series of differences (Fig. 4a). Eight compounds (Fig. 3, Table 1) were found in significantly greater amounts (on average) in the less-attractive group compared with the attractive group, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, unknown, nonanal, naphthalene, decanal, and geranylacetone [(*E*)-6,10-dimethyl-5,9-undecadien-2-one] (compound numbers: 2, 3, 4, 8, 10, 16, 17, 20, respectively in Fig. 4a; Table 1). There were no significant differences among the other compounds found in both the attractive and less-attractive groups. Mean relative amounts of 6-methyl-5-hepten-2-one, octanal, and decanal also differed, the less attractive being higher in each case (Fig. 4b). CVA, including the EAG-active compounds in Table 1, revealed statistically significant (P<0.05) discrimination of the attractive and less-attractive groups, based on the ratio of between- to within-group variability in the amounts of the 24 EAG-active chemicals (Fig. 4c).

Behavioral Assays–Human Odors Plus Compounds Five of the EAG compounds produced in greater quantities by less-attractive individuals (6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone) were tested in behavioral trials. They were each tested in conjunction with the standard hand to see if they affected attractiveness. Upwind flight activity, relative attraction, and probing activity of *Aedes aegypti*, were recorded (Figs. 5, 6, and 7).

Although flight activity to the standard hand was shown to be significantly greater than to the clean air control, there was reduced flight activity to the hand plus 6-methyl-5-hepten-2-one (Fig. 5). This was significant for all five doses tested (P=0.039 for 1×10^{-4} g, P=0.04 for 1×10^{-5} g,

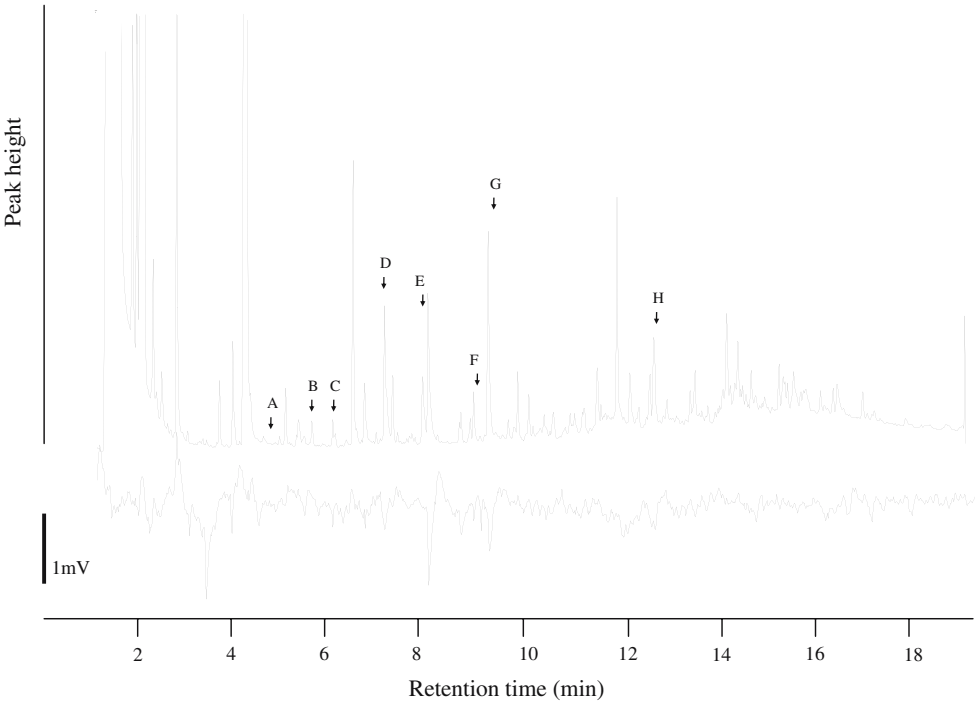

Fig. 3 Example of a coupled GC-EAG trace from *Aedes aegypti* showing antennal responses (lower trace) to a human standard, attractive air entrainment extract. The upper trace corresponds to the FID detector on the GC. Letters denote compounds with higher EAG activity in 'less attractive' individuals. **a** Benzaldehyde, **b** 6-methyl-5-hepten-2-one, **c** octanal, **d** unknown, **e** nonanal and linalool, **f** menthol and naphthalene, **g** decanal, **h** geranylacetone

P=0.019 for 1×10^{-6} g, and P<0.001 for 1×10^{-7} g, and 1×10^{-8} g). However, for all doses, flight activity was greater than to the clean air solvent control (P<0.001 for doses $\geq1\times10^{-6}$ g; P=0.004 and P=0.02 for 1×10^{-7} g and 1×10^{-8} g, respectively; Fig. 5). For octanal, all doses except 1×10^{-4} g gave reduced upwind flight activity (P<0.01), but again, there was still greater activity than to the clean air solvent control (P<0.01). Three doses of nonanal and decanal (1×10^{-6}, 1×10^{-5}, and 1×10^{-4} g) caused less flight activity (P<0.01), and the same treatments caused a greater amount of flight activity than the clean air solvent control (P<0.001 for all doses). For geranylacetone, flight activity was significantly less than to the standard hand alone (P< 0.001 for all doses), but in this case, only two, 1×10^{-5} and 1×10^{-4} g, induced greater upwind flight activity than the clean air solvent control (P<0.001).

When relative attraction of the mosquitoes was measured (Fig. 6), 6-methyl-5-hepten-2-one did not affect the attractiveness of the standard hand, but it did cause greater relative attraction compared with the clean air solvent control (P<0.001 for all concentrations). All octanal doses, except 1×10^{-4} g, gave less relative attraction than to the standard hand alone (P<0.01), and the 1×10^{-4} g gave greater relative attraction toward the treated arm than the clean air solvent control (P<0.001). For nonanal, relative attraction was lower than the standard arm alone in response to two doses (1×10^{-6} and 1×10^{-5} g; P=0.044 and P=0.013, respectively), and all doses induced greater relative attraction toward the treated arm than to the clean air control (P<0.001). Decanal at 1×10^{-6}, 1×10^{-5}, and 1×10^{-4} g gave a reduced relative attraction compared with the standard hand alone (P<0.001), and the 1×10^{-4} g dose reduced relative attraction to that of the clean air control (P=0.46). All geranylacetone doses caused a reduced relative attraction (P<0.001, except 1×10^{-8} g where P=0.02), but again, relative attraction was greater than the clean air solvent control (P<0.01 for all doses except 1×10^{-6} g where P=0.074).

Overall, probing activity differed among all odor stimuli (P<0.001; Fig. 7) and was reduced at the lower concentrations of 6-methyl-5-hepten-2-one, for doses 1×10^{-8} and 1×10^{-6} g (P=0.003 and P=0.022, respectively). Octanal, nonanal, and decanal had no effect on probing activity, and responses were not different from the standard hand alone (P=0.371, P=0.476, and P=0.077, respectively). Probing activity did differ between the standard hand and hand with four geranylacetone doses 1×10^{-8}, 1×10^{-7}, 1×10^{-6}, 1×10^{-5} g (P<0.001). Only one dose of geranylacetone, 1×10^{-4} g, did not reduce probing activity compared with the standard hand alone (P=0.33).

Taking results of the behavioral studies together (Figs. 5, 6, 7), it is clear that all five compounds significantly inhibited flight responses to a normally attractive hand at certain concentrations. 6-Methyl-5-hepten-2-one inhibited flight activation and probing activity but not relative attraction over a range of doses (1×10^{-8}–1×10^{-4} and 1×10^{-8}–1×10^{-6} g) and octanal reduced flight activation but not relative attraction or probing activity over a 1×10^{-8}–1×10^{-5} g range. Nonanal reduced both flight activity and relative attraction at 1×10^{-6}–1×10^{-5} g, but probing behavior was not significantly affected. Decanal reduced both flight activity and relative attraction over the range

Table 1 Names and Retention Indices for 24 EAG-Active Compounds with Loadings from the CVA

Compound No.	Retention Index	Compound Name	Latent Vector (Loadings)
1	776	Hexanal	0.00044
2[a]	932	Benzaldehyde	0.00133
3[a]	965	6-Methyl-5-hepten-2-one	−0.00427
4[a]	980	Octanal	0.00062
5	988	1,4-Dichlorobenzene	0.00126
6	1010	2-Ethylhexanol	0.00117
7	1022	Limonene	−0.0005
8[a]	1030	Unknown	0.00016
9	1057	Dihydromyrcenol	−0.00082
10[a]	1080	Nonanal	−0.00047
11	1083	Linalool	0.00047
12	1100	Undecane	0.0001
13	1135	(*E*)-2-Nonenal	0.00011
14	1151	Octanoic acid	0.00056
15	1160	Menthol	0.00097
16[a]	1160	Naphthalene	−0.00102
17[a]	1184	Decanal	−0.00245
18	1256	Indole	−0.00233
19	1384	Dodecanal	−0.00122
20[a]	1430	Geranylacetone	0.00042
21	1480	α-Isomethylionone	0.00037
22	1500	Pentadecane	−0.00067
23	1600	Hexadecane	−0.00024
24	1681	Heptadecene	0.00115

Latent vector is the complete set of loadings, i.e. the coefficients for the linear combination formed using the original set of variates (amounts of EAG-active compounds). The latent vector maximizes the ratio of between-group to within-group variation.
[a] Compounds found in greater amounts in extracts collected from less attractive individuals compared to attractive individuals.

1×10^{-6}–1×10^{-4} g but did not affect probing behavior. Geranylacetone gave reduced flight activity over all doses tested (1×10^{-8}–1×10^{-4} g) with relative attraction being reduced over the range 1×10^{-8}–1×10^{-4} g, and probing activity reduced over a range 1×10^{-8}–1×10^{-5} g. Indeed, geranylacetone could reduce flight activity toward the standard hand down to that found with clean air only at levels of 1×10^{-8}–1×10^{-7} g.

Discussion

In this study, we investigated the chemical basis of differences in the attraction of *Aedes aegypti* to individual human hosts. Previous studies had demonstrated that variation in attractiveness is mediated by volatile organic compounds that emanate from the skin, the actions of microorganisms, or the breath, but none has successfully identified any contributing compounds. The experiments in this study were designed to determine whether there are differences in particular compounds produced by less-attractive or attractive individuals. A new approach was developed to collect volatiles from human subjects, and for the first time, these volatile extracts were shown to elicit behavioral responses in mosquitoes that corresponded with responses initiated by the presence of human hands. Extracts from the four least attractive hands were no more attractive than an air control, suggesting that the volatiles alone, represented by the extracts, are responsible for the observed differences in behavioral responses of mosquitoes toward the hands.

Coupled GC-EAG was used to locate the volatile compounds within the extracts that are physiologically relevant to the insects, i.e. EAG-active. This identified 6-methyl-5-hepten-2-one, octanal, nonanal, decanal, and geranylacetone. Ten compounds remain unidentified, although only one, named as unknown with a Retention Index of 1030 (Table 1), could have been associated with unattractiveness. This was the only unidentified compound found in significantly greater amounts in the unattractive individuals. Care was taken to remove contamination of the extracts by controlling the washing regimes and the banning of the use of industrial or cosmetic chemicals by the individuals taking part in the trials. Despite this, some compounds including 1,4-dichlorobenzene, limonene, linalool, menthol, and 2-ethylhexanol were found, and these could be contaminants from cosmetics or food. However, limonene and naphthalene have been identified previously as human odor components (Sastry et al. 1980; Curran et al. 2005), and naphthalene has been previously identified in cattle odor (Birkett et al. 2004).

Some compounds identified in this study have been reported previously as human odor components. For example, studies of human odor samples (Schreck et al. 1981; Bernier et al. 2000; Qiu et al. 2004) have identified 346 compounds, 9 of which (octanoic acid, octanal, nonanal, decanal, benzaldehyde, pentadecane, hexadecane, 6-methyl-5-hepten-2-one, and indole) were EAG-active in our study. Hexanal, limonene, octanal, nonanal, octanoic acid, decanal, (*E*)-2-nonenal, geranylacetone, heptadecene, hexadecane, and naphthalene have also been identified previously from axillary sweat samples (Sastry et al. 1980; Zeng et al. 1991; Cork and Park 1996; Curran et al. 2005).

To test the hypothesis that the EAG-active compounds were potentially conferring low attractiveness, we tested the response of *Aedes aegypti* mosquitoes to the five compounds in a Y-tube olfactometer. All interfered significantly with the attractiveness of a normally attractive standard hand. Octanal, nonanal, and decanal reduced flight activity and relative attraction toward the standard hand at certain concentrations, and interestingly, these compounds, as well

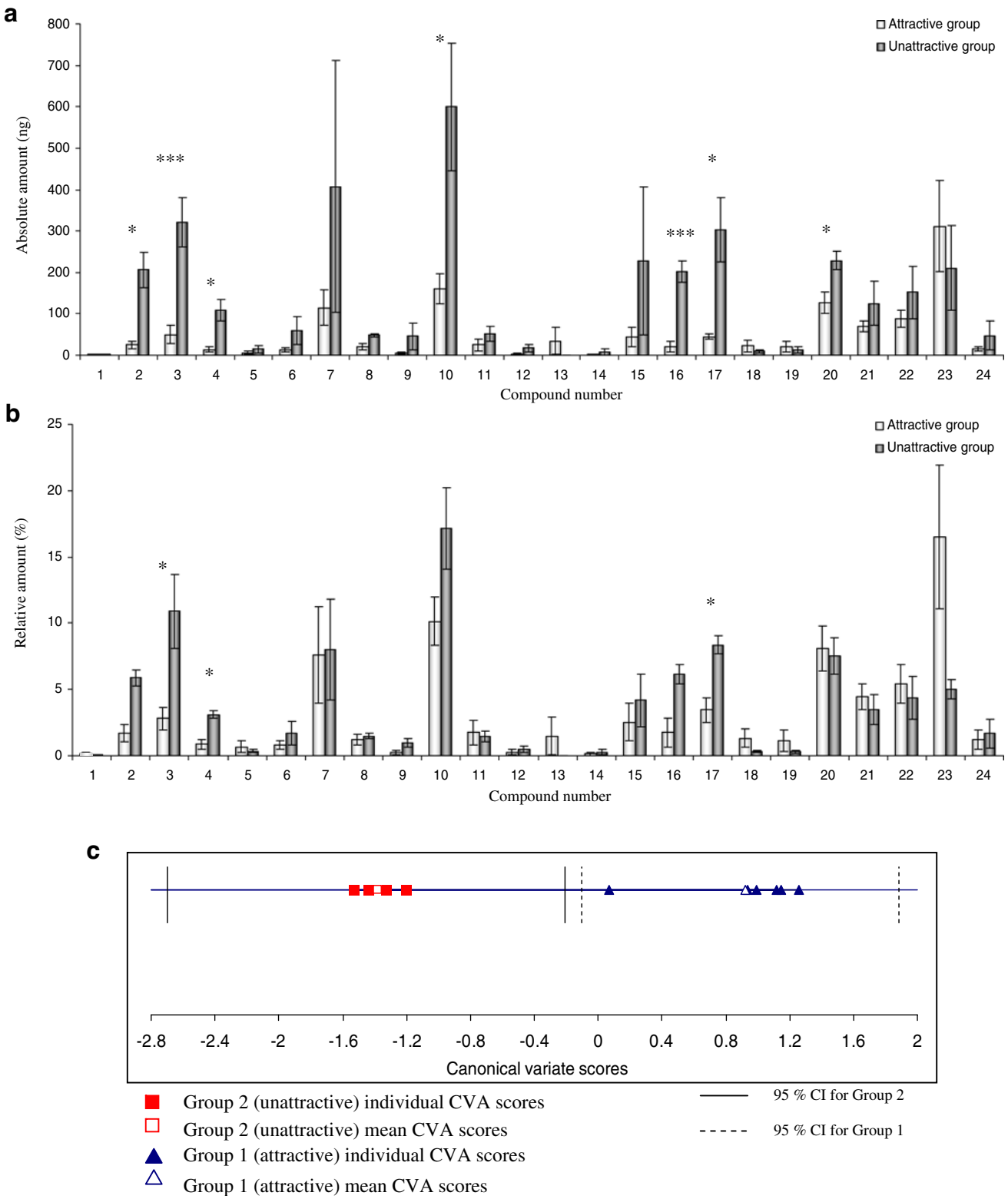

Fig. 4 Quantitative analysis of EAG-active compounds listed in Table 1. **a** Absolute amount (ng)+SE collected over 2 hr and **b** relative mean amounts of total compounds (%)+SE of EAG-active compounds collected from whole-body air entrainments of individuals within the 'attractive' group (N=6) and 'less attractive' group (N=4). Asterisks indicate statistically significant differences between the two means (ANOVA, *P<0.05; **P<0.01, ***P<0.001). **c** Plot of canonical variate scores based on the amounts of 24 EAG-active compounds within air entrainment extracts for all individuals

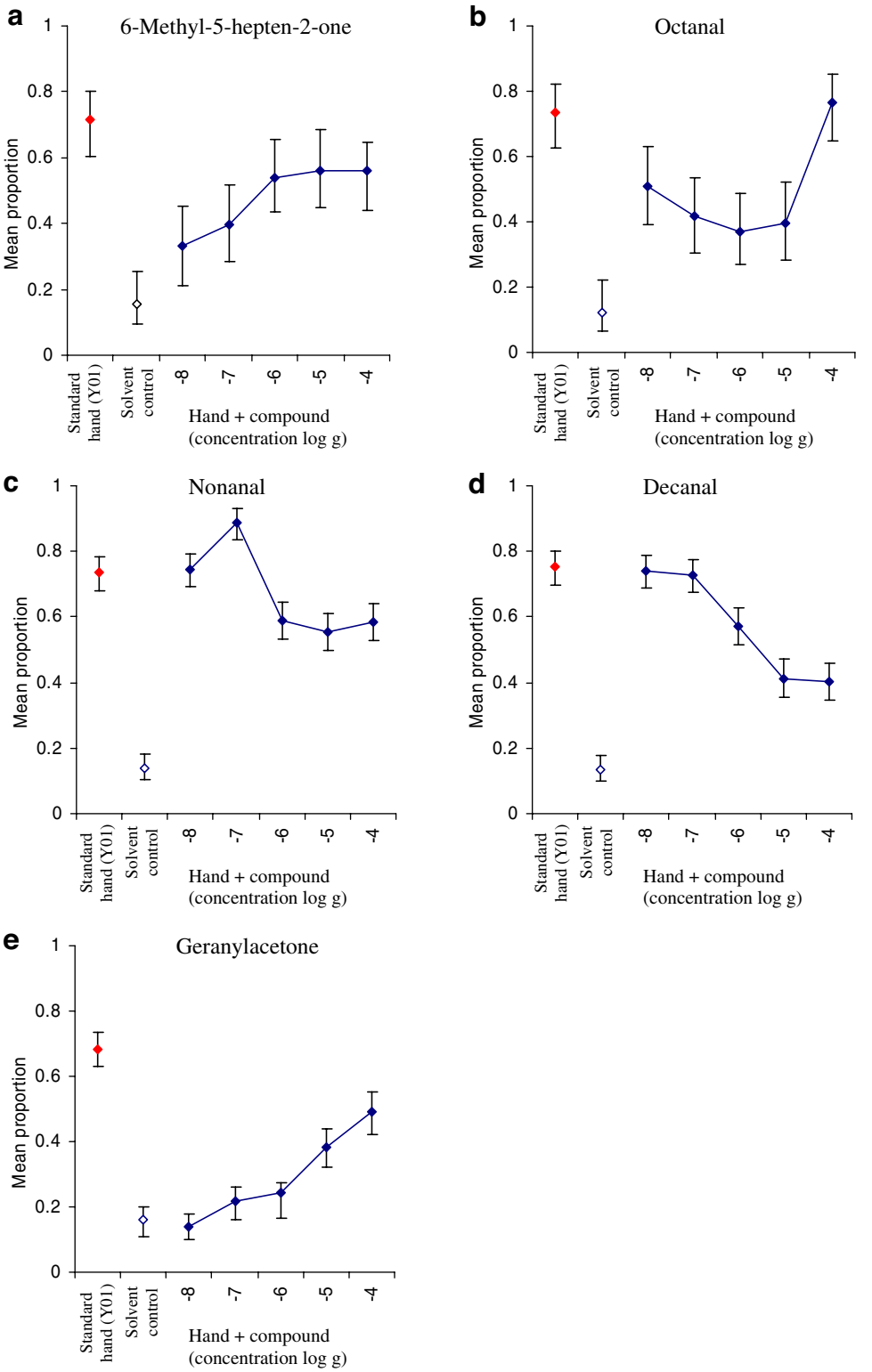

Fig. 5 Mean proportion of *Aedes aegypti* female mosquitoes showing upwind flight activity in response to the standard hand (in one side of the Y-tube with filter paper control in the other) and the hand plus differing concentrations of **a** 6-methyl-5-hepten-2-one, **b** octanal, **c** nonanal, **d** decanal, and **e** geranylacetone treatments (standard hand+chemical in one side of the Y-tube and filter paper plus solvent control in the other). Means with 95% confidence intervals (based on the binomial distribution) back-transformed from the logistic scale are shown (N=10 tests per concentration, total mosquitoes per compound, 200–227, and total number of mosquitoes, 1404–1589). Significant differences were determined using a GLM (see text)

as other aldehydes, have been suggested as a form of defense against ectoparasites in seabirds, and recently, octanal was demonstrated to be repellent to *Aedes aegypti* mosquitoes (Douglas et al. 2005). Similarly, giraffes may produce octanal and nonanal as bacteriostatic or fungistatic agents that may also repel ectoparasites (Wood and Weldon 2002).

One significant finding of our study is the reduction in flight activity and probing activity observed in response to 6-methyl-5-hepten-2-one at low concentrations, i.e., natu-

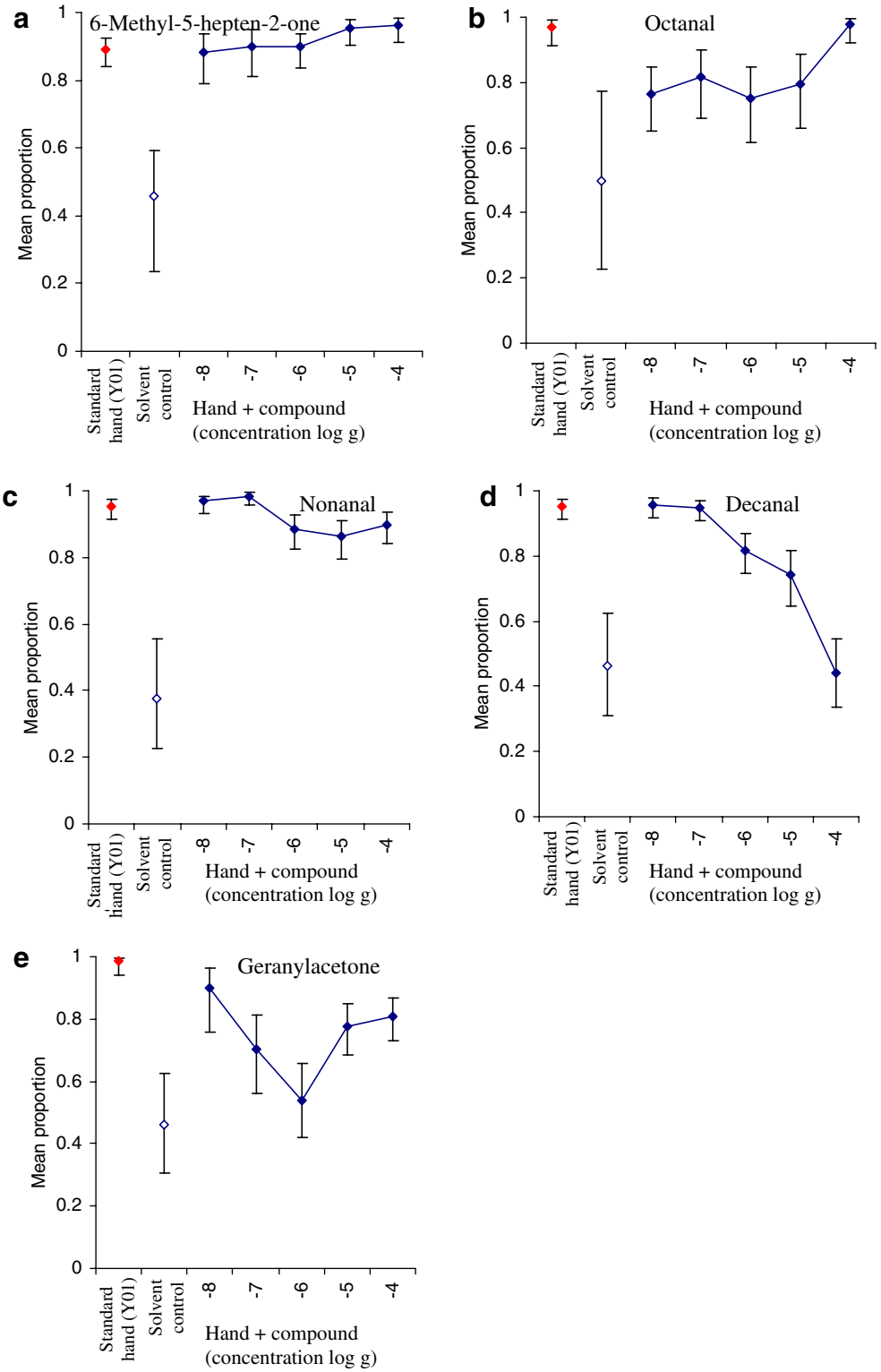

Fig. 6 Mean proportion of *Aedes aegypti* female mosquitoes showing relative attraction in response to the standard hand (in one side of the Y-tube with filter paper control in the other) and the hand plus differing concentrations of **a** 6-methyl-5-hepten-2-one, **b** octanal, **c** nonanal, **d** decanal, and **e** geranylacetone chemical treatments (standard hand + chemical in one side of the Y-tube and filter paper plus solvent control in the other). Means with 95% confidence intervals (based on the binomial distribution) back-transformed from the logistic scale are shown (N=10 tests per concentration of compound, total mosquitoes per compound, 200–227, and total number of mosquitoes, 1404–1589). Significant differences were determined using a GLM (see text)

rally occurring levels (Table 1). The doses used in the Y-tube behavior assay range from 1×10^{-8} g (10 ng) to 1×10^{-4} g (10,000 ng) applied to filter paper. The experiment lasted for 90 sec, and preliminary studies (Logan 2006—which used the same experimental procedure and equipment) have shown that the release rate of 6-methyl-5-hepten-2-one from the filter paper is 0.15 ng for the lowest concentration tested (1×10^{-8} g) and 1,500 ng for the highest (1×10^{-4} g) over the 90 sec testing period. As measured from GC analysis, 6-methyl-5-hepten-2-one was

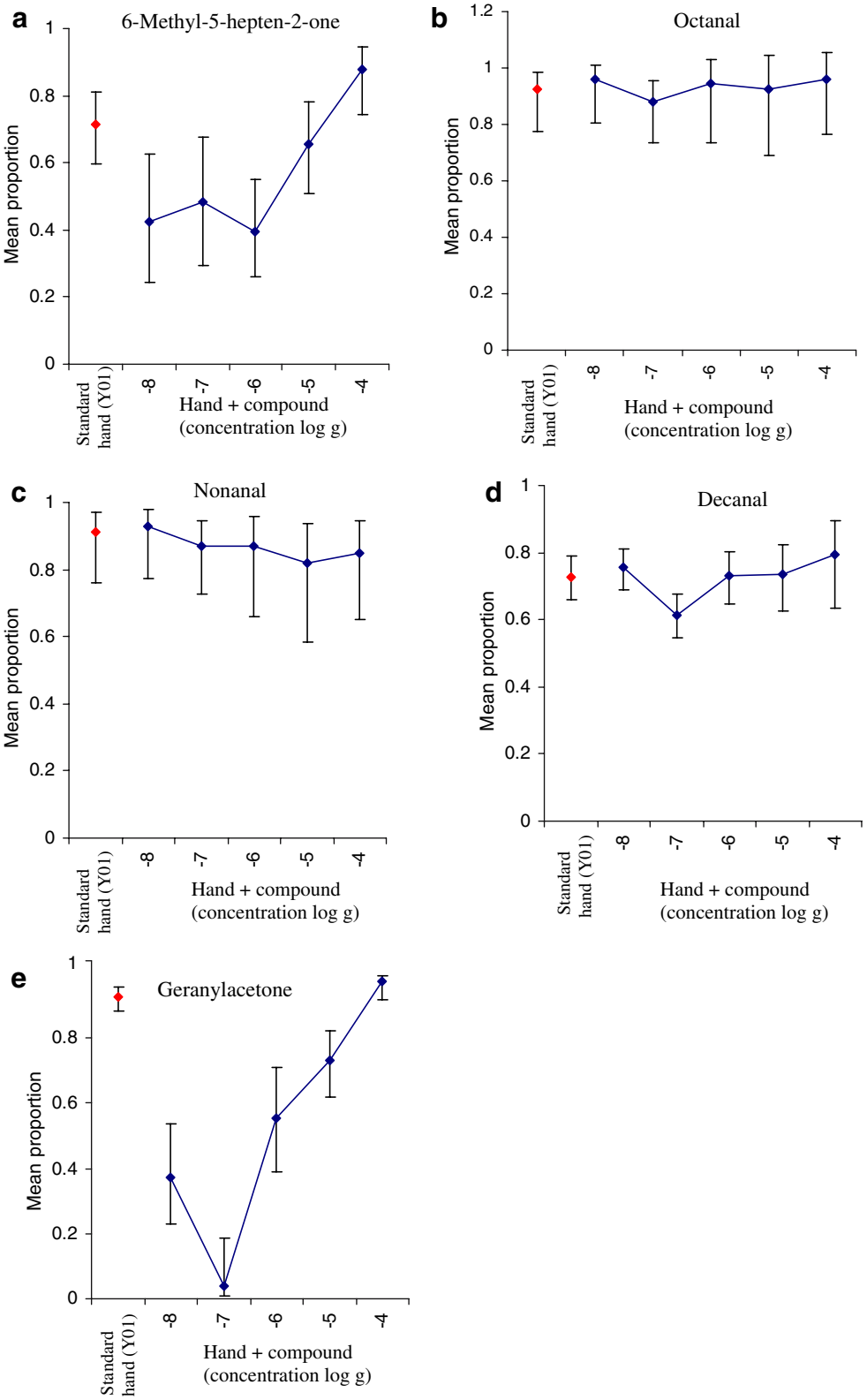

Fig. 7 Mean proportion of *Aedes aegypti* female mosquitoes showing probing activity in response to the standard hand (in one side of the Y-tube and filter paper control in the other) and the hand plus differing concentrations of **a** 6-methyl-5-hepten-2-one, **b** octanal, **c** nonanal, **d** decanal, and **e** geranylacetone chemical treatments (standard hand + chemical in one side of the Y-tube and filter paper plus solvent control in the other). Means with 95% confidence intervals (based on the binomial distribution) back-transformed from the logistic scale are shown (N=10 tests per concentration of compound, total mosquitoes per compound, 200–227, and total number of mosquitoes, 1404–1589). Significant differences were determined using a GLM (see text)

released from our volunteers at a rate of between 7 and 235 ng hr^{-1}, which is the equivalent of between 0.2 and 6 ng per 90 sec (i.e. the duration of the experiment). Therefore, the lower concentrations tested in this study are likely to represent naturally occurring levels. The effects of 6-methyl-5-hepten-2-one at these low levels indicate that the chemical has an effect on behavior at naturally occurring levels. This suggests that a small increase in this

chemical within the "normal" ratio of host odors could prevent mosquitoes from recognizing that an appropriate host is present. It is interesting that the few mosquitoes that did show flight activity were not then affected by the presence of 6-methyl-5-hepten-2-one during flight. This suggests that the compound affects behavioral responses to a human host only at the beginning and end of the mosquito's sequence of behaviors that lead to the successful location of a host. This may explain the reduced flight and probing activity observed to the human hands and extracts by less attractive individuals. However, it does not explain the differences observed in relative attraction and, therefore, is unlikely to play a role in discriminatory behavior between hosts. To our knowledge, this is the first time that 6-methyl-5-hepten-2-one has been shown to affect mosquito behavioral responses toward a normally attractive human host, and in this study, we have shown that inhibitory effects occur at lower rather than higher concentrations.

Bernier et al. (2002) suggested that 6-methyl-5-hepten-2-one plays a role in differential attraction and reported it to be present in lower quantities on the day that an individual was more attractive to mosquitoes. However, they also showed that 6-methyl-5-hepten-2-one was present in greater quantities on an attractive individual than an unattractive individual. Thus, a definitive role was not established. 6-Methyl-5-hepten-2-one has also been implicated in differential attraction of individual cattle to cattle flies, *Musca autumnalis* (de Geer) (Diptera: Muscidae) and *Haematobia irritans* (L.) (Diptera: Muscidae) (Birkett et al. 2004).

Compounds such as 6-methyl-5-hepten-2-one that are electrophysiologically and behaviorally active at low physiologically relevant levels are the most likely to be involved in a natural ecological system. At such levels, it is unknown whether they act as true repellents or have a "masking" effect that prevents attraction. The concept of "masking" is not fully defined, but could describe a process whereby insects no longer respond to their normal odor cues rather than actually being repelled. Interestingly, 6-methyl-5-hepten-2-one also affects phytophagous insect behavior toward host plants. For example, its production by wheat seedlings interferes with the location of wheat by *Rhopalosiphum padi* and *Sitodiplosis mosellana*, where it is thought to have repellent or "masking" properties (Bruce, 2005, personal communication; Quiroz et al. 1997).

Another important finding of our study is that geranylacetone, octanal, nonanal, and decanal had a significant effect on relative attraction and flight (at certain concentrations) of mosquitoes and, therefore, could be involved in orientation toward or away from a host. All doses of geranylacetone caused significantly less flight activity. Indeed, one dose reduced the response down to a level that was not significantly different from the solvent control. It also affected all three behaviors studied and was the most effective compound at reducing host location responses to a hand.

What remains unclear from our studies is whether the responses observed result from a direct repellent effect or another mechanism whereby the insects are failing to respond to normally attractive cues (i.e., "masking"). The Y-tube olfactometer used does not allow discrimination between the two possibilities, and this needs further study. Further trials are also required to determine whether these compounds could have an application in the field, for example, to develop a "push-pull" control strategy whereby the chemicals are used to prevent host location by "pushing" biting insects away from human hosts. At the same time, chemicals such as CO_2 and 1-octen-3-ol could be used as baits in traps to "pull" the insects away from hosts. The methodologies could also be applied to explore the interaction between human beings and other biting insects. This would include the malaria mosquito, *Anopheles gambiae* and the Scottish biting midge, *Culicoides impunctatus*. Although this study has shown that single human-derived compounds interfere with mosquito behavior, it is also important to understand whether mixtures and ratios play a role. We are currently doing field trials to test different mixtures and ratios against a broad range of hematophagous insects and other arthropods.

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References

ACREE, F. Jr., TURNER, R. B., GOUCK, H. K., BEROZA, M., and SMITH, N. 1968. L-Lactic acid—a mosquito attractant isolated from humans. *Science* 161:1346.

ALLAN, S. A., DAY, J. F., and EDMAN, J. D. 1987. Visual ecology of biting flies. *Annu. Rev. Entomol.* 32:297–316.

BERNIER, U. R., KLINE, D. L., BARNARD, D. R., SCHRECK, C. E., and YOST, R. A. 2000. Analysis of human skin emanations by gas chromatography-mass spectrometry. 2. Identification of volatile compounds that are candidate attractants for the yellow fever mosquito (*Aedes aegypti*). *Anal. Chem.* 72:747.

BERNIER, U., KLINE, D. L., SCHRECK, C. E., YOST, R. A., and BARNARD, D. R. 2002. Chemical analysis of human skin emanations: comparison of volatiles from humans that differ in attraction of *Aedes aegypti* (Diptera: Culicidae). *J. Am. Mosq. Control Assoc.* 18:186.

BERNIER, U. R., KLINE, D. L., POSEY, K. H., BOOTH, M. M., YOST, R. A., and BARNARD, D. R. 2003. Synergistic attraction of *Aedes aegypti* (L.) to binary blends of L-lactic acid and acetone,

dichloromethane, or dimethyl disulfide. *J. Med. Entomol.* 40:653–656.

BESANSKY, N. J., HILL, C. A., and COSTANTINI, C. 2004. No accounting for taste: host preference in malaria vectors. *Trends Parasitol.* 20:249–251.

BHASIN, A., MORDUE (LUNTZ), A. J., and MORDUE, W. 2000. Responses of the biting midge *Culicoides impunctatus* to acetone, CO_2 and 1-octen-3-ol in a wind tunnel. *Med. Vet. Entomol.* 14:300–307.

BHASIN, A., MORDUE (LUNTZ), A. J., and MORDUE, W. 2001. Field studies on efficacy of host odour baits for the biting midge *Culicoides impunctatus* in Scotland. *Med. Vet. Entomol.* 15:147–156.

BIRKETT, M. A., AGELOPOULOS, N., JENSEN, V., JESPERSEN, M. B., PICKETT, J. J. A., PRIJS, J., TRAPMAN, H. T. G. J., WADHAMS, J. L. J., and WOODCOCK, C. M. 2004. The role of volatile semiochemicals in mediating host location and selection by nuisance and disease-transmitting cattle flies. *Med. Vet. Entomol.* 18:313–322.

BRADY, J., COSTANTINI, C., SAGNON, N., GIBSON, G., and COLUZZI, M. 1997. The role of body odours in the relative attractiveness of different men to malarial vectors in Burkina Faso. *Ann. Trop. Med. Parasitol.* 91:121.

BRAKS, M. A. H., and TAKKEN, W. 1999. Incubated human sweat but not fresh sweat attracts the malaria mosquito *Anopheles gambiae* sensu stricto. *J. Chem. Ecol.* 25:663–672.

BRAKS, M. A. H., MEIJERINK, J., and TAKKEN, W. 2001. The response of the malaria mosquito, *Anopheles gambiae*, to two components of human sweat, ammonia and L-lactic acid, in an olfactometer. *Physiol. Entomol.* 26:142–148.

BROUWER, R. 1960. Variations in human body odour as a cause of individual differences of attraction for malaria mosquitoes. *Trop. Geogr. Med.* 12:186.

BURKOT, T. R. 1988. Non-random host selection by *Anopheline* mosquitoes. *Parasitol. Today* 4:156.

CORK, A., and PARK, K. C. 1996. Identification of electrophysiologically-active compounds for the malaria mosquito, *Anopheles gambiae*, in human sweat extracts. *Med. Vet. Entomol.* 10:269–276.

COSTANTINI, C., GIBSON, G., and COLUZZI, M. 1997. The role of body odours in the relative attractiveness of different men to malarial vectors in Burkino Faso. *Ann. Trop. Med. Parasitol.* 91:s121–s122.

COSTANTINI, C., SAGNON, N., DELLATORRE, A., DIALLO, M., BRADY, J., GIBSON, G., and COLUZZI, M. 1998. Odor-mediated host preferences of West African mosquitoes, with particular reference to malaria vectors. *Am. J. Trop. Med. Hyg.* 58:56–63.

COSTANTINI, C., BIRKETT, M. A., GIBSON, G., ZIESMANN, J., SAGNON, N. F., MOHAMMED, H. A., COLUZZI, M., and PICKETT, J. A. 2001. Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. *Med. Vet. Entomol.* 15:259–266.

CURRAN, A. M., RABIN, S. I., PRADA, P. A., and FURTON, K. G. 2005. Comparison of the volatile organic compounds present in human odor using SPME-GC-MS. *J. Chem. Ecol.* 31:1607–1619.

DEKKER, T., TAKKEN, W., and CARDÉ, R. T. 2001. Structure of host-odour plumes influences catch of *Anopheles gambiae* s.s. and *Aedes aegypti* in a dual-choice olfactometer. *Physiol. Entomol.* 26:124–134.

DEKKER, T., GEIR, M., and CARDÉ, R. T. 2005. Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odours. *J. Exp. Biol.* 208:293–2972.

DOUGLAS, H. D., CO, J. E., JONES, T. H., CONNER, W. E., and DAY, J. F. 2005. Chemical odorant of colonial seabird repels mosquitoes. *J. Med. Entomol.* 42:647–651.

EIRAS, A. E., and JEPSON, P. C. 1994. Responses of female *Aedes aegypti* (Diptera: Culicidae) to host odors and convection currents using an olfactometer bioassay. *Bull. Entomol. Res.* 84:207–211.

FREYVOGEL, T. A. 1961. Ein Beitrag zu den Problemen um die Blutmahlzeit von Stechmücken. *Acta Trop.* 18:201–251.

GEIER, M., and BOECKH 1999. A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odours. *J. Entomol. Exp. Appl.* 92:9.

GEIER, M., SASS, H., and BOECKH, J. 1996. A search for components in human body odour that attract females of *Aedes aegypti*. *Ciba F Symp.* 200:132–148.

GIKONYO, N. K., HASSANALI, A., NJAGI, P. G. N., GITU, P. M., and MIDIWO, J. O. 2002. Odor composition of preferred (buffalo and ox) and nonpreferred (waterbuck) hosts of some savanna tsetse flies. *J. Chem. Ecol.* 28:969–981.

GIKONYO, N. K., HASSANALI, A., NJAGI, P. G. N., and SAINI, R. K. 2003. Responses of *Glossina morsitans morsitans* to blends of electroantennographically active compounds in the odors of its preferred (buffalo and ox) and nonpreferred (waterbuck) hosts. *J. Chem. Ecol.* 29:2331–2345.

HAMILTON, J. G. C., and RAMSOONDAR, T. M. C. 1994. Attraction of *Lutzomyia longipalpis* to human skin odors. *Med. Vet. Entomol.* 8:375–380.

HEALY, T. P., COPLAND, M. J. W., CORK, A., PRZYBOROWSKA, A., and HALKET, J. M. 2002. Landing responses of *Anopheles gambiae* elicited by oxocarboxylic acids. *Med. Vet. Entomol.* 16:126–132.

JENSEN, K. M. V., JESPERSEN, J. B., BIRKETT, M. A., PICKETT, J. A., THOMAS, G., WADHAMS, L. J., and WOODCOCK, C. M. 2004. Variation in the load of the horn fly, *Haematobia irritans*, in cattle herds is determined by the presence or absence of individual heifers. *J. Med. Entomol.* 18:275–280.

KHAN, A. A., MAIBACH, H. I., STRAUSS, W. G., and FENLEY, W. R. 1966. Quantitation of effect of several stimuli on approach of *Aedes aegypti*. *J. Econ. Entomol.* 59:690.

KHAN, A. A., MAIBACH, H. I., and STRAUSS, W. G. 1968. Role of convection currents in mosquito attraction to human skin. *Mosq. News* 28:462–464.

KLINE, D. L., TAKKEN, W., WOOD, J. R., and CARLSON, D. A. 1990. Field studies on the potential of butanone, carbon-dioxide, honey extract, 1-octen-3-ol, L-lactic acid and phenols as attractants for mosquitoes. *Med. Vet. Entomol.* 4:383–391.

LINDSAY, S. W., ADIAMAH, J. H., MILLER, J. E., PLEASS, R. J., and ARMSTRONG, J. R. 1993. Variation in attractiveness of human-subjects to malaria mosquitoes (Diptera: Culicidae) in the Gambia. *J. Med. Entomol.* 30:368–373.

LOGAN, J. G. 2006. Differential responses of the yellow fever mosquito, Aedes aegypti and the Scottish biting midge, *Culicoides impunctatus* to human host odours. PhD dissertation, Rothamsted Research and University of Aberdeen.

MAYER, M. S., and JAMES, J. D. 1969. Attraction of *Aedes aegypti* (L)—responses to human arms carbon dioxide and air currents in a new type of olfactometer. *B. Entomol. Res.* 58:629.

PICKETT, J., WADHAMS, L. J., and WOODCOCK, C. M. 1998. Mate and host location by insect model systems for exploiting olfactory interactions. *The Biochemist*, August, 8–13.

QIU, Y. T., SMALLEGANGE, R. C., HOPPE, S., VAN, LOON, J. J., BAKKER, E. J., and TAKKEN, W. Y. T. 2004. Behavioural and electrophysiological responses of the malaria mosquito *Anopheles gambiae* Giles *sensu stricto* (Diptera: Culicidae) to human skin emanations. *Med. Vet. Entomol.* 18:429.

QUIROZ, A., PETTERSSON, J., PICKETT, J. A., WADHAMS, L. J., and NIEMEYER, H. M. 1997. Semiochemicals mediating spacing behavior of bird cherry-oat aphid, *Rhopalosiphum padi* feeding on cereals. *J. Chem. Ecol.* 23:2599–2607.

SASTRY, S. D., BUCK, K. T., JANAK, J., DRESSLER, M., and PRETI, G. 1980. Volatiles emitted by humans. In Biochemical Applications of Mass Spectrometry. Wiley, New York.

SCHOFIELD, S. W., and SUTCLIFFE, J. F. 1996. Human individuals vary in attractiveness for host-seeking black flies (Diptera: Simuliidae) based on exhaled carbon dioxide. *J. Med. Entomol.* 33:102–108.

SCHRECK, C. E., SMITH, N., CARLSON, D. A., PRICE, G. D., HAILE, D., and GODWIN, D. R. 1981. A material isolated from human hands that attracts female mosquitoes. *J. Chem. Ecol.* 8:429–438.

SCHRECK, C. E., KLINE, D. L., and CARLSON, D. A. 1990. Mosquito attraction to substances from the skin of different humans. *J. Am. Mosq. Control Assoc.* 6:406–410.

STEELMAN, C. D., GBUR, E. E., TOLLEY, G., and BROWN, A. H. 1993. Individual variation within breeds of beef-cattle in resistance to horn fly (Diptera: Muscidae). *J. Med. Entomol.* 30:414–420.

STEIB, B. M., GEIER, M., and BOECKH, J. 2001. The effect of lactic acid on odour-related host preference of yellow fever mosquitoes. *Chem. Senses* 26:523–528.

TAKKEN, W. 1991. The role of olfaction in host-seeking of mosquitoes - a review. *Insect Sci. Appl.* 12:287–295.

TAKKEN, W., and KNOLS, B. G. J. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol.* 44:131–157.

WOOD, W. F., and WELDON, P. J. 2002. The scent of the reticulated giraffe (*Giraffa camelopardalis reticulata*). *Biochem. Sys. Ecol.* 30:913–917.

ZAR, J. H. 1984. Biostatistical Analysis. Prentice Hall, New Jersey.

ZENG, X. N., LEYDEN, J. J., LAWLEY, H. J., SAWANO, K., NOHARA, I., and PRETI, G. 1991. Analysis of characteristic odors from human male axillae. *J. Chem. Ecol.* 17:1469–1492.

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Accumulation of Glucosinolates by the Cabbage Aphid *Brevicoryne brassicae* as a Defense Against Two Coccinellid Species

Corin Pratt · Tom W. Pope · Glen Powell · John T. Rossiter

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Abstract *Brassica nigra* plants, characterized by high levels of sinigrin, and artificial aphid diets to which sinigrin was selectively added were used to rear the crucifer specialist, *Brevicoryne brassicae*. Aphids were provided as a food source to two species of polyphagous ladybird, *Adalia bipunctata* and *Coccinella septempunctata*. First instar *A. bipunctata* were unable to survive when fed with *B. brassicae* reared on *B. nigra* or diets containing 0.2% sinigrin, but when fed with aphids reared on diets containing 0% sinigrin, survival rates were high. By contrast, first instar *C. septempunctata* were able to survive when fed with aphids reared on *B. nigra* or artificial diets containing up to 1% sinigrin. However, the presence of sinigrin in the aphid diet decreased larval growth and increased the time necessary for larvae to reach second instar for this species of ladybird. These results indicate that the presence of sinigrin in the diet of *B. brassicae* makes this aphid unsuitable as a food source for *A. bipunctata* but not for *C. septempunctata*, although for this ladybird species, there appear to be costs associated with feeding on aphids that contain this secondary metabolite.

Keywords *Adalia bipunctata* · *Coccinella septempunctata* · Glucosinolate · Chemical defense · Tritrophic interactions

Introduction

Secondary metabolites are important for plant survival in the environment, forming a chemical defense against pests and diseases (Wink 1988; Jander et al. 2001; Kliebenstein et al. 2005). Glucosinolates (anionic thioglucosides) are the main secondary metabolites accumulated by cruciferous plants (Brassicaceae). The plants also possess a myrosinase (β-thioglucoside glucohydrolase, EC 3.2.3.1; Bones and Rossiter 1996, 2006; Halkier and Gershenzon 2006). These two components are spatially segregated (Kelly et al. 1998; Koroleva et al. 2000) but are brought together upon attack by a pest or pathogen; glucosinolates are then hydrolyzed to biologically active products including nitriles, epithionitriles, thiocyanates, and isothiocyantes (Bones and Rossiter 1996, 2006; Halkier and Gershenzon 2006).

Despite this potent defense, crucifer specialists have evolved in several insect orders with counter adaptive biochemical mechanisms that allow feeding on plants that contain glucosinolates (Ratzka et al. 2002; Wittstock et al. 2004). The cabbage aphid *Brevicoryne brassicae* (L.) and the turnip aphid *Lipaphis pseudobrassicae* (=*erysimi*) (Kaltenbach) are not only able to feed on crucifers but have also developed a chemical defense system that exploits and mimics that of their host plants (Bridges et al. 2002; Kazana et al. 2007). These two aphid species are able to accumulate glucosinolates from their host plants and produce their own myrosinase that is compartmentalized into crystalline microbodies, thus avoiding internal glucosinolate hydrolysis under normal conditions. However, tissue damage as a result of attack by a predator results in production of hydrolysis products such as isothiocyanates (Francis et al. 2001; Kazana et al. 2007). Therefore, the aphid mimics the chemical defense system of its host plants and probably derives benefit in terms of protection from natural enemies. In addition, isothiocyanates also have been shown to synergize the response of *L. erysimi* to the aphid alarm pheromone, *E*-β-farnesene (Dawson et al. 1987) and may play a role in dispersing aphid colonies after attack.

Aphidophagous coccinellids have been used extensively in biological control programs for the control of aphid pests

C. Pratt · T. W. Pope · G. Powell (✉) · J. T. Rossiter
Division of Biology, Faculty of Natural Sciences,
Imperial College London,
Wye Campus, Ashford,
Kent TN25 5AH, UK
e-mail: g.powell@imperial.ac.uk

(Obrycki and Kring 1998). Indeed, the seven-spot ladybird, *Coccinella septempunctata* (L.), is commonly found predating on *B. brassicae* (Acheampong and Stark 2004). By contrast, larval survival rates of the two-spot ladybird, *Adalia bipunctata* (L.), fed with *B. brassicae* reared on a range of crucifer host-plants were low (Francis et al. 2001). However, *A. bipunctata* larvae were able to develop when fed with peach-potato aphids, *Myzus persicae* (Sulzer), reared on the same species of crucifer. This result may reflect the fact that whereas *B. brassicae* accumulates glucosinolates from its host, *M. persicae* appears to excrete these compounds in the honeydew (Weber et al. 1986; Merritt 1996). In addition, *M. persicae* lacks myrosinase activity and, therefore, does not produce toxic hydrolysis products when attacked (Francis et al. 2001).

The use of a range of crucifer host plants on which *B. brassicae* were reared and fed to *A. bipunctata* larvae indicates that the myrosinase–glucosinolate system may be central to the aphids' defense against this natural enemy (Francis et al. 2001). In this study, an artificial aphid diet system was used to manipulate the levels of glucosinolate ingested by *B. brassicae.* The aphids were fed to *A. bipunctata* larvae to investigate the effects of glucosinolates on the next trophic level. This approach was extended to include *C. septempunctata* to determine what, if any, defense glucosinolate accumulation affords *B. brassicae* against this natural enemy.

Methods and Materials

Artificial Diets Parafilm© "M" (American Can Company, Greenwich, CT, USA) was stretched over circular curtain rings of 25 mm diameter. Three hundred microliters of an artificial aphid diet (see Diet B in Dadd 1967), to which 0.0, 0.2, 0.4, 0.6, 0.8, or 1.0% sinigrin had been added, was applied to the surface of the Parafilm. A second sheet of Parafilm was stretched over the ring to create a 'sachet' containing the diet. Excess Parafilm was removed, and diet rings were stored in a freezer until required.

Insects B. brassicae and *M. persicae* cultures were each reared on *Brassica nigra* (L.) Koch. Each plant was enclosed within a perforated bread bag and maintained at 18°C with a 16L/8D photoperiod.

B. brassicae were reared on artificial diet sachets by first transferring 5–10 adult wingless aphids to the underside of a diet ring. Diet rings were then placed into Petri dishes, which were covered with semi-transparent green discs to mimic leaf spectral reflectance. The adults were left for 72 hr before being removed. Aphid nymphs produced during this 72-hr period were transferred to fresh diet rings. Aphids used in experiments were undifferentiated nymphs (wing buds not visible), aged between 3–6 d. Aphids were reared on diet rings at 18°C with a 16L/8D photoperiod.

A. bipunctata and *C. septempunctata* cultures were each maintained at 20°C with a 16L/8D photoperiod. Adult ladybirds were kept, in groups of approximately 20, within ventilated sandwich boxes and were fed daily with an excess of pea aphids, *Acyrthosiphon pisum* (Harris), which were reared on tic bean (*Vicia faba* var. *minor* L.) seedlings. Ladybird eggs were collected by transferring three to four *C. septempunctata* or five to six *A. bipunctata* to ventilated 90 mm diameter Petri dishes and were provided with an excess of *A. pisum*. Adult ladybirds were removed after 24 hr, and any eggs laid were checked daily until they had hatched.

Effect of Sinigrin on Growth of B. brassicae Nymphs Adult wingless *B. brassicae* were transferred onto diet rings containing 0% sinigrin, as previously described, except that they were removed after 24 hr. Ten nymphs were selected at random and weighed before being returned to a fresh diet, also containing 0% sinigrin for 5 d. Similarly, 10 *B. brassicae* nymphs were selected, weighed, and then reared for 5 d on diet rings containing 1% sinigrin. All aphids were kept at 18°C with a 16L/8D photoperiod. After 5 d, the aphids reared on the 0 and 1% sinigrin diets were reweighed.

Growth and Survival of A. bipunctata and C. septempunctata Larvae Newly hatched *A. bipunctata* larvae were selected at random from various egg clutches and weighed (Mettler Toledo MX5, Switzerland) and isolated to separate glass tubes (internal diameter 18 mm, height 51 mm). Each larva was assigned a treatment and supplied with an excess of aphids (approximately 7 for *A. bipunctata* larvae and 10 for *C. septempunctata*). Ladybird larval weight and survival was recorded every 24 hr until the first molt was reached or until the larva died. After weighing, larvae were transferred to a clean tube, and fresh aphids were provided. Ten replicates of the experiment were carried out for each treatment. Experiments were conducted at 21°C, 16 hr light-phase/18°C, 8 hr dark-phase regime.

The following three experiments were completed with each ladybird species:

I. Larvae were fed mixed aged *B. brassicae* or *M. persicae* nymphs (aphids had been reared on *B. nigra*).
II. Larvae were fed 3- to 6-d-old *B. brassicae* nymphs reared on artificial diets containing either 0 or 1% sinigrin. An additional 'starved' treatment was included in this experiment to compare the effects of sinigrin with complete absence of food.

III. Larvae were fed 3- to 6-d-old *B. brassicae* nymphs reared on artificial diets containing 0, 0.2, 0.4, 0.6, 0.8, or 1% sinigrin.

Analysis Data were analyzed by using Student's *t* test and chi-square tests with Yates correction with the exception of the experiments investigating the effect of a range of sinigrin concentrations in the artificial aphid diet on ladybird growth, where an analysis of variance was completed using GenStat 8th Edition. Ladybird growth was analyzed by comparing larval weight after 24 hr. There was some variation in the weights of newly hatched ladybird larvae among different experiments, presumably because some batches may have hatched earlier than others, with more opportunity for egg-case consumption and sibling egg cannibalism (Omkar et al. 2007). However, initial larval weight did not differ significantly among treatments within each experiment (data not shown).

Results

Effect of Sinigrin on Growth of *B. brassicae*

Twenty *B. brassicae* nymphs were reared for 5 d on two artificial aphid diets that were identical with the exception that sinigrin was present at one of two levels, 0 and 1%, respectively (Table 1). Initial mean weights of *B. brassicae* nymphs (<24 hr old) born on either artificial diet did not differ significantly. Similarly, after 5 days continued feeding on these two artificial diets, mean nymph weights were not different.

Growth and Survival of *A. bipunctata* and *C. septempunctata* Larvae

Experiment I Weight gain and survival data of newly hatched *A. bipunctata* and *C. septempunctata* larvae fed with either *B. brassicae* or *M. persicae* nymphs reared on *B. nigra* were recorded (Table 2). The weight of *A. bipunctata* larvae fed with *M. persicae* nymphs was significantly higher (t=3.48, P=0.003) than that for larvae fed with *B. brassicae* nymphs after the first 24 hr of the experiment. Survival of *A. bipunctata* larvae was also affected by the aphid species provided as a food source, with 90% of *M. persicae*-fed ladybird larvae surviving to second instar, compared with 0% of larvae fed with *B. brassicae* nymphs. For *C. septempunctata*, the weight of larvae fed *M. persicae* nymphs was also higher (t=3.28, P=0.005) than for larvae fed *B. brassicae* nymphs. By contrast with survival data for *A. bipunctata*, an equal number, 90%, of *C. septempunctata* larvae survived to second instar when fed either *M. persicae* or *B. brassicae*. However, larvae fed *B. brassicae* nymphs took longer (t=3.16, P=0.006) than larvae fed *M. persicae* to reach this stage.

Table 1 Weight gain of *Brevicoryne brassicae* nymphs reared on artificial diets containing 0% or 1% sinigrin

	Sinigrin Content in Aphid Artificial Diet		Significance
	0%	1%	
Mean weight in mg (0 hr)	0.035± 0.002	0.034± 0.002	t=0.32, P=0.753
Mean weight in mg (120 hr)	0.122± 0.006	0.107± 0.006	t=1.74, P=0.100

Mean weights±SE, N=10

Experiment II Newly hatched *A. bipunctata* and *C. septempunctata* larvae were starved or fed with *B. brassicae* nymphs reared on artificial diets containing 0 or 1% sinigrin (Table 3). During the first 24 hr, whether *A. bipunctata* were starved or fed with *B. brassicae* nymphs reared on artificial diets containing 0 or 1% sinigrin significantly affected larval weights (F=16.59, P<0.001). Individual contrasts between means, using least significant difference (LSD) indicated that the mean weight of larvae (after 24 hr) fed *B. brassicae* reared on diet containing 0% sinigrin was significantly higher than for larvae that were starved or fed *B. brassicae* reared on the 1% sinigrin diet. Larvae that were starved or fed *B. brassicae* nymphs reared on diet containing 1% sinigrin had mean weights that did not differ significantly. Of the *A. bipunctata* larvae fed with *B. brassicae* nymphs reared on diet containing 0% sinigrin, 100% survived to second instar. By contrast, 0% of larvae that were starved or fed with *B. brassicae* reared on the 1% diet reached their first molt. Growth of *C. septempunctata* larvae during the first 24 hr of the experiment was also affected (F=36.08, P<0.001) by the food source provided. Analysis of differences between means, using least significant difference (LSD), indicates that, as for *A. bipunctata* larvae, weights after 24 hr for *C. septempunctata* larvae fed with *B. brassicae* reared on artificial diet containing 0% sinigrin were significantly higher than for larvae that were starved or fed *B. brassicae* reared on the 1% sinigrin diet. Weights of larvae fed *B. brassicae* nymphs reared on the 1% sinigrin diet were not significantly different from starved larvae. Although a greater number of *C. septempunctata* larvae fed with *B. brassicae* reared on the 0% sinigrin diet reached second instar compared with larvae fed with *B. brassicae* nymphs reared on the 1% sinigrin diet, this difference was not significant. In addition, *C. septempunctata* larvae took less time to reach second instar when fed with *B. brassicae* reared on the 0% sinigrin diet

Table 2 Survival, mean weight after 24 hr and time to second instar data for *Adalia bipunctata* or *Coccinella septempunctata* first instar larvae fed *Brevicoryne brassicae* or *Myzus persicae* reared on *Brassica nigra*

	Aphid Species		Significance
	Myzus persicae	*Brevicoryne brassicae*	
Adalia bipunctata			
% survival to second instar	90	0	χ^2=11.99, P<0.001
Mean weight after 24 hr (mg)	0.216±0.021	0.134±0.007	t=3.48, P=0.003
No. days to second instar	2.9±0.1	-	N/A
Coccinella septempunctata			
% survival to second instar	90	90	χ^2=0, P=NS
Mean weight after 24 hr (mg)	0.332±0.014	0.276±0.010	t=3.28, P=0.005
No. days to second instar	3.0±0.0	3.6±0.2	t=3.16, P=0.006

Mean weights±SE, N=10; *N/A* not applicable

compared to larvae fed *B. brassicae* reared on diet containing 1% sinigrin (t=6.52, P<0.001). Again, no larvae that were starved reached second instar.

Experiment III Weight gain and survival of *A. bipunctata* and *C. septempunctata* larvae fed with *B. brassicae* nymphs reared on artificial diets containing a range of sinigrin concentrations were recorded (Table 4). After 24 hr of feeding, growth of *A. bipunctata* larvae fed *B. brassicae* reared on artificial diets containing 0, 0.2, 0.4, 0.6, 0.8, or 1% sinigrin differed significantly (F=31.59, P<0.001). Individual contrasts between means, using LSD, indicates that the 24-hr weights of larvae fed *B. brassicae* nymphs reared on the 0% sinigrin diet were higher than for larvae fed *B. brassicae* reared on all other diets. Mean weight of larvae fed *B. brassicae* reared on diet containing 0.2% sinigrin was also higher than for larvae fed aphids reared on diets containing 0.4% sinigrin. However, larval weights did not differ significantly among the other treatments. Only larvae fed with *B. brassicae* reared on artificial diet containing 0% sinigrin reached second instar (90%). Sinigrin content of artificial diets used to rear *B. brassicae* nymphs, which were fed to *C. septempunctata* larvae, did not affect larval weights (F=1.05, P>0.05). This lack of overall significance suggests that individual contrasts between means are not appropriate. However, time to second instar was affected (F=5.20, P<0.001). Individual contrasts between means, using LSD, indicates that *C. septempunctata* larvae fed aphids reared on the 0% sinigrin diet reached second instar significantly faster than larvae fed aphids reared on other diets. However, survival was not affected, with similar numbers of larvae reaching second instar, regardless of the sinigrin content of the diet used to rear the aphids provided as a food source.

Discussion

Survival of *A. bipunctata* larvae fed with *B. brassicae* is known to be affected by the species of cruciferous plant on which the aphids were reared (Francis et al. 2001). This effect has been correlated with glucosinolate content of the host plant. In this study, we demonstrated through the use

Table 3 Survival, mean weight after 24 hr and time to second instar data for *Adalia bipunctata* or *Coccinella septempunctata* either starved or fed *Brevicoryne brassicae* reared on artificial diets containing 0 or 1% sinigrin

	Starved	Sinigrin Content in Aphid Artificial Diet		Significance
		0%	1%	
Adalia bipunctata				
% survival to second instar	0	100	0	χ^2=16.20, P<0.001
Mean weight after 24 hr (mg)	0.092±0.005	0.172±0.014	0.114±0.004	F=16.59, P<0.001
No. days to second instar	-	2.8±0.1	-	N/A
Coccinella septempunctata				
% survival to second instar	0	100	80	χ^2=0.56, P<0.456
Mean weight after 24 hr (mg)	0.150±0.002	0.415±0.036	0.209±0.014	F=36.08, P<0.001
No. days to second instar	-	2.7±0.2	5.4±0.4	t=6.52, P<0.001

Mean weights±SE, N=10; *N/A* not applicable.

Table 4 Survival, mean weight after 24 hr and time to second instar data for *Adalia bipunctata* or *Coccinella septempunctata* fed *Brevicoryne brassicae* reared on artificial diets containing a range of concentrations of sinigrin

	Sinigrin Content in Aphid Artificial Diet						Significance
	0%	0.2%	0.4%	0.6%	0.8%	1.0%	
Adalia bipunctata							
% survival to second instar	90	0	0	0	0	0	N/A
Mean weight after 24 hr (mg)	0.220±0.013	0.130±0.006	0.101±0.008	0.116±0.004	0.108±0.007	0.108±0.005	F=31.59, P<0.001
No. days to second instar	2.8±0.1	–	–	–	–	–	N/A
Coccinella septempunctata							
% survival to second instar	100	90	90	80	70	80	χ^2=1.57, P=0.905
Mean weight after 24 hr (mg)	0.324±0.039	0.289±0.029	0.263±0.019	0.264±0.020	0.256±0.022	0.256±0.022	F=1.05, P=NS
No. days to second instar	3.7±0.2	4.4±0.3	4.7±0.2	5.0±0.2	4.9±0.1	5.0±0.3	F=5.20, P<0.001

Mean weights±SE, N=9 for *A. bipunctata* and 10 for *C. septempunctata*; *N/A* not applicable

of artificial aphid diets how the presence of a single glucosinolate, sinigrin, affects survival of first instar *A. bipunctata*. By contrast, survival of larvae of a second polyphagous species of ladybird, *C. septempunctata*, was not compromised by the presence of sinigrin in the diet of *B. brassicae*. However, more subtle costs are apparent with extended development times in larvae fed *B. brassicae* reared on diets containing sinigrin compared with larvae fed with aphids reared on diets without sinigrin.

When aphids were reared on *B. nigra*, survival rates of first instar *A. bipunctata* larvae were 90% when fed with *M. persicae*, but 0% when fed with *B. brassicae*, confirming the earlier findings of Francis et al. (2001). The apparent suitability of the generalist *M. persicae* as a food source for *A. bipunctata* may reflect the fact that this species of aphid, although able to colonize crucifers, does not accumulate glucosinolates (Weber et al. 1986). Indeed, when *M. persicae* were fed to *A. bipunctata*, the species of cruciferous plant on which the aphids were reared did not significantly affect larval mortality (Francis et al. 2001). By contrast, the crucifer specialist, *B. brassicae*, not only accumulates glucosinolates in the hemolymph (Kazana et al. 2007) but also possesses, like its host plants, the ability to hydrolyze these secondary metabolites to biologically active products including isothiocyanates (MacGibbon and Allison 1968; Bridges et al. 2002). As these hydrolysis products are known to be toxic to both insects and fungi, it has been suggested that they may provide a direct defense against generalist natural enemies (Bridges et al. 2002; Bones and Rossiter 1996, 2006) as appears to be the case in this study for *A. bipunctata*. Air entrainments show that aphids reared on a glucosinolate-containing diet certainly release isothiocyanate when attacked by foraging ladybirds (Kazana et al. 2007).

Bridges et al. (2002) also suggested that as with specialist crucifer-feeding insects, natural enemies of these herbivores are probably adapted to toxic glucosinolate hydrolysis products. The polyphagous ladybird *C. septempunctata* is known to successfully predate upon *B. brassicae* (Blackman 1967), and results presented in this paper confirm that first instar *C. septempunctata* are able to develop successfully on both *M. persicae* and *B. brassicae*. However, *C. septempunctata* larvae performed better, in terms of weight after the first 24 hr of the experiment and time to second instar, when fed *M. persicae* as opposed to *B. brassicae*.

Results from Experiment I and the earlier work by Francis et al. (2001) suggest a possible direct defensive role for glucosinolates accumulated and hydrolyzed by *B. brassicae*. However, by providing *M. persicae* and *B. brassicae* as a food source, it is not possible to discriminate between aphid morphology, behavior, or chemical composition as possible explanations for the observed differences in ladybird performance (Omkar 2005). Therefore, subsequent experiments were completed where *A. bipunctata* and *C. septempunctata* larvae were fed with *B. brassicae* reared on artificial aphid diets to which a glucosinolate, sinigrin, was selectively added.

Results from these experiments confirm that the presence of sinigrin in the diet of *B. brassicae* results in this aphid becoming unsuitable as prey for *A. bipunctata* larvae. Indeed, whereas 100% of larvae tested were able to reach second instar when fed *B. brassicae* nymphs reared on a diet containing 0% sinigrin, no larvae were able to develop when fed nymphs reared on a 1% sinigrin diet. The presence of sinigrin in aphid diets had such a strong inhibitory effect on *A. bipunctata* that larval growth and survival were similar to those insects that were assigned to the starved treatment and completely deprived of aphid food. To confirm the suitability of *B. brassicae* reared on a diet containing 0% sinigrin for *A. bipunctata*, a small number of larvae were allowed to continue feeding on this group of aphids, and the predators then successfully completed their development (unpublished observations).

C. septempunctata larvae were able to predate upon *B. brassicae*, when fed aphids reared on diets containing either 0 or 1% sinigrin. However, the presence of the glucosinolate in the aphid diet appears to have consequences for the performance of this ladybird species. Larval weights were greater and time required to reach second instar shorter for *C. septempunctata* larvae supplied with *B. brassicae* reared on a 0% sinigrin diet compared with larvae fed aphids reared on a 1% sinigrin diet. However, there was no difference in survival of first instars fed with *B. brassicae* reared on diets containing either 0 or 1% sinigrin. By contrast with *A. bipunctata*, first instar *C. septempunctata* appear to have a mechanism that at least partially negates the effects of sinigrin or the toxic hydrolysis products produced by *B. brassicae* after tissue damage. It is, however, unclear from these data whether the mechanism involved is based on tolerance or detoxification. Interestingly, glutathione transferase levels in *A. bipunctata* increase after exposure to isothiocyanates (Francis et al. 1999).

A. bipunctata larvae did not reach second instar when fed *B. brassicae* nymphs reared on artificial diets containing 0.2, 0.4, 0.6, 0.8, or 1% sinigrin. Again, only when fed aphids reared on the 0% sinigrin diet were *A. bipunctata* larvae able to develop. The higher weight of larvae fed *B. brassicae* reared on a diet containing 0.2% sinigrin compared with larvae fed aphids reared on diet containing 0.4% sinigrin indicates that *A. bipunctata* can perhaps tolerate low levels of allyl isothiocyanate to some extent. However, given an estimated phloem sinigrin content of >0.4% in *B. nigra* (Merritt 1996), it is perhaps not surprising that *A. bipunctata* larvae were unable to survive when fed with *B. brassicae* reared on this host-plant.

Growth (after 24 hr) and survival of *C. septempunctata* larvae fed *B. brassicae* was not significantly affected by sinigrin content of the diet on which the aphids were reared. However, costs were apparent (in terms of extended development time) when larvae were fed *B. brassicae* reared on any of the diets containing sinigrin. There is some evidence that this cost increased with increasing concentration of sinigrin added to the artificial diet. Indeed, when diets containing 0, 0.2, and 0.4% sinigrin are considered, times to second instar were 3.7, 4.4, and 4.7 days, respectively. Interestingly, these trends appear to level out when larvae were fed *B. brassicae* reared on diets containing higher concentrations of sinigrin. Thus, the level of defense afforded to 3- to 6-d-old *B. brassica* nymphs through the accumulation of sinigrin appears to be a function, over a limited range, of the concentration of sinigrin present in the aphid's diet. The precise relationship between dietary sinigrin concentration and the level of this glucosinolate in aphid body tissues has not been investigated. It has been shown that wingless *B. brassicae* contain approximately 3.5 times higher levels of sinigrin when reared on 1% than on 0.1% (Kazana et al. 2007). Over the range of sinigrin concentrations tested, it is qualitative aspects of host-plant chemistry that determine the interaction between *B. brassicae* and *A. bipunctata*, whereas quantitative factors may be important in determining the interaction between this species of aphid and *C. septempunctata*.

By contrast with the results for the two species of ladybird, rearing *B. brassicae* on artificial diets that contain either 0 or 1% sinigrin had no effect on the weight gain of nymphs. Studies have previously demonstrated that sinigrin acts as a strong phagostimulant to *B. brassicae* (Wensler 1962; Nault and Styer 1972). However, the fact that aphids did not perform better when feeding on diet containing 1% sinigrin vs 0% sinigrin diet may reflect the artificial conditions encountered by aphids probing through Parafilm. Indeed, when probing plants, *B. brassicae* may be able to recognize host plants when the stylets make contact with mesophyll tissue, before the phloem is reached (Gabrys and Tjallingii 2002).

Results from this study indicate that accumulation of sinigrin and production of allyl isothiocyanate by *B. brassicae* appears to affect negatively the performance of *C. septempunctata* by slowing development. Use of crops with lower levels of glucosinolates may, therefore, enhance the performance of *C. septempunctata*, making this species perhaps more effective as a biological control agent for *B. brassicae*. However, crops with lower levels of glucosinolates may bring an increased risk from generalist herbivores (Raybould and Moyes 2001).

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References

ACHEAMPONG, S., and STARK, J. D. 2004. Can reduced rates of pymetrozine and natural enemies control the cabbage aphid, *Brevicoryne brassicae* (Homoptera: Aphididae), on broccoli? *Int. J. Pest Manag* 50:275–279.

BLACKMAN, R. 1967. The effects of different aphid foods on *Adalia bipunctata* L. and *Coccinella 7-punctata* L. *Ann. Appl. Biol* 59:207–219.

BONES, A. M., and ROSSITER, J. T. 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiol. Plantarum* 97:194–208.

BONES, A. M., and ROSSITER, J. T. 2006. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry* 67:1053–1067.

BRIDGES, M., JONES, A. M. E., BONES, A. M., HODGSON, C., COLE, R., BARTLET, E., WALLSGROVE, R., KARAPAPA, V. K., WATTS, N., and ROSSITER, J. T. 2002. Spatial organization of the glucosinolate-myrosinase system in brassicae specialist aphids is similar to that of the host plant. *Proc. Royal Soc. London* 269:187–191.

DADD, R. H. 1967. Improvement of synthetic diet for the aphid *Myzus persicae* using plant juices, nucleic acids, or trace metals. *J. Ins. Physiol* 13:763–778.

DAWSON, G. W., GRIFFITHS, D. C., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, W. M. 1987. Plant-derived synergists of alarm pheromone from turnip aphid, *Lipaphis (Hyadaphis) erysimi* (Homoptera, Aphididae). *J. Chem. Ecol* 13:1663–1671.

FRANCIS, F., HAUBRUGE, E., and GASPAR, C. 1999. Effects of isothiocyanates on the glutathione S-transferases activity from *Adalia bipunctata* L. (Coleoptera: Coccinellidae). *Med. Fac. Landbouww. Univ. Gent* 64/3a:297–303.

FRANCIS, F., LOGNAY, G., WATHELET, J. P., and HAUBRUGE, E. 2001. Effects of allelochemicals from first (Brassicaceae) and second (*Myzus persicae and Brevicoryne brassicae*) trophic levels on *Adalia bipunctata*. *J. Chem. Ecol* 27:243–256.

GABRYS, B., and TJALLINGII, W. F. 2002. The role of sinigrin in host plant recognition by aphids during initial plant penetration. *Ent. Exp. Appl* 104:89–93.

HALKIER, B. A., and GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol* 57:303–333.

JANDER, G., CUI, J., NHAN, B., PIERCE, N. E., and AUSUBEL, F. M. 2001. The TASTY locus on chromosome 1 of *Arabidopsis* affects feeding of the insect herbivore *Trichoplusia ni*. *Plant Physiol* 126:890–898.

KAZANA, E., POPE, T. W., TIBBLES, L., BRIDGES, M., PICKETT, J. A., BONES, A. M., POWELL, G., and ROSSITER, J. T. 2007. The cabbage aphid: a walking mustard oil bomb. *Proc. R. Soc. London Ser. B* 274:2271–2277.

KELLY, P. J., BONES, A., and ROSSITER, J. T. 1998. Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. *Planta* 206:370–377.

KLIEBENSTEIN, D. J., ROWE, H. C., and DENBY, K. J. 2005. Secondary metabolites influence Arabidopsis/*Botrytis* interactions: variation in host production and pathogen sensitivity. *The Plant Journal* 44:25–36.

KOROLEVA, O. A., DAVIES, A., DEEKEN, R., THORPE, M. R., TOMOS, A. D., and HEDRICH, R. 2000. Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. *Plant Physiol* 124: 599–608.

MACGIBBON, D. B., and ALLISON, R. M. 1968. A glucosinolase system in the cabbage aphid *Brevicoryne brassicae*. *New Zealand J. Sci* 11:440–446.

MERRITT, S. Z. 1996. Within-plant variation in concentrations of amino acids, sugar and sinigrin in phloem sap of black mustard, *Brassica nigra* (L.) Koch (Cruciferae). *J. Chem. Ecol* 22:1133–1145.

NAULT, L. R., and STYER, W. E. 1972. Effects of sinigrin on host selection by aphids. *Ent. Exp. Appl* 15:423–437.

OBRYCKI, J., and KRING, T. J. 1998. Predaceous coccinellidae in biological control. *Annu. Rev. Entomol* 43:295–321.

OMKAR 2005. Preference-performance of a generalist predatory ladybird: A laboratory study. *Biological Control* 34:187–195.

OMKAR, PERVEZ, A., and GUPTA, A. K. 2007. Sibling cannibalism in aphidophagous ladybirds: its impact on sex-dependent development and body weight. *J. Appl. Entomol* 131:81–84.

RAYBOULD, A. F., and MOYES, C. L. 2001. The ecological genetics of aliphatic glucosinolates. *Heredity* 87:383–391.

RATZKA, A., VOGEL, H., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and KROYMANN, J. 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U.S.A* 99:11223–11228.

WEBER, G., OSWALD, S., and ZOLLNER, U. 1986. Suitability of rape cultivars with different glucosinolate content for *Brevicoryne brassicae* (L.) and *Myzus persicae* (Sultzer) (Hempitera, Aphididae). *J. Plants Diseases Protection* 93:113–124.

WENSLER, R. J. D. 1962. Mode of host selection by an aphid. *Nature* 195:830–831.

WINK, M. 1988. Plant breeding: importance of plant secondary metabolites for protection against pathogens and herbivores. *Theoret. Appl. Gen* 75:225–233.

WITTSTOCK, U., AGERBIRK, N., STAUBER, E. J., OLSEN, C. E., HIPPLER, M., MITCHELL-OLDS, T., GERSHENZON, J., and VOGEL, H. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U.S.A* 101:4859–4864.

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Experience-Induced Habituation and Preference Towards Non-Host Plant Odors in Ovipositing Females of a Moth

Hua Wang · Wen-Fei Guo · Peng-Jun Zhang · Zhi-Yi Wu · Shu-Sheng Liu

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Abstract In phytophagous insects, experience can increase positive responses towards non-host plant extracts or induce oviposition on non-host plants, but the underlying chemical and behavioral mechanisms are poorly understood. By using the diamondback moth, *Plutella xylostella*, its host plant Chinese cabbage, and a non-host plant *Chrysanthemum morifolium*, as a model system, we observed the experience-altered olfactory responses of ovipositing females towards volatiles of the non-host plant, volatiles of pure chemicals (*p*-cymene and α-terpinene) found in the non-host plant, and volatiles of host plants treated with these chemicals. We assessed the experience-altered oviposition preference towards host plants treated with *p*-cymene. Naive females showed aversion to the odors of the non-host plant, the pure chemicals, and the pure chemical-treated host plants. In contrast, experienced females either became attracted by these non-host odors or were no longer repelled by these odors. Similarly, naive females laid a significantly lower proportion of eggs on pure chemical-treated host plants than on untreated host plants, but experienced females laid a similar or higher proportion of eggs on pure chemical-treated host plants compared to untreated host plants. Chemical analysis indicated that application of the non-host pure chemicals on Chinese cabbage induced emissions of volatiles by this host plant. We conclude that induced preference for previously repellent compounds is a major mechanism that leads to behavioral changes of this moth towards non-host plants or their extracts.

Keywords Experience · *Plutella xylostella* · Non-host plant odor · Olfactory response · Oviposition preference

H. Wang · P.-J. Zhang · Z.-Y. Wu · S.-S. Liu (✉)
Institute of Insect Sciences, Zhejiang University, Hangzhou 310029, China
e-mail: shshliu@zju.edu.cn

W.-F. Guo
Department of Chemistry, Zhejiang University, Hangzhou 310027, China

Introduction

Studies of phytophagous insects have demonstrated a range of learned behavior that includes habituation, associative learning, food aversion learning, and induced preference (Papaj and Prokopy 1989; Schoonhoven et al. 2005). Experience-altered behavior has been observed in feeding (e.g., Jermy 1987; Cunningham et al. 1998a, 2004; del Campo et al. 2001; Held et al. 2001) and oviposition (e.g., Cunningham et al. 1998b; Rojas and Wyatt 1999). Earlier investigations on experience-altered host selection and oviposition behavior in adults were usually done with a range of host plants and generally showed that experience could enhance foraging efficiency in a changing host environment (Stanton 1984; Stephens 1993; Landolt and Molina 1996; Cunningham et al. 1998b). More recent investigations done with non-host plants or their extracts have shown that early adult experience could induce oviposition on non-host plants or a preference for host plants treated with non-host extracts (Liu et al. 2005; Liu and Liu 2006; Zhang et al. 2007). These recent findings have implications in both insect–plant evolution (Cunningham et al. 2001) and behavioral control of insect pests (Cunningham et al. 1999; Jallow et al. 2004) because oviposition on non-host plants is an essential step for host range expansion, and an induced preference for non-host plant-derived repellents may render these chemicals ineffective. However, the

chemical and behavioral mechanisms for this kind of experience-altered behavior towards non-host plants or their extracts are only superficially understood.

Odors emitted by a plant or its crude extract are a complex blend of individual volatile components, each with a potential effect on insect behavior (Schoonhoven et al. 2005). Host and non-host plants of a given insect may share common volatile compounds (Bengtsson et al. 2006). In some cases, it is possible that the odors of a non-host plant or its extract contain repellent as well as attractive compounds, and an experience-altered behavior may be due to a complex of chemical, neurophysiological, and behavioral causes. For example, preference for non-host plant odors by ovipositing females following experience may result from either an induced preference for the repellent compounds or a habituation to the repellents combined with an unchanged response to the attractive compounds. It has been suggested that these alternative explanations can be investigated by experiments that use individual pure chemicals found in non-host plants (Liu et al. 2005).

By using the diamondback moth *Plutella xylostella*, its host plant Chinese cabbage, and a non-host plant (*Chrysanthemum morifolium*) as a model system, we have shown that experience of the non-host plant extract results in the moths being more attracted to host plants treated with the non-host plant extracts and depositing a higher proportion of eggs on host plants treated with the extract than on untreated host plants (Liu et al. 2005). The same model system is used in this study. Here, we first identified two chemicals, α-terpinene and *p*-cymene, from the volatiles of *Chrysanthemum*. We next observed the olfactory responses of naive and experienced *P. xylostella* females to odors of the non-host plant, odors of the two chemicals, and odors of host plants treated with the two chemicals. Finally, we observed the oviposition preference of naive and experienced females towards chemical-treated host plants. These results demonstrate that induced preference for repellent chemicals is a major mechanism leading to behavioral changes towards non-host plants or their extracts.

Methods and Materials

Insects and Plants The *P. xylostella* culture was established from a field collection on a cabbage farm in a suburb of Hangzhou, China, in October 2004 and maintained on common cabbage, *Brassica oleracea* L. var. *capitata*, cultivar Jing-feng No. 1 in a temperature-controlled room at 26±1°C, 60–70% RH and 14L/10D photoperiod. The culture was replenished once a year with field-collected material.

A non-host plant of *P. xylostella*, *C. morifolium* cultivar Xiaoyangju, was used in this study. Plants of *C. morifolium* were first collected from Tongxiang, Zhejiang, China, and their shoots were transplanted in a potting mix (a mixture of peat moss, vermiculite, organic fertilizer, and perlite in a 10:10:10:1 ratio) in 1.5 l pots and grown in a greenhouse under ambient temperature and humidity and natural lighting. Plants of *C. morifolium* were used at the vegetative stage bearing about 20 leaves. The host plant, Chinese cabbage *Brassica campestris* L. ssp. *pekinensis*, cultivar Zaoshu No. 5, was used in the olfactory and oviposition tests. Chinese cabbage plants were cultivated in the above-mentioned potting mix in 1.5 l pots in greenhouses under ambient temperature and humidity and natural lighting to the five to six fully extended true-leaf stage when used in tests.

Collection and Analysis of Volatile Compounds of C. morifolium The volatile compounds of *C. morifolium* were collected by using solid-phase microextraction (SPME, Supelco, Shanghai, China) and analyzed with a gas chromatograph and mass spectrometer (GC-MS, Agilent HP6890N-HP5973, Agilent, Palo Alto, CA, USA).

The SPME holder had fiber covered with 100 μm polydimethylsiloxane. Before each session of extraction, the fiber was conditioned at 250°C for 30 min. For volatile extraction by SPME, the plant sample was enclosed in a glass container (16 cm diameter and 35 cm height). The SPME needle was injected through the septum of the sample container as well as the extended plunger to expose the fiber. Following equilibration between the fiber and the volatile sample for 30 min, the fiber was retracted into the needle. The SPME device was removed from the container and inserted directly into the GC for desorption and analysis. The port was operated in splitless mode. After 3 min, the split valve was opened, and the fiber was removed at the same time. This treatment and analysis were replicated twice.

Chemical constituents of the volatiles were identified following GC-MS analysis on an HP 6890N Series GC System connected to an HP 5973 Network Mass Selective Detector with an HP-5 column (30 m×0.32 mm i.d., 0.25 μm film thickness). The temperature program for GC was as follows: the initial temperature was maintained at 40°C, held for 2 min, heating up at 8°C/min to 120°C, and then at 1.5°C/min to 160°C. The carrier gas was helium (1 ml/min). The mass spectrum was repetitively scanned from 35 to 450 a.m.v. every 2 s. The MS was used in election impact ionization mode at 70 eV.

Chemical constituents of the volatile were identified by mass spectrum matching in the NIST98 library as well as by comparison of retention time and the fragmentation patterns of the mass spectra with those of authentic commercial samples.

Individual Pure Chemicals The pure chemicals, α-terpinene and *p*-cymene, were purchased from Sigma-Aldrich Chem-

icals, Poole, UK. Chemicals were dissolved in distilled water (containing 2% acetone) to make the test solutions. Solutions of α-terpinene or *p*-cymene were always applied at a concentration of 500 mg/l.

Analysis of Cabbage Plant Volatiles Headspace analysis was conducted to investigate volatile emission by cabbage plants that were untreated (control) or treated with a solution of pure chemicals. Cabbage plants were removed from the pots, and care was taken to ensure that roots were not damaged; then, the entire soil and root system was covered in aluminum foil. Preliminary tests showed that this treatment had little effect on plant growth and volatile collection. A volume of 10 ml solution of either α-terpinene or *p*-cymene was applied to each of the clean, undamaged cabbage plants. Plants that served as controls were sprayed with 10 ml of distilled water (containing 2% acetone). The plants were left to dry for 1 h before being placed into the collection vessels.

Volatiles were collected in a system modified from Turlings et al. (1998). Air was first pushed through a glass bottle with water to be humidified, a flow meter to measure and regulate the air flow, and a charcoal filter to purify the air. The moist and purified air then entered a glass cylinder (16 cm diameter and 35 cm height) at 400 ml·min^{-1}. To create a laminar flow, the air was forced through a glass frit at the top of the cylinder. Approximately 4 cm above the bottom, there was a 25 mm vertical female ground–glass connector for a collection trap. The trap was a glass tube (10 cm long, 5 mm diameter) that contained 30 mg of 80/100 mesh Super-Q (Altech, USA). The air passing over the plant was pulled through Super-Q adsorbent and vented out. The air-inlet, air-outlet, filter, and sampling vessel were connected with Teflon tubing. Each collection session lasted for 24 h. After each collection session, each trap was rinsed with 1 ml methylene dichloride, and the solution was concentrated to 100 μl by a stream of pure nitrogen. Volatile samples of control and chemical-treated cabbage plants were refrigerated at −4°C until they were analyzed. Each treatment was replicated twice.

Compounds were analyzed by using HP6890N GC equipped with an HP-5 column (30 m×0.32 mm i.d., 0.25 μm film thickness) and a flame ionization detector. A volume of 1 μl of sample was injected in splitless mode with nitrogen (1.6 ml/min) as the carrier gas. The column temperature was programmed to maintain at 40°C for 4 min and then increase at 8°C/min to 220°C. The temperature of the injector slit was 250°C.

Experience Treatment Test cohorts of *P. xylostella* were reared on common cabbage from egg to pupation in a temperature-controlled room at 26±1°C, 60–70% RH and 14L/10D photoperiod. Pupae were collected 2–3 days after pupation, taken out of their cocoons, and thoroughly washed in distilled water using the procedure described by Liu and Liu (2006). The pupae were placed in a clean, ventilated cage (45×45×45 cm) for adult emergence and mating. Adults were provided with 15% honey-water as food upon emergence but were not exposed to any plant odors (away from the rearing room) until either being used as untreated controls or being exposed to various experience treatments at 2–3 days post-emergence.

To investigate the effects of experience on the olfactory and oviposition preference of *P. xylostella* females, we treated the test insects by using the following procedures:

(1) Experience with non-host plant: For this treatment, a group of individuals from the test cohorts were moved manually (with a fine soft brush) at the late fourth instar from common cabbage to *C. morifolium* to force them to pupate on the non-host plants. The insects were left to emerge naturally on the plants. Adults were provided with 15% honey-water and enclosed with the non-host plants in a cage until being used in the olfactometer bioassay at 2–3 days post-emergence. A group of untreated adults reared from common cabbage were bioassayed as the "No experience" (naive) control for this experience treatment.

(2) Experience with pure chemicals: We used a clear, cylindrical, and ventilated plastic container (14.5 cm in height, with a top of 9 cm diameter and a base of 13 cm diameter) as the arena for experience treatments. The inner surface of the container was sprayed evenly with 1 ml of a chemical solution. A piece of filter paper (9 cm diameter) was placed at the bottom of the container, and 2 ml of a chemical solution was applied evenly onto the filter paper with a small pipette. The container with the treated filter paper was dried for 1 h, and then four females were introduced to experience the chemical for 10 min before they were used immediately in olfactory or oviposition preference tests. "No experience" control females were handled in the same manner except that the arena for experience was treated with distilled water (containing 2% acetone) only.

(3) Experience with chemical-treated host plants: Prior to each test, a cabbage plant was sprayed with 10 ml of a chemical solution and placed in a ventilated cage (45×45×45 cm) to air dry for 1 h. A group of untreated females was then transferred from the emergence cage onto the treated plant in the cage to experience the treated plant for 30 min before they were used immediately in olfactory or oviposition preference tests. A group of untreated females was taken from the emergence cage to be bioassayed as "No experience" control adults.

Basic Procedure for Testing Olfactory Preference We used a Y-tube olfactometer to observe the olfactory responses of

P. xylostella females (for details of the olfactometer, see Zhang et al. 2007). The two arms were connected to two separate glass containers holding the odor sources. The size of the glass containers varied from 6.5 cm diameter×8.5 cm height for holding a piece of filter paper to 16×35 cm height for holding a plant. A flow meter was connected to each arm of the olfactometer. A pump was used to draw air over a charcoal filter and then through the olfactometer at a rate of 400 ml·min^{-1}. As moths were most active at night, we conducted all observations in a dark room (lit with only a 15-W red light) at 26±1°C between 1900 and 2300 hours.

For each replicate of a given treatment, four females were released into the stem of the olfactometer and left in the olfactometer to respond for 15 min. When a moth walked up to the upwind end of an olfactometer arm, either within 4 cm to the blocking screen or into the corresponding moth-trapping bulb at the end of 15 min, it was recorded as making a choice for that odor. When a moth did not reach the end of either arm within 15 min, it was recorded as "no response". Each moth was used only once. After running five replicates, the connections of the odor sources to the olfactometer arms were exchanged to remove any asymmetrical bias in the set-up. The olfactometer tube and glass container were thoroughly washed with alcohol at the end of the observations for a given treatment and dried overnight in an oven at 200°C.

Olfactory Response to the Non-host Plant C. morifolium Females of *P. xylostella* were taken haphazardly from the "Experience with non-host plant" and the corresponding "No experience" control treatments and observed by using the Y-tube olfactometer procedure described above. One arm of the Y-tube olfactometer was connected to a glass container that held a fresh *C. morifolium* plant and the other arm to an empty glass container. For each of the two treatments, we conducted 20 replicates to bioassay a total of 80 females. The test plants were replaced by new ones of the corresponding categories after every five replicates.

Olfactory Response to Pure Chemicals For bioassays of females with prior experience of either α-terpinene or *p*-cymene, 2 ml solution of a chemical were applied evenly onto a piece of filter paper (9 cm diameter) with a pipette. The treated filter paper was air-dried for 1 h. Another piece of filter paper was treated similarly with 2 ml of distilled water (containing 2% acetone) as the control. The treated and control pieces of paper were placed separately into the two odor source containers of the Y-tube olfactometer. Four females of a given treatment were then observed in the olfactometer by using the procedure described above. For each experience treatment or its corresponding "No experience" control, we conducted 10 or 15 replicates to bioassay a total of 40 or 60 females, and fresh odor sources (i.e., fresh treated and control pieces of papers) were used for each replicate.

Olfactory Response to Cabbage Plants Sprayed with a Pure Chemical Prior to each bioassay, one cabbage plant was sprayed with 10 ml solution of either α-terpinene or *p*-cymene, and another cabbage plant was sprayed with 10 ml of distilled water (containing 2% acetone) as the control. The treated and control plants were air-dried for 2 h and then placed separately into the two odor-source containers of the Y-tube olfactometer. Four females of a given treatment were then observed in the olfactometer by using the procedure described above. For each experience treatment or its corresponding "No experience" control, we conducted 10 replicates to bioassay a total of 40 females. The test plants were replaced by new ones of the corresponding categories after every five replicates.

Oviposition Preference A dual-choice bioassay was used to determine whether the responses of ovipositing females would change following prior experiences of *p*-cymene. Cabbage plants were first sprayed with a solution of *p*-cymene (see above), while the control plants were sprayed with distilled water (containing 2% acetone), 10 ml solution per plant. Sprayed plants were air-dried for 2 h. One treated plant and one control plant were placed in diagonally opposite corners of a cage (55×55×55 cm), and the position of the plants adjusted so that the foliage of the two plants was approximately 20 cm apart at the closest point. Three *P. xylostella* females were then released into the center of each cage between 1900 and 2000 hours. After 12 h in darkness at 26±1°C, the moths were removed, and the eggs deposited on each of the two plants were counted.

We tested the oviposition preference of females of each of the following three experience treatments towards control and *p*-cymene-treated cabbage plants: no experience, experience of *p*-cymene-treated filter paper, and experience of *p*-cymene-treated cabbage. We conducted 11–13 replicates (each in a separate cage) for each of the treatments.

Statistical Analysis For the olfactometer bioassays, the number of females choosing each of the two odor arms in each of the experience or "No experience" treatments was compared with a replicated *G* test of goodness-of-fit with the null hypothesis of no preference, and for the observations on each odor source, a *G* test of a 2×2 table of independence was used to compare the relative proportions of responding and non-responding females between naive and experienced treatments. For the oviposition preference tests, a replicated goodness-of-fit *G* test was used to compare the numbers of eggs deposited on each pair of plants in a given plant treatment with the null hypothesis of no preference, and a *G*

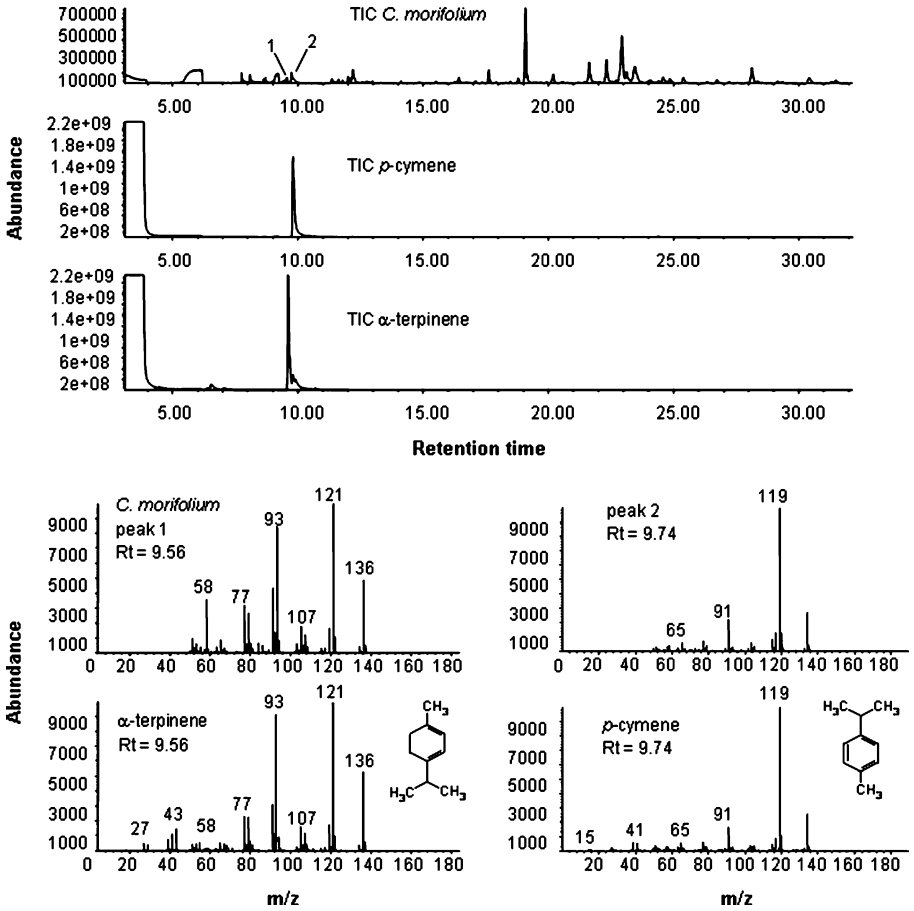

Fig. 1 GC-MS analysis of volatile compounds emitted from *C. morifolium* using authentic standards for comparison of retention times and mass spectra. Total ion current chromatogram of *C. morifolium* compared with that of two authentic standards. The mass spectra of peak 1 and peak 2, identified as α-terpinene and *p*-cymene, respectively, are shown together with that of the respective authentic standards

test of a 2×2 table of independence was used to compare the overall relative proportions of eggs laid on the control and treated host plants between females from any two experience treatments (Sokal and Rohlf 1995).

Results

Chemical Analysis of Plant Volatiles Identification of volatile compounds of *C. morifolium* (Fig. 1) was done by comparing GC-MS fragmentation patterns for the major peaks with authentic standards and NIST98 libraries. The mass spectra of peak 1 and peak 2 were identified as α-terpinene and *p*-cymene, respectively. The remaining peaks were not identified due to lack of authentic standards at the time.

Analysis of the volatiles released from control cabbage plants and cabbage plants sprayed with a solution of α-terpinene or *p*-cymene indicated that qualitative differences occurred in the volatiles collected following treatment with each of the two chemicals (Fig. 2). α-Terpinene- or *p*-cymene-treated cabbage plants emitted more volatiles in addition to the pure chemical applied to the plants compared to the volatile compounds from untreated plants.

Olfactory Response In the olfactometer choice tests with volatiles of "*C. morifolium* vs. air", a significantly lower proportion of females in the "No experience" (naive) control made their first choice towards the nonhost plant than air; in contrast, the proportions of females making their first choice towards *C. morifolium* or air did not differ significantly when the females had a prior experience of the nonhost plant (Table 1).

In the olfactometer choice tests with "α-terpinene vs. air", a significantly lower proportion of females in the naive

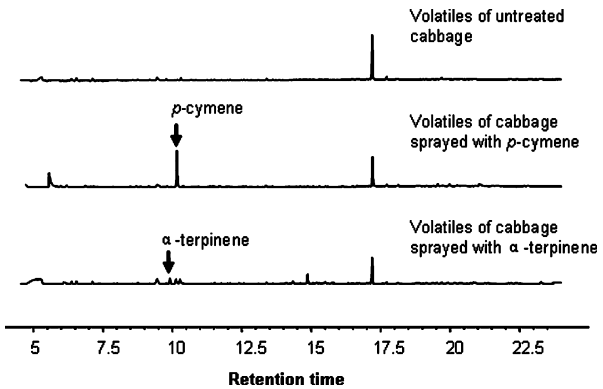

Fig. 2 GC-FID profiles of volatiles emitted from untreated cabbage plants compared with those of plants treated with *p*-cymene or α-terpinene solution

Table 1 Effect of prior experience of *C. morifolium* on choice responses of *P. xylostella* females to volatiles of "*C. morifolium* vs. air" in a Y-tube olfactometer

Experience of *C. morifolium* Plant	Number of Replicates[a]	Total Number of Choices to Volatiles		Mean % of Choices to Volatiles		Replicated *G* Test of Goodness-of-fit[b]	
		Air	*C. morifolium*	Air	*C. morifolium*	G_P	G_H
No	20	33	13	71.7	28.3	9.0*	26.0 NS
Yes	20	27	34	44.3	55.7	0.8 NS	27.6 NS

NS Non-significant
*$P<0.05$
[a] Four females per replicate, and non-responding females were excluded from the analysis.
[b] G_P *G*-pooled, $df=1$; G_H *G*-heterogeneity between replicates, $df=19$

control made their first choice towards α-terpinene; in contrast, a significantly higher proportion of females made their first choice towards α-terpinene when the females had a prior experience of the chemical (Table 2).

In the olfactometer choice tests that compared volatiles of "α-terpinene-treated cabbage vs. air", a significantly lower proportion of females in the naive control made their first choice towards α-terpinene-treated cabbage than air; in contrast, the proportions of females making their first choice towards α-terpinene-treated cabbage or air were similar when the females had a prior experience with α-terpinene-treated cabbage plants (Table 3).

In the olfactometer choice tests with "*p*-cymene vs. air", a significantly lower proportion of females in the naive control made their first choice towards *p*-cymene than air, whereas a significantly higher proportion of females made their first choice towards *p*-cymene than air when the females had a prior experience of the chemical (Table 4).

Similarly, for "*p*-cymene-treated cabbage vs. air", a significantly lower proportion of females in the naive control made their first choice towards *p*-cymene-treated cabbage than air; in contrast, a significantly higher proportion of females made their first choice towards *p*-cymene-treated cabbage than air when the females had a prior experience of *p*-cymene-treated cabbage plants (Table 5).

Prior experience reduced the relative proportion of non-responding females in all cases, although the reductions reached a significant level only in the treatments with two odor sources (Fig. 3).

Oviposition Preference In the choice tests that used *p*-cymene-treated and control cabbages, the numbers (mean± SE) of eggs laid per replicate (i.e., for three females) were 114.4±10.9, 85.0±12.5, and 79.3±14.2 for the naive females, females with prior experience of *p*-cymene, and females with prior experience of *p*-cymene-treated plants, respectively. These means did not differ significantly among the three groups of females (one-way analysis of variance, $F_{2,33}=2.74$, $P=0.079$). The naive females laid a significantly higher proportion of eggs (55.6%) on the control plants than on the *p*-cymene-treated plants (44.4%; $G_p=18.6$, $df=1$, $P<0.001$; $G_H=104.2$, $df=12$, $P<0.001$). In contrast, females with a prior experience of *p*-cymene deposited a significantly higher proportion of eggs (57.6%) on the *p*-cymene-treated plants than on the control plants (42.4%; $G_p=21.9$, $df=1$, $P<0.001$; $G_H=126.2$, $df=10$, $P<0.001$). However, the proportions of eggs laid on the *p*-cymene-treated and control plants (50.7% vs. 49.3%) did not differ significantly for females with prior experience of *p*-cymene-treated plants ($G_p=0.2$, $df=1$, $P=0.67$; $G_H=179.4$, $df=11$, $P<0.001$; Fig. 4). As indicated by the G_H

Table 2 Effect of prior experience of α-terpinene on choice responses of *P. xylostella* females to volatiles of "α-terpinene vs. air" in a Y-tube olfactometer

Experience of α-terpinene	Number of Replicates[a]	Total Number of Choices to Volatiles		Mean % of Choices to Volatiles		Replicated *G* Test of Goodness-of-fit[b]	
		Air	α-terpinene	Air	α-terpinene	G_P	G_H
No	15	29	15	65.9	34.1	4.5*	15.2 NS
Yes	15	12	35	25.5	74.5	11.6**	9.0 NS

NS Non-significant
*$P<0.05$, **$P<0.01$
[a] Four females per replicate, and non-responding females were excluded from the analysis.
[b] G_P *G*-pooled, $df=1$; G_H *G*-heterogeneity between replicates, $df=14$

Table 3 Effect of prior experience of α-terpinene-treated cabbage plant on choice responses of *P. xylostella* females to volatiles of "α-terpinene-treated cabbage vs. air" in a Y-tube olfactometer

Experience of α-terpinene- Treated Cabbage	Number of Replicates[a]	Total Number of Choices to Volatiles		Mean % of Choices to Volatiles		Replicated *G* Test of Goodness-of-fit[b]	
		Air	Cabbage	Air	Cabbage	G_P	G_H
No	10	20	3	87.0	13.0	14.1**	11.2 NS
Yes	10	11	14	44.0	56.0	0.4 NS	17.3*

NS Non-significant
*$P<0.05$, ** $P<0.01$
[a] Four females per replicate, and non-responding females were excluded from the analysis.
[b] G_P *G*-pooled, $df=1$; G_H *G*-heterogeneity between replicates, $df=9$

statistic, the variations among replicates were large in all three treatments.

Discussion

Odor compositions of host plants are taxon-specific, and the olfactory system of an insect has the capacity to distinguish these odors from others and use the plant chemical cues in host foraging (Schoonhoven et al. 2005). *C. morifolium* is a non-host plant of *P. xylostella* and produces many kinds of volatile compounds (Fig. 1). These volatile compounds can be obtained by various methods that involve extraction of the plant material. However, sampling air from around intact plants gives a more accurate picture of the volatile profile (Tholl et al. 2006). Our previous study showed that the extract of dried leaves of *C. morifolium* had a repellent effect on naive *P. xylostella* females, but experienced females were not repelled, and they were attracted instead by host plants treated with the non-host plant extract (Liu et al. 2005). In the present study, naive females of this insect showed an aversion to volatiles emitted from intact plants of *C. morifolium*, but females with a prior experience with the plant were not repelled by the plant volatiles (Table 1). In another study, *P. xylostella* females with a prior experience of pea, another non-host plant of the insect, showed preference for the non-host plant volatiles (Zhang et al. 2007). Thus, depending on the plant species, *P. xylostella* females can exhibit habituation or induced preference for volatiles emitted from non-host plants.

GC-MS analysis of volatile compound emitted from *C. morifolium* plants (by comparison with authentic standards) showed the presence of α-terpinene and *p*-cymene in the blend (Fig. 1). Naive females of *P. xylostella* showed aversion to both these volatiles at the concentration tested, but experienced females became attracted by them (Tables 2 and 4). Experience-altered responses towards volatiles of Chinese cabbage treated with each of the two compounds were similar to those towards the pure compounds, although the females with a prior experience of α-terpinene-treated plants only lost their aversion to the plant volatiles and did not become attracted by them (Tables 3 and 5). The somewhat different responses towards α-terpinene- and α-terpinene-treated plants were not surprising, as volatiles from α-terpinene-treated plants contained many more compounds in addition to α-terpinene (Fig. 2). The oviposition preference tests showed similar experience-altered behaviors to those revealed by the olfactory bio-assays. Naive females showed a preference for oviposition on untreated plants, whereas experienced females either showed a preference for oviposition on *p*-cymene-treated plants or were no longer repelled by the treated plants

Table 4 Effect of prior experience of *p*-cymene on choice responses of *P. xylostella* females to volatiles of "*p*-cymene vs. air" in a Y-tube olfactometer

Experience of *p*-cymene	Number of Replicates[a]	Total Number of Choices to Volatiles		Mean % of Choices to Volatiles		Replicated *G* Test of Goodness-of-fit	
		Air	*p*-cymene	Air	*p*-cymene	G_P	G_H
No	10	20	6	77.0	23.0	8.0*	16.0 NS
Yes	10	4	26	13.3	86.7	17.1**	12.3*

NS Non-significant
*$P<0.05$, ** $P<0.01$
[a] Four females per replicate, and non-responding females were excluded from the analysis.
[b] G_P *G*-pooled, $df=1$; G_H *G*-heterogeneity between replicates, $df=9$

Table 5 Effect of prior experience of *p*-cymene-treated cabbage on choice responses of *P. xylostella* females to volatiles of "*p*-cymene-treated cabbage vs. air" in a Y-tube olfactometer

Experience of *p*-cymene-Treated Cabbage	Number of Replicates[a]	Total Number of Choices to Volatiles		Mean % of Choices to Volatiles		Replicated *G* Test of Goodness-of-fit[b]	
		Air	Cabbage	Air	Cabbage	G_P	G_H
No	10	19	5	79.2	20.8	6.1*	13.0 NS
Yes	10	4	29	13.8	86.2	21.4**	13.3 NS

NS Non-significant
*$P<0.05$, ** $P<0.01$
[a] Four females per replicate, and non-responding females were excluded from the analysis.
[b] G_P *G*-pooled, $df=1$; G_H *G*-heterogeneity between replicates, $df=9$

(Fig. 4). The combined results of olfactory and oviposition bioassays demonstrate that experience-induced preference for previously repellent compounds is a major mechanism that leads to behavioral changes of ovipositing females towards non-host plants or their extracts and that increased acceptance of non-host plants or their extracts following experience does not have to involve initially attractive compounds (Liu et al. 2005). It is likely that similar behavioral changes towards repellents were involved in experience-induced preference for oviposition on substrates treated with neem-based products (Liu and Liu 2006).

Although experience-induced preference for repellents in ovipositing females of phytophagous insects has been demonstrated in only a few cases, it is likely a widespread phenomenon in view of the commonly observed evidence for habituation and induced preference to feeding deterrents in phytophagous larvae (Jermy 1987; Papaj and Prokopy 1989; Schoonhoven et al. 2005). In addition, experience-induced habituation and preference for repellents have been recorded in several saprophagous and blood-sucking dipterans (Jaenike 1982; McCall and Eaton 2001; Kaur et al. 2003). Experience-induced preference for repellents allows the insects to retain a degree of flexibility to more successfully deal with uncertainty in the environment, including ovipositing on poor host plants when highly suitable plants are not available (Stephens 1993). It may also play an important role in allowing insects to explore new host plants, a necessary precursor for the evolution of host range expansion (Janz et al. 2006; Zhang and Liu 2006; Zhang et al. 2007). For deployments of non-host plant-derived chemicals in behavioral manipulation of insect pests, effective strategies and techniques have to be developed to mitigate the decrease in responses to the repellents (Gould 1991; Akhtar and Isman 2003).

Charleston et al. (2006) found that cabbage plants treated with extract of the syringa tree *Melia azedarach* emitted large quantities of volatiles as well as some additional compounds that were not detected in either the syringa extract or the untreated plants, suggesting that the syringa extract induced the emission of cabbage volatiles. Such enhanced emission of volatiles caused by interactions between a plant and the extract of another plant was observed also in this study (Fig. 2). As the enhanced emission of volatiles by the treated plant may influence the

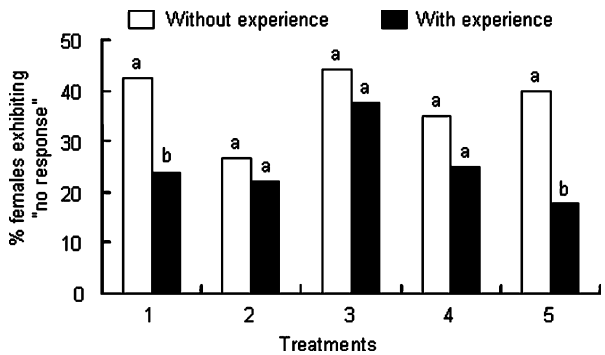

Fig. 3 Percentages of *P. xylostella* females without or with prior experience exhibiting "no response" in the Y-tube olfactometer in various treatments: *1* without or with prior experience of *C. morifolium* plant (see Table 1 for sample sizes); *2* without or with prior experience of α-terpinene-treated paper (see Table 2 for sample sizes); *3* without or with prior experience of α-terpinene-treated plant (see Table 3 for sample sizes); *4* without or with prior experience of *p*-cymene-treated paper (see Table 4 for sample sizes); *5* without or with prior experience of *p*-cymene-treated plant (see Table 5 for sample sizes). *Bars* in the same group with different letters differ significantly at $P=0.05$ (*G* test of a 2×2 table of independence)

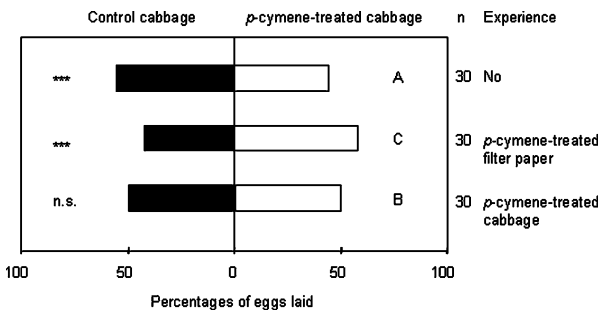

Fig. 4 Percentages of eggs laid on control and *p*-cymene-treated cabbage plants by *P. xylostella* females with various prior experiences in choice tests. *Asterisks on the left* indicate significant difference from no preference within a choice test (***$P<0.001$, *n.s.* not significant), and the *bars* of different treatments followed by *different letters on the right* indicate significant difference ($P<0.01$) between them

behavior of the herbivores as well as their natural enemies (Charleston et al. 2005, 2006), this kind of response has obvious implications in the deployment of non-host plant chemicals for behavioral manipulation of insect pests (Gould 1991; Isman 2006; Liu et al. 2006).

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References

AKHTAR, Y., and ISMAN, M. B. 2003. Binary mixtures of feeding deterrents mitigate the decrease in feeding deterrent response to antifeedants following prolonged exposure in the cabbage looper, *Trichoplusia ni* (Lepidoptera: Noctuidae). *Chemoecology* 13:177–182.

BENGTSSON, M., JAASTAD, G., KNUDSEN, G., KOBRO, S., BACKMAN, A. C., PETTERSSON, E., and WITZGALL, P. 2006. Plant volatiles mediate attraction to host and non-host plant in apple fruit moth, *Argyresthia conjugella*. *Entomol. Exp. Appl.* 118:77–85.

CHARLESTON, D. S., KFIR, R., VET, L. E. M., and DICKE, M. 2005. Behavioural responses of diamondback moth *Plutella xylostella* (Lepidoptera: Plutellidae) to extracts derived from *Melia azedarach* and *Azadirachta indica*. *Bull. Entomol. Res.* 95:457–465.

CHARLESTON, D. S., GOLS, R., HORDIJK, K. A., KFIR, R., VET, L. E. M., and DICKE, M. 2006. Impact of botanical pesticides derived from *Melia azedarach* and *Azadirachta indica* plants on the emission of volatiles that attract parasitoids of the diamondback moth to cabbage plants. *J. Chem. Ecol.* 32:325–349.

CUNNINGHAM, J. P., WEST, S. A., and WRIGHT, D. J. 1998a. Learning in the nectar foraging behaviour of *Helicoverpa armigera*. *Ecol. Entomol.* 23:363–369.

CUNNINGHAM, J. P., JALLOW, M. F. A., WRIGHT, D. J., and ZALUCKI, M. P. 1998b. Learning in host selection in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae). *Anim. Behav.* 55:227–234.

CUNNINGHAM, J. P., ZALUCKI, M. P., and WEST, S. A. 1999. Learning in *Helicoverpa armigera* (Lepidoptera: Noctuidae): a new look at the behavior and control of a polyphagous pest. *Bull. Entomol, Res.* 89:201–207.

CUNNINGHAM, J. P., WEST, S. A., and ZALUCKI, M. P. 2001. Host selection in phytophagous insects: a new explanation for learning in adults. *Oikos* 95:537–543.

CUNNINGHAM, J. P., MOORE, C. J., ZALUCKI, M. P., and WEST, S. A. 2004. Learning, odor preference and flower foraging in moths. *J. Exp. Biol.* 207:87–94.

DEL CAMPO, M. L., MILES, C. I., SCHROEDER, F. C., MUELLER, C., BOOKER, R., and RENWICK, J. A. 2001. Host recognition by the tobacco hornworm is mediated by a host plant compound. *Nature* 411:186–189.

GOULD, F. 1991. Arthropod behavior and the efficacy of plant protectants. *Annu. Rev. Entomol.* 36:305–330.

HELD, D. W., EATON, T., and POTTER, D. A. 2001. Potential for habituation to a neem-based feeding deterrent in Japanese beetles, *Popillia japonica*. *Entomol. Exp. Appl.* 101:25–32.

ISMAN, M. B. 2006. Botanical insecticides, deterrents, and repellents in modern agriculture and an increasingly regulated world. *Annu. Rev. Entomol.* 51:45–66.

JAENIKE, J. 1982. Environmental modification of oviposition behavior in Drosophila. *Am. Nat.* 119:784–802.

JALLOW, M. F. A., CUNNINGHAM, J. P., and ZALUCKI, M. P. 2004. Intra-specific variation for host plant use in *Helicoverpa armigera* (Hübner) (Lepidoptera: Noctuidae): implications for management. *Crop Prot.* 23:955–964.

JANZ, N., NYLIN, S., and WAHLBERG, N. 2006. Diversity begets diversity: host expansions and the diversification of plant-feeding insects. *BMC Evol. Biol.* 6:4 DOI 10.1186/1471-2148-6-4.

JERMY, T. 1987. The role of experience in the host selection of phytophagous insect, pp. 143–157, in R. F. Chapman, E. A. Bernays, and J. G. Stoffolano (eds.). Perspectives in Chemoreception and BehaviorSpringer-Verlag, New York.

KAUR, J. S., LAI, Y. L., and GIGER, A. D. 2003. Learning and memory in the mosquito *Aedes aegypti* shown by conditioning against oviposition deterrence. *Med. Vet. Entomol.* 17:457–460.

LANDOLT, P. J., and MOLINA, O. 1996. Host-finding by cabbage looper moth (Lepidoptera: Noctuidae): learning of host odor upon contact with host foliage. *J. Insect Behav.* 9:899–908.

LIU, T. X., and LIU, S. S. 2006. Experience-altered oviposition responses to a neem-based product, Neemix®, by the diamondback moth, *Plutella xylostella*. *Pest Manag. Sci.* 62:38–45.

LIU, S. S., LI, Y. H., LIU, Y. Q., and ZALUCKI, M. P. 2005. Experience-induced preference for oviposition repellents derived from a non-host plant by a specialist herbivore. *Ecol. Lett.* 8:722–729.

LIU, S. S., LI, Y. H., and LOU, Y. G. 2006. Non-host plant extracts reduce oviposition of *Plutella xylostella* (Lepidoptera: Plutellidae) and enhance parasitism by its parasitoid *Cotesia plutellae* (Hymenoptera: Braconidae). *Bull. Entomol. Res.* 96:373–378.

MCCALL, P. J., and EATON, G. 2001. Olfactory memory in the mosquito *Culex quinquefasciatus*. *Med Vet. Entomol.* 15:197–203.

PAPAJ, D. R., and PROKOPY, R. J. 1989. Ecological and evolutionary aspects of learning in phytophagous insects. *Annu. Rev. Entomol.* 34:315–359.

ROJAS, J. C., and WYATT, T. D. 1999. The role of pre- and post-imaginal experience in the host-finding and oviposition behavior of the cabbage moth. *Physiol. Entomol.* 24:83–89.

SCHOONHOVEN, L. M., VAN LOON, J. J. A., and DICKE, M. 2005. Insect–Pant Biology. 2nd edn.Oxford University Press, New York.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry: The Principle and Practice of Statistics, in Biological Research. 3rd edn.W. H. Freeman and Company, New York.

STANTON, M. L. 1984. Short-term learning and the searching accuracy of egg-laying butterflies. *Anim. Behav.* 32:33–40.

STEPHENS, D. W. 1993. Learning and behavioral ecology: incomplete information and environmental predictability, pp. 195–218, in D. R. Papaj, and A. C. Lewis (eds.). Insect Learning: Ecological and Evolutionary Aspects. Chapman & Hall, New York.

THOLL, D., BOLAND, W., HANSEL, A., LORETO, F., RÖSE, U. S. R., and SCHNITZLER, J.-P. 2006. Practical approaches to plant volatile analysis. *Plant J.* 45:540–560.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta* 207:146–152.

ZHANG, P. J., and LIU, S. S. 2006. Experience induces a phytophagous insect to lay eggs on a nonhost plant. *J. Chem. Ecol.* 32:745–753.

ZHANG, P. J., LIU, S. S., WANG, H., and ZALUCKI, M. P. 2007. The influence of early adult experience and larval food restriction on responses towards non-host plants in moths. *J. Chem. Ecol.* 33:1528–1541.

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Structure–Activity Relationships of Phenylpropanoids as Antifeedants for the Pine Weevil *Hylobius abietis*

B. Bohman · G. Nordlander · H. Nordenhem · K. Sunnerheim · A.-K. Borg-Karlson · C. R. Unelius

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Abstract Ethyl cinnamate has been isolated from the bark of *Pinus contorta* in the search for antifeedants for the pine weevil, *Hylobius abietis*. Based on this lead compound, a number of structurally related compounds were synthesized and tested. The usability of the Topliss scheme, a flow diagram previously used in numerous structure–activity relationship (SAR) studies, was evaluated in an attempt to find the most potent antifeedants. The scheme was initially followed stepwise; subsequently, all compounds found in the scheme were compared. In total, 51 phenylpropanoids were tested and analyzed for SARs by using arguments from the field of medicinal chemistry (rational drug design). Individual Hansch parameters based on hydrophobicity, steric, and electronic properties were examined. The effects of position and numbers of substituents on the aromatic ring, the effects of conjugation in the molecules, and the effects of the properties of the parent alcohol part of the esters were also evaluated. It proved difficult to find strong SARs derived from single physicochemical descriptors, but our study led to numerous new, potent, phenylpropanoid antifeedants for the pine weevil. Among the most potent were methyl 3-phenylpropanoates monosubstituted with chloro, fluoro, or methyl groups and the 3,4-dichlorinated methyl 3-phenylpropanoate.

Keywords Antifeedant · Pine weevil · *Hylobius abietis* · Structure–activity · SAR · Phenylpropanoid · Phenylpropanoate · Phenylacrylate

B. Bohman · C. R. Unelius (✉)
School of Pure and Applied Natural Sciences,
University of Kalmar,
SE-391 82 Kalmar, Sweden
e-mail: rikard.unelius@hik.se

G. Nordlander · H. Nordenhem
Department of Ecology,
Swedish University of Agricultural Sciences,
P.O. Box 7044, SE-750 07 Uppsala, Sweden

K. Sunnerheim
Department of Natural Sciences, Mid Sweden University,
SE-851 70 Sundsvall, Sweden

A.-K. Borg-Karlson
KTH Chemistry, Organic Chemistry,
Ecological Chemistry Group, Royal Institute of Technology,
SE-100 44 Stockholm, Sweden

Introduction

The pine weevil *Hylobius abietis* (L.) is a severe pest in areas of Europe where clear-cutting of conifer forests with subsequent replanting is practiced (Långström and Day 2004) because newly planted seedlings are frequently killed by the feeding of adult weevils on the stem bark (Day et al. 2004). There is a strong demand in Sweden and other European countries for new methods that prevent this pest from damaging forest plantations without the use of pesticides (Långström and Day 2004). Pine weevils walking on the ground locate the seedlings by responding to both olfactory and visual stimuli (Björklund et al. 2005).

Previously, it has been shown that lodgepole pine, *Pinus contorta* Douglas ex Loudon, is not as severely attacked as Scots pine, *P. sylvestris* L. (Bratt et al. 2001). This observation led to the hypothesis that the bark of *P. contorta* contains secondary metabolites that act as natural antifeedants against *H. abietis*. This was confirmed in laboratory bioassays where bark extracts from *P. contorta* deterred feeding more than bark extracts from *P. sylvestris*. Bioassay-guided fractionation of *P. contorta* bark led to the

isolation and identification of two compounds, ethyl cinnamate and ethyl 2,3-dibromo-3-phenylpropanoate, which possessed antifeedant activity (Bratt et al. 2001). Ethyl cinnamate and structurally related esters are more potent antifeedants than the corresponding carboxylic acids (Sunnerheim et al. 2007). The results of an investigation of benzoate derivatives as antifeedants for *H. abietis* indicated that even small changes in the structure of a derivative might induce dramatic effects on the antifeedant activity (Unelius et al. 2006). Therefore, it is important to understand the relation between the substitution pattern of the aromatic ring and also the antifeedant effect in phenylpropanoids.

When looking for new bioactive compounds, it might not be feasible to test a large number of structures to find the most potent one. Under these circumstances, it is beneficial to test compounds for activity as they are synthesized and to use the results to select the next analog to be tested. These ideas are applied in the Topliss approach (Topliss 1972), which we used as a tool in search for the phenylpropanoid with the highest antifeedant effect. A Topliss scheme is a flow diagram designed such that the optimal substituent can be found expediently (Topliss 1977), and it gives recommendations on how to proceed after each compound has been tested. The substituents in the Topliss scheme have been chosen based on physico-chemical properties in combination with ease of synthesis. The test approach drawn up by Topliss is a modified and simplified quantitative SAR (QSAR) method developed from the Hansch method for structure–activity correlations. In the Hansch method, mathematical functions are used to correlate biological activity to chemical structure. For instance, the biological activities of a number of substituted aromatic compounds on bacteria, insects, and mammals have been examined and correlated to the properties of the substituents (Hansch and Fujita 1963). Depending on the structural configurations of the receptor active sites, different factors may affect efficiency. In this study, the relationships between individual Hansch parameters and potency of antifeedants against the pine weevil are presented.

To further explore the effects of structural changes in the aromatic rings of compounds such as phenylpropanoids, the number of substituents and the substitution pattern on the ring can be examined and evaluated in relation to activity, i.e., antifeedant efficacy. Certain positions are expected to be beneficial for a strong interaction to a target receptor. In addition to monosubstituted analogs, compounds with two or three substituents can also be tested and evaluated. As biological activity is potentially dependent upon the electronic properties of the entire molecule, it is important to investigate whether acrylates or propanoates are the superior antifeedants. The choice of parent alcohol for the ester is also likely to be important (Unelius et al. 2006).

The overall goal of the present study was to find new effective antifeedants related to ethyl cinnamate that could be used for conifer seedling protection. Throughout this process, we wanted to evaluate the Topliss approach for finding more potent antifeedants by using a low number of syntheses and biological tests. Individual Hansch parameters were also evaluated, as were the number, pattern, and properties of substituents on the aromatic ring. Furthermore, a number of 3-phenylpropanoates and 3-phenylacrylates were compared in search of a potential effect of conjugation. In addition, methyl, ethyl, propyl, and butyl esters of selected 3-phenylpropanoic and 3-phenylacrylic acids were included to determine the importance of the parent alcohol part of the esters. Finally, we compared the results from structural alterations on the antifeedant activity of the phenylpropanoids with the results from a similar previous study on benzoic acid derivatives (Unelius et al. 2006) in search for similarities and differences in selectivity.

Methods and Materials

Collection and maintenance of weevils Both sexes of *H. abietis* were collected during spring migration at a saw mill in southern Sweden where they landed in large numbers. After collection, weevils were stored in darkness at 10°C and provided with fresh branches of Scots pine, *Pinus sylvestris*, as food. These storage conditions interrupted the reproductive development so that females did not begin ovipositing until about a week after they had been transferred to the experimental conditions, i.e., a light regime of L18/D6 at 22°C. This transfer was made about 10 d before the insects were used in the following bioassay.

Laboratory bioassay Compounds were tested for their antifeedant effect on *H. abietis* by means of a two-choice laboratory bioassay (Bratt et al. 2001; Legrand et al. 2004; Borg-Karlson et al. 2006; Unelius et al. 2006). For each test, 40 pine weevils (20 females+20 males) were starved for 24 hr before the test period. Each weevil was placed in a Petri dish (142 mm diameter) and provided with a Scots pine twig (prepared as described below) placed on a moistened filter paper. One day before the test, the twigs were wrapped in aluminum foil, and two holes (diameter 5 mm; 25 mm apart) were punched in the foil with sharp-edged metal rings. After the removal of the aluminum foil inside the rings, one of the two areas was treated with 100 μl of a 50- or 5-mM methyl acetate solution of the compound to be tested, and the other was treated with the same amount of pure solvent as control. When the solvent had evaporated on the following day, the metal rings were removed and the bioassay started. After 24 hr, the proportion of treated bark and control area of each test

twig that had been consumed was recorded. There was generally no significant difference in response between the sexes, and the data presented have been pooled.

The effects of the various treatments are described by two alternative antifeedant indices (AFI) (Blaney et al. 1984) with the general formula: $100\times(C-T)/(C+T)$. The two variants are: (1) antifeedant index, area (AFIa) where C represents the mean area of the control surfaces consumed and T represents the mean area of the treated surfaces consumed and (2) antifeedant index, number (AFIn) where C represents the number of the control surfaces with feeding scars and T represents the number of the treated surfaces with feeding scars.

Thus, AFIa is a measure that captures the reduction in feeding, whereas AFIn is a measure of the frequency of complete inhibition of the initiation of feeding. The two indices were fairly well correlated, but AFIa tended to be higher than AFIn because the antifeedant substances generally affected both the initiation of feeding and the amount of bark consumed if feeding commenced. For both indices, positive values (up to a maximum of 100) reflect an antifeedant effect, whereas negative ones (down to a minimum of −100) indicate a stimulant effect.

Test chemicals The origins of the compounds tested are given in Tables 1, 2, and 3. Final purities ranged from 96% to 99%. When necessary, compounds were purified by preparative chromatography (Baeckström et al. 1987) or flash chromatography on silica gel (Merck 60, 0.040–0.063 mm, Darmstadt, Germany). The tested esters were synthesized by methods A–F (see "Synthesis" below), purchased from Sigma-Aldrich Sweden AB, Sweden or obtained from previous work by H. Erdtman and T. Norin at KTH Chemistry, Organic Chemistry, Stockholm. All cinnamic acid derivatives had the (*E*)-configuration.

Synthesis Commercial phenylpropanoic and phenylacrylic acids were either esterified by using methods A or B or the esters were formed according to methods C–F.

Method A: Acid-catalyzed esterification from corresponding commercial phenylpropanoic or -phenylacrylic acids. A typical procedure is given for the synthesis of methyl 3-(4-methylphenyl)acrylate: 3-(4-methylphenyl)acrylic acid (1.2 mmol) was dissolved in methanol (11 ml). Concentrated sulfuric acid (three drops) was added. The mixture was refluxed for 4 hr, concentrated with a rotary evaporator, diluted with water, and extracted with ethyl ether. The ether phase was washed with Na_2CO_3 (aq), dried over $MgSO_4$, and evaporated to give white crystals (1.15 mmol, 93%).

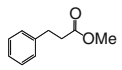
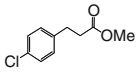
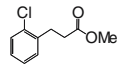
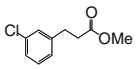
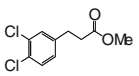
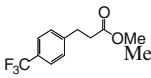
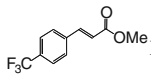
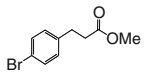
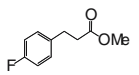
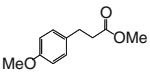
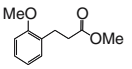
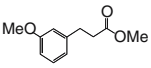
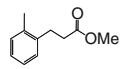
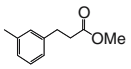
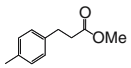
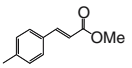
Method B: Preparation of esters by reaction of the carboxylic acid with dicyclohexylcarbodiimide (DCC), 4-methylaminopyridin (DMAP), and methanol in CH_2Cl_2. A typical example is the synthesis of methyl-3-(4-(dimethylamino)phenyl)acrylate: 3-(4-(dimethylamino)phenyl) acrylic acid (3.15 mmol) and DCC (3.67 mmol) were dissolved in methanol (35 ml). A solution of DMAP (1.23 mmol) in CH_2Cl_2 (5 ml) was added, and the reaction mixture was refluxed for 2 hr, concentrated with a rotary evaporator, dissolved in CH_2Cl_2, filtered, washed with water, and dried over $MgSO_4$. Evaporation of the solvent and purification by silica gel chromatography yielded the product (2.44 mmol, 77%) as yellow crystals.

Method C: Preparation of propanoates by catalytic hydrogenation of acrylate analogs (Vogel 1989). A typical example is the formation of methyl 3-(4-(dimethylamino)phenyl)propanoate: Methyl 3-(4-(dimethylamino)phenyl)acrylate (1.32 mmol) was dissolved in methanol (30 ml). To the solution, 39 mg Pd/C (10%) were added, and the reaction mixture was set under an atmosphere of hydrogen. When the reaction was completed after 45 min, the catalyst was filtered off, and the solvent evaporated. Methyl 3-(4-(dimethylamino) phenyl)propanoate was obtained (0.83 mmol, 63%) as slightly yellow crystals.

Method D: Preparation of propanoates by *O*-alkylation of hydroxyl-substituted analogs (Legrand et al. 2004): Isopropyl 3-(4-methoxyphenyl)propanoate was obtained by reacting isopropyl 3-(4-hydroxyphenyl)propanoate with methyl iodide and potassium carbonate in acetone. Methyl 3-(4-butoxyphenyl)propanoate was obtained by reacting methyl 3-(4-hydroxyphenyl)propanoate with potassium hydroxide and butyl iodide. Methyl 3-(3,4-dimethoxyphenyl)propanoate was obtained by reacting methyl 3-(3,4-dihydroxyphenyl)propanoate with sodium hydride and methyl iodide in THF according to the standard procedure (Vogel 1989).

Method E: Preparation of 3-phenylacrylates by Knoevenagel reaction (Harwood et al. 1998) followed by acid-catalyzed esterification. A typical example is the synthesis of methyl 3-(4-ethylphenyl)acrylate: Malonic acid (10.4 mmol) was dissolved in pyridine (3 ml) with heating. 4-Ethylbenzaldehyde (10.2 mmol) and a catalytic amount of piperidine (10 drops) were added while stirring. The reaction mixture was refluxed until the production of carbon dioxide

Table 1 Structures of mono-substituted phenylpropanoids and physical properties - Hansch parameter values for substituents on the aromatic rings (Hansch et al. 1973; Topliss 1972; Swain and Lupton 1968)

Entry	Structure	Name of compound	σ	π	F	R	MR	MW
1		Methyl 3-phenylpropanoate [a]	0	0	0	0	0	1
2		Methyl 3-(4-chlorophenyl)propanoate [a]	0.23	0.71	0.41	-0.15	6.03	35
3		Methyl 3-(2-chlorophenyl)propanoate [a]	N/A	0.71	0.41	-0.15	6.03	35
4		Methyl 3-(3-chlorophenyl)propanoate [a]	0.37	0.71	0.41	-0.15	6.03	35
5		Methyl 3-(3,4-dichlorophenyl)propanoate [a]	0.60	1.42	0.82	-0.30	12.1	71
6		Methyl 3-(4-trifluoromethylphenyl)propanoate [b]	0.54	0.88	0.38	0.19	5.02	69
7		Methyl 3-(4-trifluoromethylphenyl)acrylate [a]	0.54	0.88	0.38	0.19	5.02	69
8		Methyl 3-(4-bromophenyl)propanoate [a]	0.23	0.86	0.44	-0.17	8.88	80
9		Methyl 3-(4-fluorophenyl)propanoate [a]	0.06	0.14	0.43	-0.34	0.92	19
10		Methyl 3-(4-methoxyphenyl)propanoate [c]	-0.27	-0.02	0.26	-0.51	7.87	31
11		Methyl 3-(2-methoxyphenyl)propanoate [a]	N/A	-0.02	0.26	-0.51	7.87	31
12		Methyl 3-(3-methoxyphenyl)propanoate [a]	0.12	-0.02	0.26	-0.51	7.87	31
13		Methyl 3-(2-methylphenyl)propanoate [a]	N/A	0.56	-0.04	-0.13	5.65	15
14		Methyl 3-(3-methylphenyl)propanoate [a]	-0.07	0.56	-0.04	-0.13	5.65	15
15		Methyl 3-(4-methylphenyl)propanoate [a]	-0.17	0.56	-0.04	-0.13	5.65	15
16		Methyl 3-(4-methylphenyl)acrylate [a]	-0.17	0.56	-0.04	-0.13	5.65	15

[a] prepared from the corresponding carboxylic acids by refluxing in the alcohol with H_2SO_4 as a catalyst: Method A

[b] prepared by catalytic hydrogenation of acrylates: Method C.

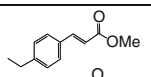
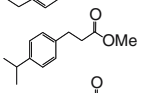
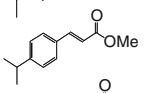
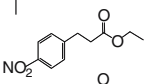
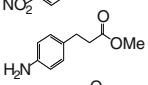
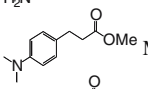
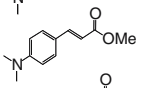
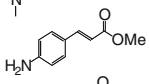
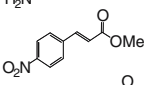
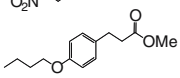
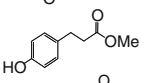
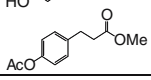
[c] obtained from previous work by H. Erdtman and T. Norin at the Dep. of Organic Chemistry, KTH, Stockholm.

abated. HCl (aq, 2 M, 10 ml) was added, and the precipitate formed was filtered off by suction and washed with HCl (aq, 2 M, 10 ml), water (10 ml), and hexane (10 ml) and dried *in vacuo* to yield methyl 3-(4-ethylphenyl)acrylic acid (7.8 mmol, 76%). The carboxylic acid was esterified according to method A.

Method F: Methyl 3-(4-acetyloxyphenyl)propanoate was prepared from methyl 3-(4-hydroxyphenyl) propanoate according to the standard procedure (Vogel 1989).

All reactions were monitored by thin layer chromatography. The spectroscopic data of the products were analyzed and compared with literature data.

Table 1 (continued)

Entry	Structural formula	Compound	σ	π	F	R	MR	MW
17		Methyl 3-(4-ethylphenyl)acrylate [d]	-0.15	1.02	-0.05	-0.10	10.3	29
18		Methyl 3-(4-isopropylphenyl)propanoate [b]	-0.15	1.53	-0.05	-0.10	15.0	43
19		Methyl 3-(4-isopropylphenyl)acrylate [b]	-0.15	1.53	-0.05	-0.10	15.0	43
20		Ethyl 3-(4-nitrophenyl)propanoate [b]	0.78	-0.28	0.67	0.16	7.36	46
21		Methyl 3-(4-aminophenyl)propanoate [e]	-0,66	-1,23	0	-0.68	5.42	16
22		Methyl 3-(4-dimethylaminophenyl)propanoate [e]	-0.83	0.18	0.10	-0.92	15.6	44
23		Methyl 3-(4-dimethylaminophenyl)acrylate [f]	-0.83	0.18	0.10	-0.92	15.6	44
24		Methyl 3-(4-aminophenyl)acrylate [b]	-0,66	-1,23	0	-0.68	5.42	16
25		Methyl 3-(4-nitrophenyl)acrylate [b]	0.78	-0.28	0.67	0.16	7.36	46
26		Methyl 3-(4-butoxyphenyl)propanoate [g]	-0.32	1.55	0.25	-0.55	21.7	73
27		Methyl 3-(4-hydroxyphenyl)propanoate [h]	-0.37	-0.67	0.29	-0.64	2.85	17
28		Methyl 3-(4-acetyloxyphenyl)propanoate [i]	0.31	-0.64	0.41	-0.07	12.5	59

[d] prepared via a Knoevenagel condensation: Method E.
[e] prepared by catalytic hydrogenation of cinnamates: Method C.
[f] prepared from the carboxylic acid by reaction with DCC, DMAP and methanol in CH_2Cl_2: Method B.
[g] prepared by O-alkylation of hydroxy-substituted analogs: Method D.
[h] purchased from SigmaAldrich Co, Sweden.
[i] prepared by O-acetylation of hydroxy-substituted analogs: Method F.

Spectroscopy H NMR (400 or 250 MHz) and C NMR (100 or 63 MHz) spectra were recorded on Varian 400, Bruker 400, or Bruker 250 instruments using the solvent signals, $CDCl_3$ or CD_3OD, as internal standards.

The Topliss scheme The first substance in the Topliss scheme possesses an unsubstituted aromatic ring (or a phenylic compound with one or more substituents not being changed over the time of the study) (Topliss 1972). In the modified Topliss scheme used, we started with methyl 3-phenylpropanoate (Fig. 1). The analogs were tested at 50 mM concentration in methyl acetate with the solvent as control treatment. As the AFI values for many substances were close to 100 at 50 mM, new tests were conducted at 5 mM concentration. The Topliss scheme was used continuously in the dynamic experimental plan, i.e., after the synthesis of each compound and after the evaluation of the test results from bioassays, the scheme was consulted for the choice of compound next to be synthesized and tested. Finally, analogs from all positions of the scheme were synthesized and tested to critically evaluate the method.

Hansch parameters The Hansch equation (Eq. 1) contains a number of parameters that reflect different properties of substituents on aromatic rings (Hansch and Deutsch 1966), such as hydrophobicity (π), electronic effects (σ, F, and R) and steric factors (molar refractivity [MR] and molecular weight [MW]). The relation between steric parameters can be found in the Lorentz–Lorentz equation (Eq. 2). One expression for the relationship between electronic effects is also shown (Eq. 3).

$$\mathrm{Log}(1/C) = k_1(\log\pi)^2 + k_2\log\pi + k_3\sigma + k_4\mathrm{MR} + k_5 \quad (1)$$

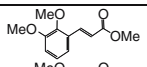
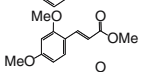
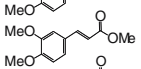
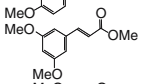
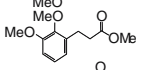
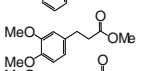
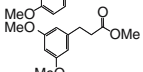
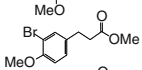
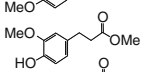
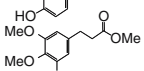
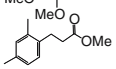
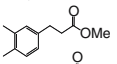
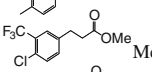
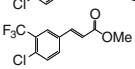
$$\mathrm{MR} = \mathrm{MW}\left(n^2-1\right)/d\left(n^2+2\right)\left[\mathrm{cm}^3/\mathrm{mol}\right] \quad (2)$$

$$R = \sigma - k_6F \quad (3)$$

C is the concentration required for biological activity, k_1–k_6 are the system-dependent constants, n is the refraction index, and d is the density.

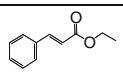
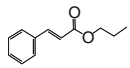
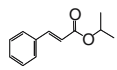
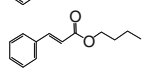
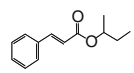
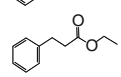
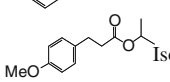
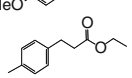
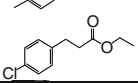
In this study, the hydrophobic parameter used was π, derived from log P values (Hansch et al. 1973). Three

Table 2 Structures of disubstituted and trisubstituted phenylpropanoids and physical properties - Hansch parameter values for substituents on the aromatic rings (Hansch et al. 1973; Topliss 1972; Swain and Lupton 1968)

Entry	Structural formula	Compound	σ	π	F	R	MR	MW
29		Methyl 3-(2,3-dimethoxyphenyl)acrylate [a]	N/A	-0.04	0.52	-1.02	15.7	62
30		Methyl 3-(2,4-dimethoxyphenyl)acrylate [a]	N/A	-0.04	0.52	-1.02	15.7	62
31		Methyl 3-(3,4-dimethoxyphenyl)acrylate [a]	-0.15	-0.04	0.52	-1.02	15.7	62
32		Methyl 3-(3,5-dimethoxyphenyl)acrylate [a]	0.24	-0.04	0.52	-1.02	15.7	62
33		Methyl 3-(2,3-dimethoxyphenyl)propanoate [b]	N/A	-0.04	0.52	-1.02	15.7	62
34		Methyl 3-(3,4-dimethoxyphenyl)propanoate [c]	-0.15	-0.04	0.52	-1.02	15.7	62
35		Methyl 3-(3,5-dimethoxyphenyl)propanoate [b]	0.24	-0.04	0.52	-1.02	15.7	62
36		Methyl (3-bromo-4-methoxyphenyl)propanoate [a]	0.12	0.84	0.70	-0.68	16.8	111
37		Methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate [a]	-0.25	-0.69	0.55	-1.15	10.7	48
38		Methyl 3-(3,4,5-trimethoxyphenyl)propanoate [a]	-0.03	-0.06	0.78	-1.53	23.6	93
39		Methyl 3-(2,4-dimethylphenyl)propanoate [a]	N/A	1.12	-0.08	-0.26	11.3	30
40		Methyl 3-(3,4-dimethylphenyl)propanoate [a]	-0.24	1.12	-0.08	-0.26	11.3	30
41		Methyl 3-(4-chloro-3-trifluoromethylphenyl)propanoate [b]	0.66	1.59	0.79	0.04	11.0	104
42		Methyl 3-(4-chloro-3-trifluoromethylphenyl)acrylate [a]	0.66	1.59	0.79	0.04	11.0	104

[a] prepared from the corresponding carboxylic acids by refluxing in the alcohol with H2SO4 as a catalyst: Method A.
[b] prepared by catalytic hydrogenation of cinnamates: Method C.
[c] prepared by O-alkylation of hydroxy-substituted analogs: Method D.

Table 3 Structures of phenylpropanoids (variations in the parent alcohol parts) and physical properties - Hansch parameter values for substituents on the aromatic rings (Hansch et al. 1973; Topliss 1972; Swain and Lupton 1968)

Entry	Structural formula	Compound	σ	π	F	R	MR	MW
43		Ethyl cinnamate [a]	0	0	0	0	0	1
44		Propyl cinnamate [a]	0	0	0	0	0	1
45		Isopropyl cinnamate [a]	0	0	0	0	0	1
46		Butyl cinnamate [a]	0	0	0	0	0	1
47		2-Butyl cinnamate [a]	0	0	0	0	0	1
48		Ethyl 3-phenylpropanoate [a]	0	0	0	0	0	1
49		Isopropyl 3-(4-methoxyphenyl)propanoate [b]	-0.27	-0.02	0.26	-0.51	7.87	31
50		Ethyl 3-(4-methylphenyl)propanoate [a]	-0.17	0.56	-0.04	-0.13	5.65	15
51		Ethyl 3-(4-chlorophenyl)propanoate [a]	0.23	0.71	0.41	-0.15	6.03	35

[a] prepared from the corresponding carboxylic acids by refluxing in the alcohol with H2SO4 (catalyst): Method A.
[b] prepared by O-alkylation of hydroxyl-substituted analogs: Method D.

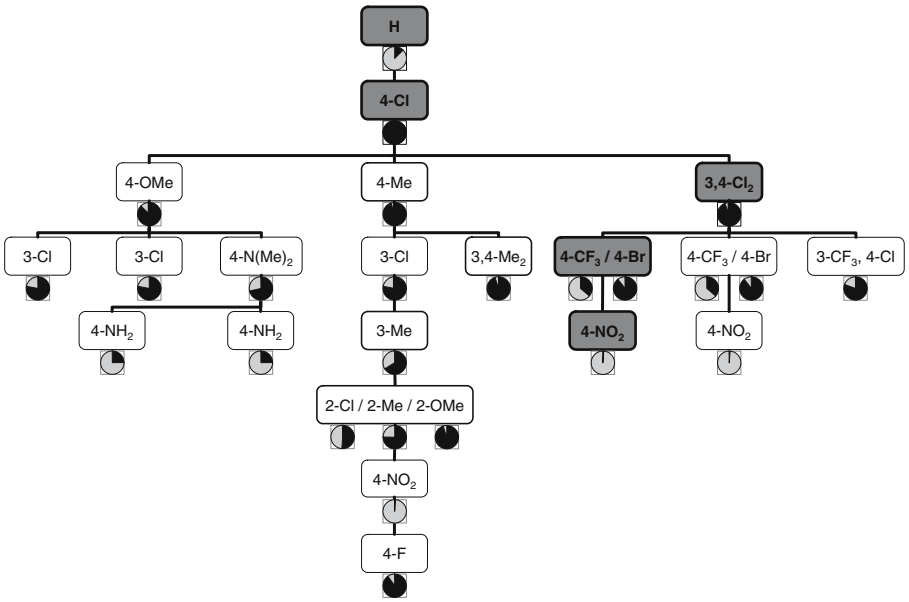


Fig. 1 The complete modified Topliss scheme used in this study. Substituents on the aromatic ring are shown for each analog. From each "parent" compound (from the top of the scheme) the choice of the next analog is shown, depending on the result of the previous test. If the potency increased for the latest tested compound, the next compound to be tested is the one to the right in the next lower level of the scheme, i.e., first, the unsubstituted compound and the 4-chloro-substituted compound is tested, and if the latter compound is more active, the 3,4-dichloro analog is tested next. If the activity is equally high, the analog straight below is tested, i.e., if the 4-Cl analog is equally active as the 4-H, the 4-Me analog is tested next. If the activity is lower, the analog below to the left is tested, i.e., if the 4-Cl analog is less active than the unsubstituted compound, the 4-OMe analog is tested next. Our results directed us to conduct the test series indicated by *shaded, bold boxes*. The results (AFIn) are shown in circle diagrams below each analog (*filled black circle* AFIn=100, *empty gray circle* AFIn=0; all tests carried out at 50 mM)

different electronic parameters were applied: σ, F, and R. The widely used σ parameter has the disadvantage that the resonance effect contributes for metasubstituted aromatic rings (σ values for orthosubstituted compounds have not been included), whereas for parasubstituted systems, the field factors affect the values of σ (Swain and Lupton 1968). For the parameters F and R, the field effects (F) and resonance effects (R) are separated. The steric parameters used to measure the "bulk" of the molecules were molecular weight (MW) and molar refractivity (MR). MR differs from MW in having an electronic contribution, as it is directly proportional to polarizability (Eq. 2; Hansch et al. 1973). The values of all parameters used in this study are available in the literature (Hansch and Deutsch 1966; Swain and Lupton 1968 and Hansch et al 1973).

Results

Evaluation of the Topliss method for finding potent methyl 3-phenylpropanoate antifeedants To determine fully the usability of this approach, the scheme was initially followed step by step (as was intended in Topliss' method); but in a second phase of the study, all compounds found in all branches of the scheme were obtained and compared.

The investigation started with methyl 3-phenylpropanoate (entry 1, Table 1). The first analog to be tested was the more hydrophobic and electron-withdrawing 4-chloro compound (entry 2, Table 1), which was more active than the unsubstituted compound. The next analog to be tested according to the scheme was the 3,4-dichloro compound (entry 5, Table 1), which was even more hydrophobic, and the ring was more electron-poor. This analog showed similar or slightly lower activity. The 4-CF_3 analog (entry 6, Table 1), which did not have a metasubstituent and was less lipophilic than 3,4-Cl but more lipophilic than 4-Cl, was tested next and was less active than the previous one. The 4-Br analog (entry 8, Table 1), an alternative to 4-CF_3, also showed low activity. Finally, the analog with the less lipophilic but more electron-withdrawing 4-NO_2 substituent, can be found in the bottom of scheme 1. Ethyl 3-(4-nitrophenyl)propanoate (entry 20, Table 1) was tested instead of methyl 3-(4-nitrophenyl)propanoate but the results can be compared with the results from ethyl 3-phenylpropanoate (entry 48, Table 3), and it is obvious that nitro analogs have relatively low activities (Fig. 2a and Table 1).

To find out if the Topliss method generated the optimal antifeedant, we decided to investigate all three branches in parallel.

Following the right branch of the Topliss scheme, all compounds except methyl 3(4-chloro-3-trifluoromethyl-phenyl)propanoate (entry 41, Table 2) were tested, as the scheme was followed systematically. This compound was less active than the analogs substituted with 4-Cl (entry 2,

Table 1), 3,4-Cl (entry 5, Table 1), and 4-Br (entry 8, Table 1) but more active than the 4-CF_3 (entry 6, Table 1) and 4-NO_2 (entry 20, Table 1) analogs (Fig. 2a).

In the center branch, the 4-methyl (entry 15, Table 1) and 3,4-dimethyl analogs (entry 40, Table 2) were tested next. Further down this branch, steric factors were examined by testing the 2- and 3-substituted analogs, i.e., the 3-chloro (entry 4, Table 1) and the 2-chloro (entry 3, Table 1), 2-methyl (entry 13, Table 1) and 2-methoxy (entry 11, Table 1) analogs. These compounds generally had lower activity than the 4-substituted analogs but the electronic factors did not seem to greatly affect the activity. The 4-F analog (entry 9, Table 1) has similar values of the Hansch parameters but showed higher potency than the parent unsubstituted methyl 3-phenylpropanoate (Fig. 2b).

For the left branch of the scheme, the choices after the electron-donating 4-methoxy analog (entry 10, Table 1) were other more electron-donating compounds: the 4-N$(Me)_2$ (entry 22, Table 1) and the 4-NH_2 (entry 21, Table 1) analogs. The dimethylamino compound had similar potency compared with the 4-methoxy compound, whereas the amino compound was much less potent (Fig. 2c). As a hypothesis, the polarity of the amino group is the cause of the low antifeedant activity.

To be able to better compare the most potent antifeedants, which all had AFI values close to 100, additional tests were carried out at 5 mM concentration. The results from these tests showed a stronger decrease in activity at the lower concentration for the methoxy analogs than for the analogs with chloro, bromo, fluoro, and methyl substituents (Fig. 3).

As potent antifeedants were found in all branches of the Topliss scheme and not preferentially among the analogs obtained when the proposed route was followed, the Topliss approach was abandoned. Consequently, we continued our survey of the SARs of phenylpropanoids by using other methods.

Effect of the position of substituents on the aromatic ring To see whether substituents on the aromatic ring should be situated in the ortho, meta, or para position for optimal activity, tests were performed for all monosubstituted isomers of the methyl (entries 13–15, Table 1), chloro (entries 2–4, Table 1), and methoxy (entries 10–12, Table 1) analogs (Fig. 3a). For the methyl-substituted compounds, the difference in position did not greatly affect the potency, neither on AFIn nor AFIa. For the monochlorinated compounds, there was a larger difference in activity in the order para>meta>ortho. For the methoxy analogs, the metasubstituted and parasubstituted compounds showed similar potency in all tests, whereas it is interesting to note that the activity of the orthosubstituted analog was almost nil at 5 mM (Fig. 3a).

Effects of unsaturation and conjugation To understand the importance of conjugation to the carbonyl group in the phenylpropanoids, several substituted methyl 3-phenylpropanoates were compared with their corresponding 3-phenylacrylates (Fig. 4). Most of the pairs of conjugated (3-phenylacrylates) and nonconjugated compounds (3-phenylpropanoates) had similar activities (Fig. 4). However, when we compared the activities of the analogs with the most electron-withdrawing and electron-donating groups, we found a trend. The activities of the compounds with electron-withdrawing groups (4-CF_3 and 4-NO_2) (entries 6–7, 20, 25, Table 1) were favored by conjugation, whereas the activity of compounds with electron-donating substituents (4-N$(CH_3)_2$ and 4-NH_2) (entries 21–24, Table 1) decreased for the conjugated analogs (Fig. 4b).

Comparison of disubstituted and trisubstituted compounds We also investigated whether it was possible to improve the activity by adding more substituents to the aromatic ring and by optimizing the substitution pattern. Consequently, several compounds with two or three substituents were tested (Fig. 3b). Different substitution patterns were compared for dimethoxy- and trimethoxy-substituted (entries 33–35, 38, Table 2) methyl 3-phenylpropanoates. For the dimethoxy-substituted compounds, there was a strong effect favoring 2,3- and 3,5-substitution over 3,4- and 3,4,5-substitution. The 2,3- and 3,5-disubstituted methoxy analogs were slightly less active compared with the monosubstituted methoxy analogs (Fig. 3b). For disubstituted methyl analogs (entries 39–40, Table 2), the 2,4-analog was slightly more potent than the 3,4-analog, but the difference was minor. The disubstituted methyl compounds were approximately as potent as the monosubstituted analogs (Fig. 3c). Furthermore, methyl 3-(4-ethylphenyl)acrylate (entry 17, Table 1) was tested to establish whether the larger and more hydrophobic ethyl group alone could enhance the activity, but this compound was less active than the ones previously tested.

The 3,4-dichlorinated analog (entry 5, Table 1) was tested and compared with the monohalogenated analogs. It showed similar potency as the 3-chloro, (entry 4, Table 1) but slightly less potency than the 4-chloro compound (entry 2, Table 1).

A series of dimethoxy-substituted 3-phenylacrylates (entries 29–32, Table 2) were also tested for the influence of substitution pattern on activity. The order of reactivity between combinations of substituents was 2,3>2,4≫3,5>3,4 (Fig. 4a). These results corresponded well with the results from the dimethoxyphenylpropanoates above.

Activity of phenylpropanoates with mixed substituents - Three analogs with mixed substituents were added to the

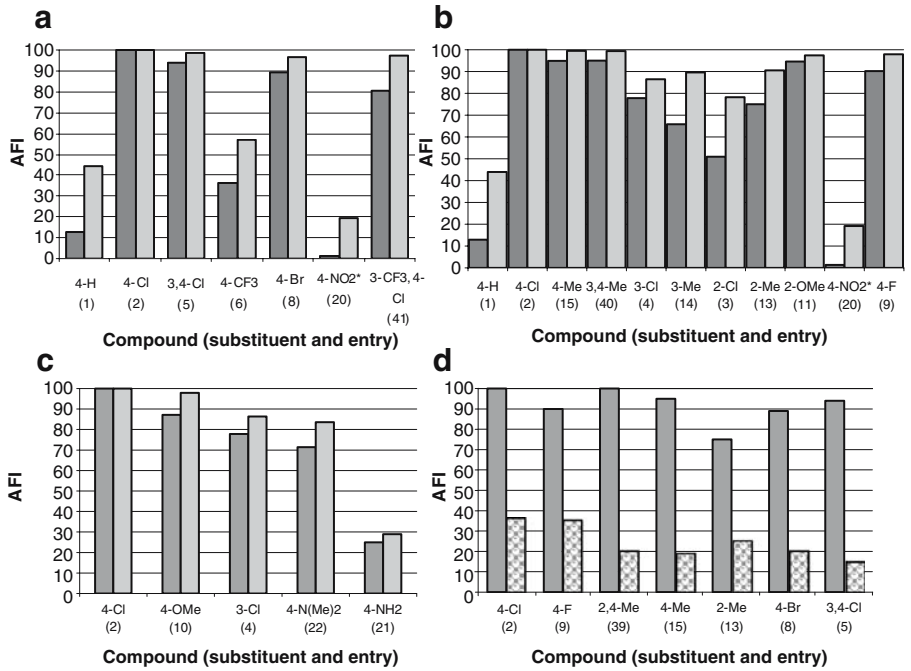


Fig. 2 AFI for substituted 3-phenylpropanoates in the Topliss scheme. AFIn is represented by *dark columns* and AFIa by *light columns*. Substituent(s) and entry number (in *parentheses*) are given beneath the *x*-axis: **a** the right branch (50 mM concentration), ethyl 3-(4-nitrophenyl)propanoate is denoted by an *asterisk*; **b** the center branch (50 mM concentration), ethyl 3-(4-nitrophenyl)propanoate is denoted by an *asterisk*; **c** the left branch (50 mM concentration); **d** AFIn for the *most potent* compounds from the Topliss scheme tested at 50 mM (*dark columns*) and 5 mM concentrations (*dotted columns*)

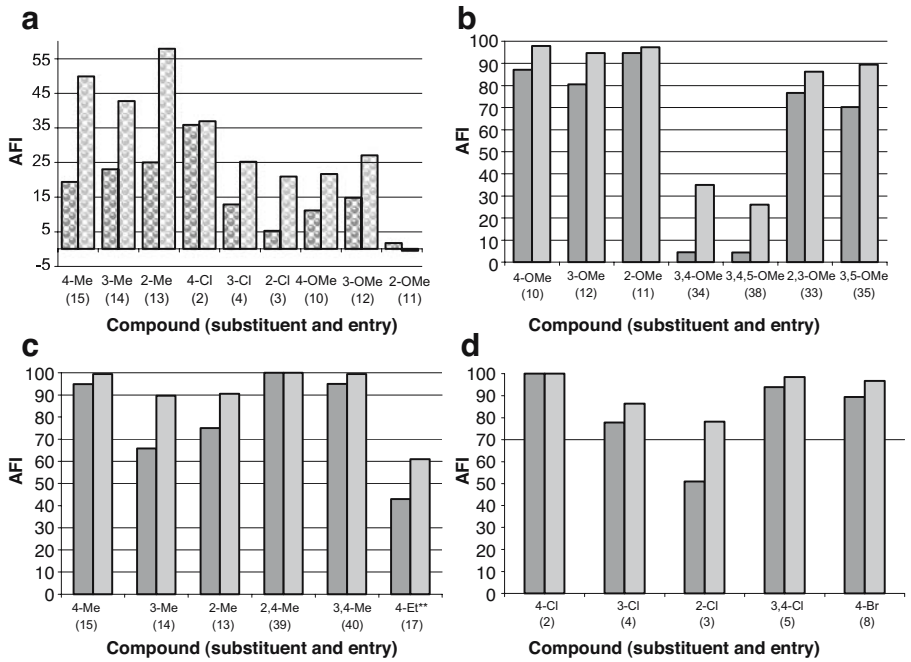


Fig. 3 AFI for substituted 3-phenylpropanoates. AFIn is represented by *dark columns* and AFIa by *light columns*. Substituent(s) and entry number (in *parentheses*) are given beneath the *x*-axis: **a** methyl-, chloro-, and methoxy-substituted analogs (5 mM concentration, *dotted columns*); **b** methoxy-substituted analogs (50 mM concentration); **c** methyl-substituted analogs (50 mM concentration), methyl 3-(4-ethylphenyl)acrylate is denoted by *two asterisks*; **d** chloro- and bromo-substituted analogs (at 50 mM concentration)

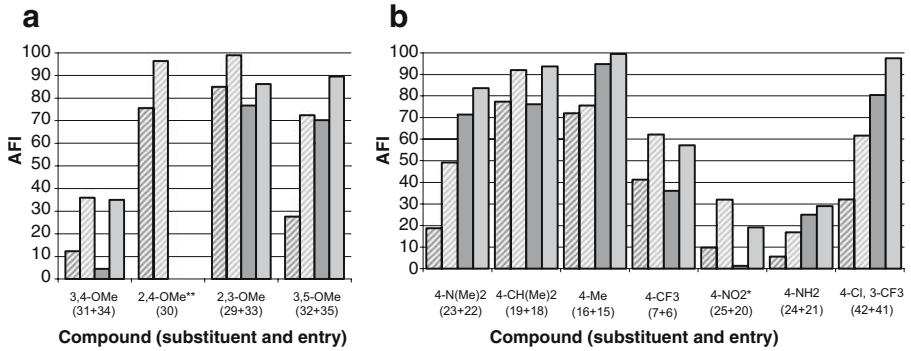


Fig. 4 AFI for methyl 3-phenylacrylates (acr; *striped columns*) and methyl 3-phenylpropanoates (pro; *plain columns*) at 50 mM concentration. AFIn is represented by *dark columns* and AFIa by *light columns*. Substituent(s) and entry number (in *parentheses*) are given beneath the *x*-axis. Each group of compounds with identical substitution pattern are shown in the following order: AFIn(acr), AFIa(acr), AFIn(pro), and AFIa(pro). Ethyl 3-(4-nitrophenyl)propanoate tested instead of methyl 3-(4-nitrophenyl)propanoate is denoted by an *asterisk*; 3-phenylpropanoate analog not tested is denoted by *two asterisks*. **a** Methoxy-substituted analogs; **b** analogs with substituents other than methoxy

test series. These were the 3-bromo-4-methoxy (entry 36, Table 2), 4-chloro-3-trifluoromethyl (entry 41, Table 2), and 4-hydroxy-3-methoxy analogs (entry 37, Table 2). The first compound was less active than the corresponding mono-substituted analogs ((3-Br, 4-OCH_3) vs. 4-Br and 4-OCH_3). The (3-CF_3, 4-Cl) analog was less potent than 4-Cl but more potent than 4-CF_3, and the (3-OCH_3, 4-OH) analog was a more active antifeedant than 4-OH (Fig. 5a). Although not all possible variations were tested, the conclusion from our test series was that no beneficial effects could be found by mixing different substituents on the aromatic ring.

Effect of the parent alcohol part of the esters on antifeedant activity of phenylpropanoids The methyl (entry 10, Table 1) and isopropyl (entry 49, Table 3) esters of 3-(4-methoxyphenyl)propanoate and five alkyl cinnamates (ethyl-, *n*-propyl-, isopropyl-, *n*-butyl-, and 2-butylcinnamate) (entries 43–47, Table 3) were compared to elucidate the effect of the parent alcohol of the esters. All compounds were tested at 50 mM concentration. Isopropyl cinnamate was the most potent of the cinnamates (Fig. 5b) while methyl 3-(4-methoxyphenyl)propanoate was more potent than the corresponding isopropyl ester analog (Fig. 5c). The butyl esters were much less active in all tests, and therefore, no esters derived from higher alcohols were tested.

Furthermore, the methyl and ethyl esters of 3-phenylpropanoic acid, (entry 1, Table 1, vs. entry 48, Table 3), 3-(4-methylphenyl)propanoic acid (entry 15, Table 1, vs. entry 50, Table 3) and 3-(4-chlorophenyl)propanoic acid (entry 2, Table 1, vs. entry 51, Table 3) were compared. Apparently, no major difference in activity between methyl and ethyl esters were detectable except for the unsubstituted 3-phenylpropanoate where the methyl ester was considerably less active compared to the ethyl ester (Fig. 5c).

Influences by Hansch parameters By comparing Hansch parameters in relation to antifeedant activity for a series of substituted methyl 3-phenylpropanoates, the effect of each parameter was analyzed. The aim was to find the importance of each parameter and thus the properties of the substituents that affect antifeedant activity. In total, 29 methyl 3-phenylpropanoates and 11 3-phenylacrylates were compared.

The Hansch parameters that were examined for correlations to antifeedant activity were σ, π, F, R, MR, and MW (Tables 1, 2, and 3). For the electronic parameter σ and the field effect (F), there was no apparent correlation to antifeedant potency. A similar result was found after analyses of the steric parameters (MR and MW), i.e., small and light molecules as well as bulky and heavy compounds showed good antifeedant activity. For the lipophilicity parameter (π), there was a correlation with antifeedant potency. If π was negative ($\pi<-0.1$), the activity was, on average, lower (Fig. 6a). The resonance factor R also showed a correlation to AFI: No compound with good antifeedant potency had $R<-0.5$ (Fig. 6b). For 3-phenylacrylates, the results were similar.

Comparison with benzoate antifeedants In a previous study, we measured the AFI of a large number of benzoates (Unelius et al. 2006). Methoxy-substituted benzoates showed high antifeedant activity. Therefore, the following analogous phenylpropanoids were tested in addition to the compounds already discussed: Methyl 3-(4-butoxyphenyl) propanoate, (entry 26, Table 1), methyl 3-(4-hydroxyphenyl) propanoate (entry 27, Table 1), methyl 3-(4-acetyloxyphenyl)propanoate (entry 28, Table 1), and methyl 3-(4-hydroxy-3-methoxyphenyl)propanoate (entry 37, Table 2). In agreement with the results from the benzoate study, the methoxy analogs (for example, 3-(4-methoxyphenyl)prop-

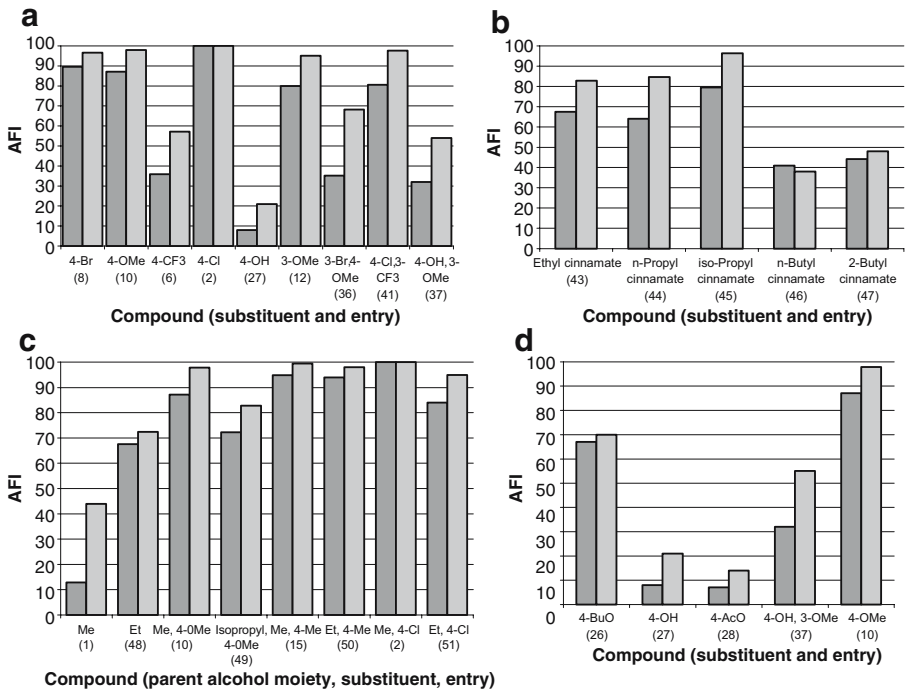


Fig. 5 AFI for 3-phenylpropanoids (at 50 mM concentration). AFIn is represented by *dark columns* and AFIa by *light columns*. Substituent (s) and entry number (in *parentheses*) are given beneath the *x*-axis: **a** methyl 3-phenylpropanoates with mono and mixed substituents; **b** cinnamates with different parent alcohol moieties; **c** 3-phenylpropanoates with different parent alcohol moieties; **d** derivatives of methyl 3-(4-hydroxyphenyl)propanoate

anoate; entry 10, Table 1) were most effective (Fig. 5d), but, in contradiction to the results from the benzoate study, the tested methyl- or halogen-substituted phenylpropanoids had similar or even higher antifeedant activity than the methoxy analogs.

Discussion

When initially applying the Topliss approach in our search for potent antifeedants, the most active compounds happened to appear early in the scheme, so the use of the remaining scheme became redundant. Normal rational design was used, therefore, during the reminder of this SAR study. Nevertheless, the Topliss approach may be a useful tool in other projects within the field of chemical ecology where an effective semiochemical is to be found in a limited time and at low costs.

To correlate the structures of the phenylpropanoids to their antifeedant activity for *H. abietis* was difficult. Apart from the result that highly polar substituents were unfavorable for activity, it was difficult to discriminate any particular factors that affected the antifeedant activity. The position of substituents on the aromatic ring does not appear to be critical for the antifeedant activity–a number of 2-, 3-, and 4-substituted analogs had similar potency. The most potent compounds tested were methyl 3-phenylpropanoates mono-substituted with chloro, fluoro, or methyl groups, and the 3,4-dichlorinated methyl 3-phenylpropanoate.

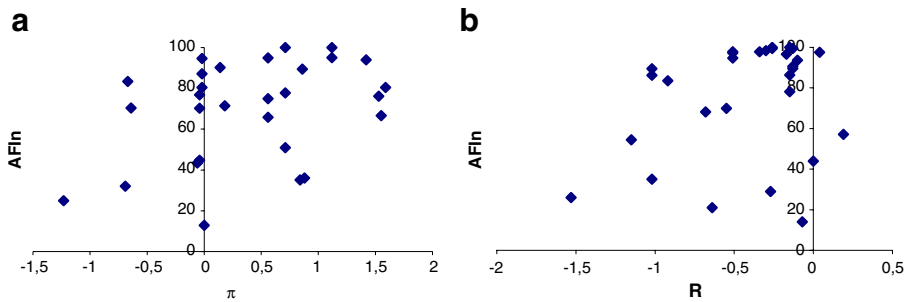


Fig. 6 AFIn for methyl 3-phenylpropanoates (at 50 mM concentration) vs. values of various Hansch parameters: **a** AFIn vs. the hydrophobicity parameter π; **b** AFIn vs. the steric parameter *R*

In a comparison of 3-phenylpropanoates and acrylates, the lower flexibility caused by the double bond in the acrylates may be an important factor that affects interactions with a receptor. Nevertheless, we focused on the electronic effects in our analyses of conjugated vs. nonconjugated analogs. In compounds with electron-withdrawing substituents on the aromatic ring, the antifeedant effect increased with conjugation, and for compounds with electron-donating substituents, the effect decreased for the conjugated analogs. Electron richness in the aromatic ring of the phenylpropanoids may be important for high activity. Alternatively, or additionally, the carbonyl moiety should be deprived of electron density. Eriksson (2006) studied the antifeedant activity of 3-phenylpropenals and 3-phenylpropanals against *H. abietis* and concluded that conjugation does not seem to be critical for activity.

In the comparison between monosubstituted and disubstituted compounds, the disubstituted methyl analogs were slightly less potent than the monosubstituted. The decreased potency cannot be correlated to a negative effect of the increased bulkiness of the compounds as this effect was not seen for the chloro analogs. The magnitude of difference observed (more than tenfold difference) between the disubstituted and trisubstituted methoxy analogs is intriguing. The 3,4- and 3,4,5-substituted saturated compounds were much less active than the 2,3- and 3,5-substituted, which is in agreement with the results from saturated analogs of cinnamic aldehyde (Eriksson 2006). When we compared the antifeedant effect of conjugated acrylate analogs (3,4-OCH_3, 2,4-OCH_3, 2,3-OCH_3, and 3,5-OCH_3), the results differed considerably. In this case, the 2,3- and 2,4-analogs were more potent than the 3,4- and 3,5-analogs, which is contradictory to the results for the corresponding phenylpropenals (Eriksson 2006). Our results indicate that there was an effect by conjugation.

For the compounds with mixed substituents, the (3-Br, 4-OCH_3) analog was less potent than the monosubstituted compounds, which implies that the increased bulk lowered activity. In contrast, the (3-CF_3, 4-Cl) compound was more potent than its monosubstituted analogs.

When esters with different alcohol parts were compared, methyl, ethyl, and isopropyl esters showed potency within the same range, whereas the butyl esters tested showed lower potency. One explanation could be that the bulkiness of the butyl analogs caused the lower antifeedant activity.

The investigation of the relation of the various Hansch parameters to antifeedant activity revealed a correlation to π and R, respectively. For π, a negative value ($\pi<-0.1$) gave a lower AFI. All eight compounds with a more negative F had AFIa<60 at 50 mM concentration. This result was expected as too highly hydrophilic compounds have not been very active in previous investigations of antifeedants for *H. abietis* (Unelius et al. 2006; Sunnerheim et al. 2007). A similar relationship was found for R where compounds having $R<-0.5$ showed low AFIn and AFIa values, i.e., a relatively high resonance potential seems to be necessary. The steric parameters, MR and MW, showed no correlation to antifeedant activity. When the steric factors for each position (ortho, meta, para) on the aromatic ring were correlated to the AFIs, no general trend was found, but the number of compounds tested was limited (10 para, 3 meta and 3 orthomonosubstituted compounds). These results may be indicative of a multireceptor response that causes the antifeedant effect, since small as well as bulky, and light as well as heavy compounds all showed similar antifeedant potency. This multireceptor hypothesis is supported by the fact that most other parameters also did not correlate to AFI.

In the relatively few studies available on the responses of other organisms to phenylpropanoids, there are both similarities and dissimilarities with our results for *H. abietis*. That esters of phenylpropanoids are more active than the corresponding alcohols and acids was shown both in the present study and in studies of derivatives of cinnamic acid as bird repellents (Jacubas et al. 1992; Watkins et al. 1999). An example of a compound with diverging effects between organisms is ethyl 3-(4-nitrophenyl)acrylate, which showed no antifeedant activity against *H. abietis* although it has previously been found to be an oviposition deterrent for the onion fly *Delia antique* (Meigen) (Cowles et al. 1990). Another related compound, cinnamaldehyde, has been proved to act as an antifeedant for grain storage insects (Huang and Ho 1998). A comparison of the phenylpropanoid vs. the benzoate antifeedants of *H. abietis* (Unelius et al. 2006) reveals a number of similarities: Carboxylic acids are generally less effective antifeedants than the corresponding esters, which also is in agreement with the results from the study on *H. abietis* by Eriksson (2006). Furthermore, the length of the alkyl chain in the parent alcohol moiety of the esters should be short, and methoxy-substituted aromatics are more potent than hydroxy-substituted in both classes of substances. On the other hand, the prerequisites of the substitution pattern for good antifeedant activity seem to differ between benzoates and phenylpropanoids. For example, the best benzoate is the 2,4-dimethoxy analog, whereas the corresponding 2,4-dimethoxy-phenylpropanoids have rather low activity, and the most effective propanoids, the halogen- or methyl-substituted compounds, have very low AFIs as benzoate analogs.

Among the 51 compounds tested in this study, a large number of potent antifeedants were found. A majority of the compounds tested was more effective than the lead compound, ethyl cinnamate, which has been identified in the inner bark of *Pinus contorta* (Bratt et al. 2001). The most potent compounds were methyl 3-phenylpropanoates monosubstituted with chloro, fluoro, or methyl groups on the aromatic

ring, and the 3,4-dichlorinated methyl 3-phenylpropanoate. These compounds are good candidates for further studies in the laboratory and in the field. A pool of over 20 highly active phenylpropanoid compounds provides many possibilities to develop mixtures of compounds that could potentially be even more effective than the single compounds tested so far. Field assays that evaluate chemical and physical properties, such as volatility and stability to resist water and UV radiation, are required to establish the effectiveness of these compounds for conifer seedling protection under field conditions.

Our findings are probably a consequence of effects from the stimuli of several taste and odor receptors processed by the insect brain. Earlier investigations (Wibe et al. 1996, 1997, 1998) have described the response of numerous receptors in the antennae of *H. abietis*, of which some are highly specific and some more generally tuned to olfactory stimuli, within groups of monoterpenes and aromatic compounds. Thus, it is likely that several receptor types are activated during feeding and that each type is tuned to molecules with different structural properties. The results from a study of structural analogs of naphthoquionones as feedants/antifeedants for the larvae of the Mexican bean beetle, *Epilachna varivestis*, are as intriguing as ours. A complex interplay of electronic, steric, electrochemical, and positional requirements was found that affected the feeding response (Weissenberg et al. 1997).

The ecological background to why feeding of *H. abietis* is affected by derivatives of phenylpropanoates is still largely unknown. Although it is possible that ethyl cinnamate is partly responsible for the fact that *H. abietis* feeds less on the bark of *P. contorta* than of *P. sylvestris* (Bratt et al. 2001), this is at least not specifically a substance used by *P. contorta* for defense against this insect, as the tree and the insect species have their natural distributions in different parts of the world. However, phenylpropanoates may be of more general importance in plant–insect interactions than presently acknowledged, as indicated by the frequently strong antifeedant responses in the pine weevil.

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References

BAECKSTRÖM, P., STRIDH, K., LI, L., and NORIN, T. 1987. Claisen rearrangements with mesityl oxide dimethyl ketal. Synthesis of ipsdienone, *E*- and *Z*-ocimenone, 2,6-dimethyl-2,7-octadien-4-one and 2,6-dimethyl-2,7-octadien-4-ol. *Acta Chem. Scand* B41:442–447.

BJÖRKLUND, N., NORDLANDER, G., and BYLUND, H. 2005. Olfactory and visual stimuli used in orientation to conifer seedlings by the pine weevil, *Hylobius abietis*. *Physiol. Entomol* 30:225–231.

BLANEY, W. M., SIMMONDS, M. S. J., EVANS, S. V., and FELLOWS, L. E. 1984. The role of the secondary plant compound 2,5-dihydroxymethyl 3,4-dihydroxypyrrolidine as a feeding inhibitor for insects. *Entomol. Exp. Appl* 36:209–216.

BORG-KARLSON, A.-K., NORDLANDER, G., MUDALIGE, A., NORDENHEM, H., and UNELIUS, C. R. 2006. Antifeedants in the feces of the pine weevil *Hylobius abietis*: Identification and biological activity. *J. Chem. Ecol* 32:943–957.

BRATT, K., SUNNERHEIM, K., NORDENHEM, H., NORDLANDER, G., and LÅNGSTRÖM, B. 2001. Pine weevil (*Hylobius abietis*) antifeedants from lodgepole pine (*Pinus contorta*). *J. Chem. Ecol* 27:2253–2262.

COWLES, R. S., MILLER, J. R., HOLLINGSWORTH, R. M., ABDEL-AAL, M. T., SZURDOKI, F., BAUER, K., and MATOLCSY, G. 1990. Cinnamyl derivatives and monoterpenoids as non-specific ovipositional deterrents for the onion fly. *J. Chem. Ecol* 16:2401–2428.

DAY, K. R., NORDLANDER, G., KENIS, M., and HALLDÓRSON, G. 2004. General biology and life cycles of bark weevils, Chapter 14, Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis. pp. 331–349, in F. Lieutier, K. R. Day, A. Battisti, J. C Grégoire, and H. F. Evans (eds.). Kluwer, Dordrecht.

ERIKSSON, C. 2006, Isolation, synthesis and structure–activity relationships of antifeedants against the pine weevil, *Hylobius abietis*. Ph.D. Dissertation, KTH, Stockholm, Sweden. ISBN: 91-7178-301-6.

HANSCH, C., and FUJITA, T. 1963. ρ–σ–π Analysis. A method for the correlation of biological activity and chemical structure. *J. Am. Chem. Soc* 86:1616–1626.

HANSCH, C., and DEUTSCH, E. W. 1966. The structure–activity relationship in amides inhibiting photosynthesis. *Biochim. Biophys. Acta* 112:381–391.

HANSCH, C., LEO, A., UNGER, S. H., KI, H. K., NIKAITANI, D., and LIEN, E. J. 1973. "Aromatic" substituent constants for structure–activity correlations. *J. Med. Chem* 16:1207–1216.

HARWOOD, L. M., MOODY, C. J., and PERCY, J. M. 1998. Experimental Organic Chemistry. 2nd edn. Blackwell Science, Oxford.

HUANG, Y., and HO, S. H. 1998. Toxicity and antifeedant activities of cinnamaldehyde against the grain storage insects, *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. *J. Stored Prod. Res* 34:11–17.

JACUBAS, W. J., SHAH, P. S., MASON, J. R., and NORMAN, D. M. 1992. Avian repellency of coniferyl and cinnamyl derivatives. *Ecol. Appl* 2:147–156.

LÅNGSTRÖM, B., and DAY, K. R. 2004. Damage, control and management of weevil pests, especially *Hylobius abietis*, Chapter 19, Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis. pp. 415–444, in F. Lieutier, K. R. Day, A. Battisti, J. C Grégoire, and H. F. Evans (eds.). Kluwer, Dordrecht.

LEGRAND, S., NORDLANDER, G., NORDENHEM, H., BORG-KARLSON, A.-K., and UNELIUS, C. R. 2004. Hydroxy-methoxybenzoic methyl esters: synthesis and antifeedant activity on the pine weevil, *Hylobius abietis*. *Z. Naturforsch* 59b:829–835.

SUNNERHEIM, K., NORDQVIST, A., NORDLANDER, G., BORG-KARLSON, A.-K., UNELIUS, C. R., BOHMAN, B., NORDENHEM, H., HELLQVIST, C., and KARLÉN, A. 2007. Quantitative structure–activity relationships of pine weevil antifeedants, a multivariate approach. *J. Agric. Food Chem* 55:9365–9372.

SWAIN, C. G., and LUPTON, E. C. 1968. Field and resonance components of substituent effects. *J. Am. Chem. Soc* 90:4328–4337.

TOPLISS, J. G. 1972. Utilization of operational schemes for analog synthesis in drug design. *J. Med. Chem* 15:1006–1011.

TOPLISS, J. G. 1977. A manual method for applying the Hansch approach to drug design. *J. Med. Chem* 20:463–469.

UNELIUS, C. R., NORDLANDER, G., NORDENHEM, H., HELLQVIST, C., LEGRAND, S., and BORG-KARLSON, A.-K. 2006. Structure–activity relationships of benzoic acid derivatives as antifeedants for the pine weevil, *Hylobius abietis*. *J. Chem. Ecol* 32:2191–2203.

VOGEL, A. I. 1989. Textbook of Practical Organic Chemistry. 5th edn. Prentice Hall, Upper Saddle River, NJ.

WATKINS, R. W., LUMLEY, J. A., GILL, E. L., BISHOP, J. D., LANGTON, S. D., MACNICOLL, A. D., PRICE, N. R., and DREW, M. G. B. 1999. Quantitative structure–activity relationships (QSAR) of cinnamic acid bird repellents. *J. Chem. Ecol* 25:2825–2845.

WEISSENBERG, M., MEISNER, J., KLEIN, M., SCHAEFFLER, I., ELIYAHU, M., SCHMUTTERER, H., and ASCHER, K. R. S. 1997. Effect of substituent and ring changes in naturally occuring naphthoquinones on the feeding response of larvae of the mexican bean beetle, *Epilachna varivestis*. *J. Chem. Ecol* 23:3–18.

WIBE, A., BORG-KARLSON, A.-K., NORIN, T., and MUSTAPARTA, H. 1996. Identification of plant volatiles activating the same receptor neurons in the pine weevil, *Hylobius abietis*. *Entomol. Exp. Appl* 80:39–42.

WIBE, A., BORG-KARLSON, A.-K., NORIN, T., and MUSTAPARTA, H. 1997. Head-space plant volatiles eliciting responses in receptor neurons of the pine weevil (*Hylobius abietis* L.). *J. Comp. Physiol* 180:585–595.

WIBE, A., BORG-KARLSON, A.-K., PERSSON, M., NORIN, T., and MUSTAPARTA, H. 1998. Enantioselectivity of receptor neurons in the pine weevil (*Hylobius abietis*). *J. Chem. Ecol* 24:273–287.

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Responses of *Helicoverpa armigera* to Tomato Plants Previously Infected by ToMV or Damaged by *H. armigera*

Li Lin · Tse-Chi Shen · Yi-Hua Chen · Shaw-Yhi Hwang

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Abstract We report the comparative inducing effects of a phytopathogen and a herbivorous arthropod on the performance of an herbivore. Tomato, *Lycopersicon esculentum* Mill., was used as the test plant, and tomato mosaic virus (ToMV) and corn earworm, *Helicoverpa armigera* Hübner, were used as the phytopathogen and herbivore, respectively. There were decreases in the efficiency of conversion of ingested food and efficiency of conversion of digested food when *H. armigera* was reared on tomato plants that had been previously inoculated with ToMV. However, virus inoculation did not affect feeding or oviposition preferences by *H. armigera*. In contrast, approximate digestibility, total consumption, relative growth rate, and relative consumption rate were lower for fourth-instar *H. armigera* that fed on plants previously damaged by the same herbivore. Feeding and oviposition were both deterred for *H. armigera* that fed on previously damaged plants. The duration of development of *H. armigera* was also prolonged under this treatment. Infection by ToMV and feeding damage by *H. armigera* increased the host plant's peroxidase and polyphenol oxidase activity, respectively, suggesting that the performance of *H. armigera* may be affected by the induced phytochemistry of the host plant. Overall, this study indicated that, in general, insect damage has a stronger effect than ToMV infection on plant chemistry and, subsequently, on the performance of *H. armigera*.

Keywords Corn earworm · *Helicoverpa armigera* · Induced plant chemistry · Peroxidase · Plant–phytopathogen–herbivore interaction · Polyphenol oxidase · Tomato mosaic virus (ToMV)

L. Lin · T.-C. Shen · Y.-H. Chen · S.-Y. Hwang (✉)
Department of Entomology, National Chung Hsing University, Taichung, Taiwan
e-mail: oleander@dragon.nchu.edu.tw

Introduction

Almost every plant species is used as a food source by a variety of phytopathogens and herbivores, and these exploiters seldom exist in isolation from each other. When considering their abundance and biodiversity (Hawksworth 1991), it is obvious that the concurrent or sequential occurrence of phytopathogens and herbivores on a host plant is common, and that interactions between phytopathogens and herbivores can be expected. Thus, plants often deal with diverse enemies (Moran 1998; Genoud and Metraux 1999; Maleck and Dietrich 1999; Paul et al. 2000; Kruess 2002; Rostás and Hilker 2002, 2003). Owing to their sedentary life traits, plants have evolved specific ways to cope with their multiple enemies. They synthesize a broad range of constitutive and induced phytochemicals that may directly affect the inducers themselves or indirectly affect subsequent intruding herbivores or phytopathogens (Moran 1998; Rostás et al. 2002; Wittstock and Gershenzon 2002; Johnson et al. 2003).

Interactions among diverse plant enemies can be direct or indirect, mutualistic, detrimental or neutral, and these effects may be exerted by induced plant chemistry (Hatcher et al. 1995; Moran 1998; Rostás and Hilker 2002, 2003; Johnson et al. 2003; Stout et al. 2006). Both phytopathogens and herbivores are biotic stress factors that induce changes in the plant metabolism, such as changes in patterns of nutrient allocation and induction of defense-related phytochemistry (Ayres 1992; Baldwin and Preston 1999; Hammerschmidt 1999; Rostás and Hilker 2002; Stout et al. 2006). Thus, phytopathogens and herbivores interact indirectly by influencing the suitability of their shared host plant.

Several studies have indicated that cross-resistance between herbivores and phytopathogens occurs, e.g., prior attacks by phytopathogenic fungi increase resistance against subsequent attacks by herbivores (Karban et al.

1987; Hatcher et al. 1994, 1995; Hatcher 1995; Siemens and Mitchell-Olds 1996; Kruess 2002; Rostás and Hilker 2002; Rostás et al. 2003; Stout et al. 2006), and previous herbivore-damaged plants become less suitable for fungi (Karban et al. 1987; Hatcher et al. 1994; Hatcher and Paul 2000; Rostás et al. 2003). Most studies of plant–phytopathogen–herbivore interactions, however, have been focused on the effects of phytopathogenic fungi (Moran 1998; Kruess 2002; Rostás and Hilker 2002; Rostás et al. 2002, 2003; Johnson et al. 2003), and relatively little is known about the effects of phytopathogenic viruses on plants and on lepidopteran insects (Stout et al. 1999; Mayer et al. 2002; McKenzie et al. 2002). In addition to the plant–phytopathogen–herbivore interactions, many studies have also revealed a plant–herbivore–herbivore interaction (Karban and Myers 1989; Stout and Duffey 1996; Karban and Baldwin 1997; Denno et al. 2000). Different herbivores of a host plant may be separated spatially or temporally, but they may interact with each other through mediation of the host plant. Most plant–herbivore–herbivore research has been concerned with the effect of plant-induced responses on herbivores (Karban and Baldwin 1997; Felton and Eichenseer 1999; Underwood 1999; Bezemer and van Dam 2005). However, diverse effects have been found for plant-mediated interaction, and this may be because of complicating interactions among the defense-related signalling pathways and the resulting induction of plant secondary compounds (Stout et al. 2006).

Corn earworm, *Helicoverpa armigera* (Hübner), has a worldwide distribution and is a highly polyphagous agricultural pest. The host plant spectrum of *H. armigera* includes important agricultural crops such as tomato, cotton, maize, chickpea, sorghum, cereals, and soybean (Fitt 1989; Cunningham et al. 1999; Gupta et al. 2003; Diongue et al. 2004). Feeding on foliage or fruiting structures by insatiable larvae usually leads to substantial economic losses (Reed and Pawar 1982). Tomato mosaic virus (ToMV) causes mosaic disease of tomato and other important crop plants (Green et al. 1987; Breman 1989; Duarte et al. 2001). The ToMV-infected tomato plants display light and dark green mottled areas of the leaves, and fruits may be reduced in size and number with irregular ripening (Green et al. 1987). In tomato plantations, both *H. armigera* and ToMV may occur simultaneously. We investigated the interactions among tomato, ToMV, and corn earworm (*H. armigera*). Specifically, this study focused primarily on plant-mediated indirect interactions between ToMV and *H. armigera*, and on the indirect interactions between insect-induced responses and *H. armigera* performance. In addition, we assessed changes in plant chemistry because of induction by herbivore damage or virus infection that might be relevant to the performance of *H. armigera*.

Methods and Materials

Plants Tomato plants (*L. esculentum* Mill. cv Tainan-Yasu No. 6) were grown from seeds in a greenhouse (27–30°C). Supplemental light was provided 16 hr/d in addition to natural daylight. Before sowing, seeds were soaked first in 5% bleach for 30 min and rinsed with distilled water three times to eliminate contamination. Seeds were potted in standard potting soil, watered daily, and fertilized once a week with commercial synthetic 25–5–20 (N–P–K) fertilizer (1/1,000 Hyponex®4). Plants were transplanted to 17.8 cm diameter pots when they had three fully expanded leaves (20 to 28 d after sowing).

H. armigera and ToMV H. armigera were obtained as eggs from the Taiwan Agricultural Chemicals and Toxic Substances Research Institute, Council of Agriculture. Larvae were reared on an artificial diet in a growth chamber (27°C, 12L/12D photoperiod). ToMV was obtained from the laboratory of Dr. F. J. Jan (Department of Plant Pathology, National Chung Hsing University, Taiwan). As the standard method, ToMV was suspended in 10 mM sodium phosphate (pH 7.0) and inoculated onto the true leaf of *Chenopodium quinoa*. For the experimental inoculation treatments, the virus suspension was obtained by grinding the infected *C. quinoa* leaves with a sodium phosphate buffer. To prevent loss of viral activity, all treatments were conducted in a cold room (4°C), and the virus suspension was used within 8 hr after suspension.

Plant-mediated interactions between ToMV and H. armigera To evaluate the effects of systemic infection by ToMV on the resistance of tomato foliage to *H. armigera*, four-leaf tomato plants were assigned in equal numbers to two treatment groups (approximately 60 plants per treatment). Plants in the first group were subjected to a localized infection by ToMV, and those in the second group were the control group. Inoculations were confined to the terminal leaflet of the third leaf. Leaflets were sprayed first with corundum powder; then, they were inoculated by gently rubbing the upper surfaces of the leaflet with a pestle saturated with a virus suspension (in phosphate buffer, pH 7.0). Plants in the control group received the same inoculation treatment, except that a phosphate buffer was used instead of the virus suspension. Three days after the inoculation, leaflets of the fourth leaf (not the treated leaf) were used to evaluate the effects, to assess the chemical changes caused, and to determine the suitability of the treatment for *H. armigera*. Chemical analyses and the bioassays used to assess the suitability of the inoculation treatment for *H. armigera* are described below.

A larval feeding preference bioassay utilized newly molted, fourth-instar *H. armigera*. One leaflet from the fourth leaf was collected from both treated and control tomato plants. Areas of these leaflets were measured first with a portable area meter (Li-3000A, Li-Cor, Lincoln, NE, USA). The leaf petiole of each leaflet was inserted into a water pik to maintain leaf turgor, and leaflets from both treatments were placed in a Petri dish (140×15 mm). Seven 4th instars were placed in the center of each Petri dish and allowed to randomly select and feed on foliage for 6 hr. After they had fed, larvae were removed and the leaf areas of both leaflets were measured again. Feeding percentage was calculated as: Feeding preference (%)=[(leaf area consumed, either treated or control leaflet)/(leaf area consumed of treated leaflet+leaf area consumed of control leaflet)]×100%. Six replicates were performed.

We also evaluated the effect of foliage quality of treated plants on the oviposition choice of adult *H. armigera*. Pupae were separated by sex, and 3 d after eclosion, 10 moths (five of each sex) were placed into a glass cylinder (90 mm long×55 mm diameter) for mating. One day after mating, these 10 moths were transferred to a nylon mesh cage (60×60×60 cm), and two plants, one representing treatment (virus infection) and the other control, were provided for the moths to deposit eggs. Seventy-two hours later, plants were removed from cages, eggs were counted, and the oviposition preference was calculated as: Oviposition preference (%)=[(egg number from either treated or control leaflet)/(egg number from treated leaflet+egg number from control leaflet)]×100%. Six replicates were performed.

Short-term feeding trials were conducted to evaluate the effect of foliage quality on growth rate, food consumption rates, and food processing efficiencies of fourth instars of *H. armigera*. Three days after the inoculation, leaflets of the fourth leaf (untreated leaf) were used. Fifty newly hatched larvae were grown on artificial diet in a Percival growth chamber (12L/12D photoperiod) at a constant 27°C until molting to fourth instars. Each assay consisted of a newly molted and weighed larva placed into a rearing cup (250 ml) that contained a leaf from a plant that had received one of the two different treatments (N=15 replicates per plant treatment). Leaves (above the fourth leaf) were changed every 1–2 d or as necessary during the bioassay. Upon molting to fifth instar, larvae were frozen, oven-dried at 50°C for 1 wk, and reweighed. Nutritional indices were calculated to evaluate insect growth, consumption, and food utilization efficiency (Haynes and Millar 1998; Schoonhoven et al. 1998). These indices were calculated from standard formulas for approximate digestibility (AD), efficiency of conversion of digested food (ECD), and efficiency of conversion of ingested food (ECI) as described by Waldbauer (1968) and Haynes and Millar (1998). The initial rather than the average weights of the larvae were used to calculate the relative growth rate (RGR) and relative consumption rate (RCR) (Farrar et al. 1989). Analysis of covariance (ANCOVA) has been suggested to be more appropriate than the use of ratio variables for the analysis of nutritional indices (Raubenheimer and Simpson 1992, 2003; Raubenheimer 1995; Packard and Boardman 1999; Thompson et al. 2005). Therefore, we performed an ANCOVA (PROC GLM; SAS Institute 1999) on the absolute growth rate (AGR) (weight gained per day) and absolute consumption rate (ACR) (food consumed per day) by using initial weights as covariates. We reported the results from both the standard (ratio) and ANCOVA approaches for a reason: any errors introduced by variation in initial larval weights are inconsequential when the range of initial weights is small, as was the case in this study. Initial dry weights of the test insects were estimated based on a wet-to-dry weight conversion factor determined from five newly molted fourth instars. Similarly, initial dry weights of leaves fed to insects were estimated by dry weight conversion using foliage collected from each plant group at the time of the bioassay. Means and standard errors were calculated for duration, RGR, AGR, RCR, ACR, total consumption (TC), AD, ECD, and ECI for insects fed on foliage from differently treated plants. During the bioassay, additional leaf material from the test plants was collected to measure their chemical content.

Plant-mediated interactions between insect damage and H. armigera To evaluate the effects of systemic induction by *H. armigera* feeding on resistance of tomato foliage to *H. armigera*, four-leaf tomato plants (28-d-old) were assigned in equal numbers to the two treatment groups (approximately 60 plants per treatment). Plants in the first group were subjected to localized feeding by *H. armigera*, and those in the second were the control group. Feeding was confined to the third leaf. One newly molted fourth-instar larva was restricted by a mesh bag and fed on the third leaf. Twenty-four hours after feeding (about half of the leaf area was removed), each larva was removed from the plant. Plants in the control group received the same bag treatment except no larva was used. Three days after feeding, the leaflets of the fourth leaf (untreated leaf) were used to evaluate the effects of the treatment on the suitability of the leaf in preference and feeding trials for *H. armigera* and to assess any chemical changes caused by the treatments. The bioassays used to assess the suitability of leaflets for *H. armigera* (preference and feeding trials) and leaf sampling were similar to those used previously (see "Plant-mediated interactions between ToMV and *H. armigera*").

Chemical analyses Concurrent with the feeding bioassay, foliar samples were collected for chemical analysis. Systemic leaves (i.e., not the virus- or insect-treated leaves, but the fourth leaf and leaves growing above the fourth leaf) from treated tomato plants (six plants), and similarly aged leaves from control plants (six plants) were harvested (0, 3, 7, 14, and 21 d after treatment). Leaves were sampled from different plants (not previously sampled) at the various sampling time intervals to insure independence of sampling. The fourth leaf was used for measuring the total protein concentration and the activities of polyphenol oxidase and peroxidase. The fifth and above leaves were flash frozen in liquid nitrogen, freeze-dried, ground, and stored in a freezer for water and nonstructural carbohydrate analyses at a later date.

Spectrophotometric assays of the activities of polyphenol oxidase and peroxidase were performed with an extract of the fourth leaf (Moran 1998; Stout et al. 1999). Leaf extract was prepared with a tissue grinder (Drill Press Stand Model 212; Dermel, Racine, WI, USA) to homogenize the whole leaf in pH 7 phosphate buffer containing 7% (*w*/*v*) polyvinylpolypyrrolidone. A volume (1.5 ml) of homogenate was removed and placed into a 1.7-ml centrifuge tube. Then, 100 µl of a 10% solution of Triton X-100 were added by mixing with the homogenate. This homogenate was centrifuged at 6,000×*g* for 15 min. The resulting supernatant was used for enzyme activity determination. Total protein was assessed with bovine serum albumin as the standard (Bradford 1976). Polyphenol oxidase and peroxidase activity were measured as in Stout et al. (1999) for the rate of formation of melanin-like material from the phenolic substrates. For polyphenol oxidase assays, 10 to 100 µl of enzyme extract were added to 500 µl of 10 mM catechol in pH 8 potassium phosphate buffer (0.1 M), and the change in absorbance of the mixture at 470 nm was recorded for 30 sec. The method for measuring peroxidase activity was similar, but the substrate for peroxidase activities consisted of 5 mM guaiacol with 0.02 mM H_2O_2 added as a cofactor. Polyphenol oxidase and peroxidase activities were reported as ΔOD_{470} min^{-1}mg fresh $weight^{-1}$ (Ryan et al. 1982).

Foliar water and total nonstructural carbohydrate contents were also quantified for each foliar sample. Differences between wet and dry weights of leaf samples were used to determine water contents. An enzymatic method was used to measure the total nonstructural carbohydrates (TNC) of each sample. Extracts of TNC (starch plus soluble carbohydrates/sugars) were incubated with amyloglucosidase to completely hydrolyze starch before assaying for reducing sugars (Madsen 1997; Liao 2003).

Statistical analyses For all bioassays, means and standard errors (SE) were calculated for the insect performance parameters (feeding and oviposition preference, growth rate, consumption rate, and food utilization efficiencies) and plant chemistry. The Student's *t* test (PROC TTEST; SAS Institute 1999) was used to compare insect performance among virus-inoculated, previously damaged (by insects), and control host plants.

Results

Plant-mediated interactions between ToMV and H. armigera Infection of tomato plants by ToMV had no effect on larval feeding and adult oviposition preference (Fig. 1). Larvae consumed almost equal amounts of foliage from both the virus-infected and the control leaves. Adult moths laid slightly more eggs on the control plants (58%) than on the virus-infected plants (42%), but this difference was not significant.

Performance (duration, growth rates, and consumption rates) of fourth-instar *H. armigera* was similar between the virus-infected and the control plants (Table 1). Analysis of covariance (rate variables) and analysis of variance (ratio variables) gave identical results for growth (AGR, RGR) and consumption (ACR, RCR) parameters (Table 1). However, larvae that fed on virus-infected plants had significantly reduced food conversion efficiencies (ECD and ECI).

Plant-mediated interactions between insect damage and H. armigera Previous feeding on tomato plants by *H. armigera* resulted in a dramatic change in preference of the fed-upon plants to subsequent feeding by *H. armigera*. Fourth-instar larvae consumed more than nine times the amount of foliage of control plants than they did of treated plants (Fig. 1). Female moths laid significantly more eggs on control plants than they did on treated plants (Fig. 1).

Performance (duration, growth rates, and consumption rates) of the fourth instars varied substantially between treated and control plants (Table 1). Analysis of covariance (rate variables) and analysis of variance (ratio variables) also gave similar results for growth (AGR, RGR) and consumption (ACR, RCR) parameters (Table 1). In contrast to the virus-infected study, growth rates (RGR) and consumption rates (RCR) varied significantly between control and treatment plants. Both growth rates (RGR) and consumption rates (RCR) were higher for insects fed on foliage of the control tomatoes (Table 1). Larval duration (DUR), however, was longer for insects that fed on insect-damaged plants. In addition, *H. armigera* larvae had higher digestibility (AD) on control than on treated host plants. The efficiencies of conversion of ingested and digested food (ECD and ECI), however, were not significantly

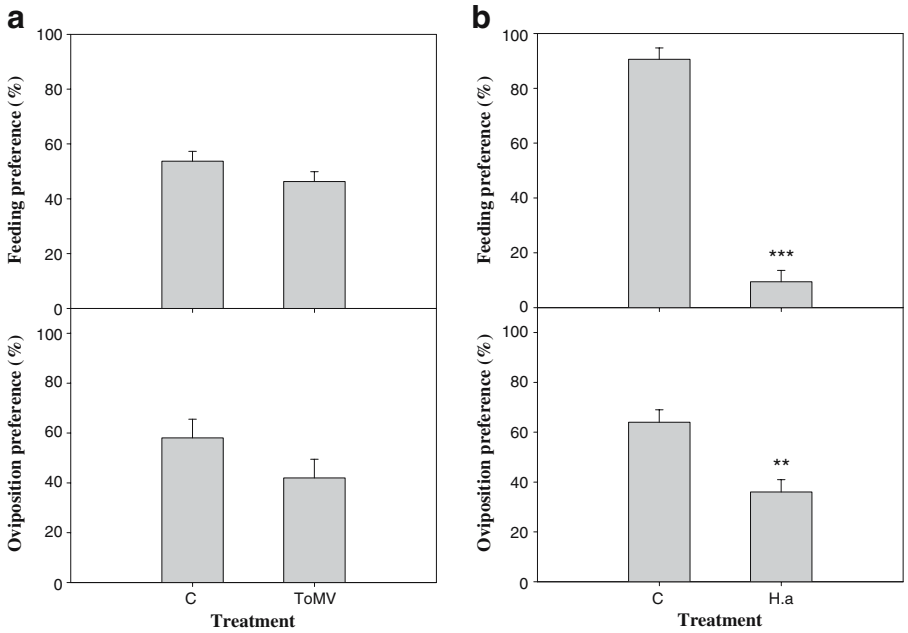

Fig. 1 Larval feeding and adult oviposition preferences (mean±SE, N=6 per treatment) of *H. armigera* in differently treated tomato leaves. **a** Foliage of tomato was inoculated with ToMV. **b** Foliage of tomato was fed on previously by *H. armigera*. *C* control, *ToMV* leaf of tomato plants that were inoculated previously with ToMV, *H. a* leaf of tomato plants that were fed on previously by *H. armigera* larvae. *Asterisks* denote significant differences (**P<0.01, ***P<0.001; Student's t test for independent samples)

different between control and treated plants. In summary, when fed on the previously fed-upon foliage, *H. armigera* larvae consumed less foliage (reduced RCR), had lower digestibility (reduced AD), and grew more slowly (reduced RGR and increased DUR).

Foliage chemistry Based on the bioassays, biochemical analyses were conducted to assess the relationship between the effect of the treatments on pest resistance and the expression of specific enzyme activities or compounds that are probably resistance mechanisms. Peroxidase activity increased slightly with leaf age in all experiments. Peroxidase activity, however, was significantly induced in tomato plants inoculated with ToMV in the systemic leaves by the seventh day after infection (Fig. 2a), and was 30% higher than in control plants. In contrast, peroxidase activity was not significantly different between the previously fed-upon plants and the controls (Fig. 2b).

Polyphenol oxidase activity also increased with leaf age of tomatoes, but the activity was not significantly different between the control and virus-infected plants (Fig. 2a). In contrast, in the insect-feeding treatment (Fig. 2b), polyphenol oxidase activity was significantly higher (30%) than in control plants on the third day after the feeding treatment.

Our results also indicated that foliar water and total protein contents were similar between the treatments and their controls (Fig. 3). Foliar water contents remained between 85% and 90% throughout the experiments. In

Table 1 Performance of corn earworm, *H. armigera*, reared on tomato leaves from plants that were infected with ToMV or infested previously with *H. armigera*

	AD (%)	ECD (%)	ECI (%)	DUR (d)	TC (mg)	RGR (mg/mg/d)	AGR (mg/d)	RCR (mg/mg/d)	ACR (mg/d)
Infected with ToMV									
C	66.35±1.90	23.80±1.27	15.73±0.80	3.33±0.13	65.16±3.55	0.53±0.03	3.08±0.20	3.43±0.19	19.61±0.81
ToMV	63.79±0.81	20.06±1.12	12.79±0.72	3.42±0.16	66.38±3.96	0.46±0.04	2.57±0.34	3.57±0.19	19.71±1.63
P[a]	0.2342	0.0477	0.0183	0.6644	0.8228	0.1128	0.1994	0.6051	0.955
Infested previously with *H. armigera*[b]									
C	60.49±3.95	33.26±3.43	19.19±1.01	3.12±0.06	49.82±2.76	0.53±0.03	3.04±0.20	2.79±0.09	15.97±0.87
H. a	44.43±2.43	42.51±4.58	18.36±1.65	3.67±0.20	39.60±2.36	0.38±0.05	2.05±0.25	2.10±0.21	11.19±1.08
P[a]	0.0029	0.132	0.6216	0.022	0.0116	0.0169	0.0014	0.0103	<0.0001

AD: approximate digestibility, *ECD*: efficiency of conversion of digest food, *ECI*: efficiency of conversion of ingested food, *DUR*: duration, *TC*: total consumption, *RGR*: relative growth rate, *AGR*: absolute growth rate, *RCR*: relative consumption rate, *ACR*: absolute consumption rate, *C*: control, *ToMV*: leaf from plant inoculated with ToMV, *H. a*: leaf from plant fed on by *H. armigera* larvae

[a] AD, ECD, and ECI values are transformed to arcsine values for analysis by t test. Values for AGR and ACR were analyzed by ANCOVA.

[b] Short-term feeding trial (mean±SE, N=15 plants, one insect per plant).

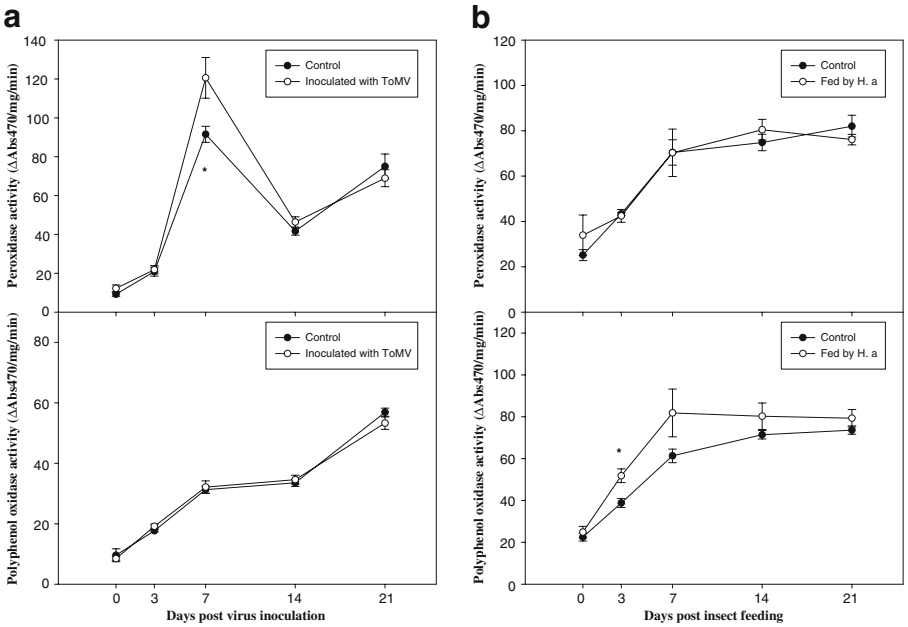

Fig. 2 Response of tomato leaf peroxidase and polyphenol oxidase activities (mean±SE, N=6 plants per treatment) to ToMV/*H. armigera* infestation. **a** Foliar peroxidase and polyphenol oxidase activities in tomato inoculated with ToMV over time. **b** Foliar peroxidase and polyphenol oxidase activities in tomato previously fed on by *H. armigera* over time. *Asterisks* denote significant differences (*P<0.05; Student's t test for independent samples)

addition, the foliar total nonstructural carbohydrate contents were similar between the virus-infected and control treatments during the experiments (Fig. 3). However, in the insect-damage treatment, the total nonstructural carbohydrate content decreased significantly on the third day after the feeding treatment (Fig. 3).

Discussion

We showed that herbivory and viral infections have varied effects on performance and food processing efficiencies of *H. armigera*. Herbivory and virus infection may also affect host plant biochemistry, and changes in host oxidase activity because of induction might affect subsequent performance of *H. armigera*.

Previous investigations have indicated that plant-mediated interactions may occur between phytopathogens and herbivores or between various herbivorous species (Moran 1998; Stout et al. 1999; Thaler et al. 1999; Kruess 2002; Mayer et al. 2002; Rostás and Hilker 2002; Rostás et al. 2002; Johnson et al. 2003). Many studies were focused on the effect of prior attacks by phytopathogens on herbivores or vice versa (Karban et al. 1987; Hatcher et al. 1994, 1995; Siemens and Mitchell-Olds 1996; Hatcher and Paul 2000; Kruess 2002; Rostás and Hilker 2002; Stout et al. 2006), or on plant–herbivore–herbivore interaction (Karban and Myers 1989; Stout and Duffey 1996; Karban and Baldwin 1997; Denno et al. 2000). Among the plant–phytopathogen–herbivore interaction studies, most were focused on the cross-effects between fungal infection and insects (Hatcher 1995; Stout et al. 1999, 2006; Rostás and Hilker 2002; Rostás et al. 2003), and only a few looked at the cross-effects between virus infection and lepidopteran insects.

In our study, we compared concurrently the relative inducing effects of phytopathogenic virus and feeding by an arthropod herbivore on herbivore performance. The larvae of *H. armigera* prefer to feed on undamaged foliage rather than on herbivore-attacked foliage, and the adults also exhibit a decreased preference for leaflets of herbivore-damaged plants. Previous studies have indicated that after a prior attack by herbivores, plants can be induced to increase their polyphenol oxidase activity, which decreases the nutrient value of the foliage and reduces the feeding preference of the insects (Felton et al. 1992; Stout and Duffey 1996; Bostock et al. 2001). Our results show that *H. armigera* prefers to feed or oviposit on the control (undamaged) foliage, which also contains the lower polyphenol oxidase activity. In contrast to the herbivore-induced results, no difference was found in the preference behavior of *H. armigera* between control (undamaged) foliage and virus-infected foliage. Although some workers have suggested that adult beetles will avoid feeding and will avoid ovipositing on fungus-infested leaves (Kruess 2002; Rostás and Hilker 2002; Rostás et al. 2002), conflicting results have also been reported (Moran 1998). The cause for these varied results is unclear. Recent literature that deals with herbivore and pathogen effects has indicated that the effects of induced resistance are often not specific to any particular attacker, and the signal transduction pathways involved in induction after attack by herbivores or pathogens may overlap (Paul et al. 2000). Hypothetically, the resistance response induced by one attacker might be effective against a range of other potential attackers. However, in our system, the induced resistances

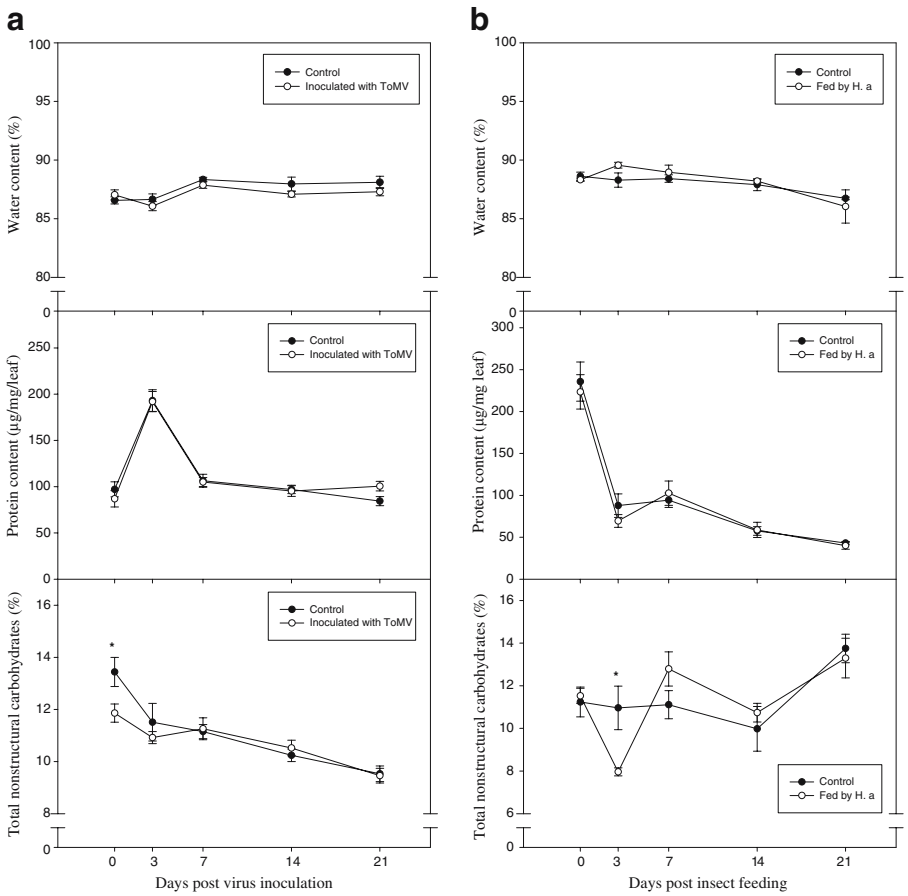

Fig. 3 Response of foliar water, protein, and total nonstructural carbohydrates (mean±SE, N=6 plants per treatment) to ToMV/*H. armigera* infestation. **a** Foliar water, protein, and total nonstructural carbohydrates contents in tomato inoculated with ToMV over time. **b** Foliar water, protein, and total nonstructural carbohydrates contents in tomato previously fed on by *H. armigera* over time. *Asterisks* denote significant differences (*P<0.05; Student's t test for independent samples)

show some degree of specificity, and resistance induced by a phytopathogenic virus may not be effective against a herbivore. In addition, studies of the role of cross-talk in tripartite interactions that involve jasmonic acid (JA) and salicylic acid (SA) pathways have demonstrated that a potential negative effect may occur between these induction-related pathways. This may lead to a reduction in resistance to the second attacker relative to plants not subject to initial attack (Stout et al. 2006).

In the short-term feeding trials, fourth-instar *H. armigera* performed differently between treatments and controls. Fourth instars grew more slowly and consumed less food on herbivore-damaged foliage than on control (undamaged) foliage. Stout and Duffey (1996) also indicated that beet armyworm, *Spodoptera exigua*, larvae grew slower and consumed less leaf tissue from corn earworm (*Helicoverpa zea*)-damaged plants than from control plants. This may be because of the decrease in the nutritional value of herbivore-damaged foliage. As previously mentioned, after having been damaged by insect feeding, plants increase their polyphenol oxidase activity and reduce their foliar carbohydrate concentrations. This increase in oxidase activity and decrease in carbohydrate content might decrease the nutritive value and thus reduce performance of subsequent feeding insects (Felton et al. 1989, 1992; Stout and Duffey 1996; Simpson and Raubenheimer 2001; Bostock et al. 2001; Lee et al. 2002). Similarly, our results of food utilization efficiencies show that larvae fed on herbivore-induced foliage have significantly lower absorption efficiency (AD). However, food conversion efficiency was not different. On the other hand, larval growth and consumption rates were not significantly different between virus-induced and control (undamaged) foliage. We found that larvae fed on virus-infected foliage had slightly lower food processing efficiencies (ECD and ECI) than those on control foliage, but the consumption and growth rates were not different. Other studies as well as ours have revealed an increase in peroxidase (POD) after insect or artificial damage in cucumber, corn, tomato, and other plants (Miller and Kelley 1989; Svalheim and Robertsen 1990; Dowd and Norton 1995; Bi et al. 1997; Moran 1998; Stout et al. 1998). Some studies have indicated that peroxidase induction is concurrent with the induction of systemic acquired resistance (SAR) against subsequent infection by the phytopathogenic fungus *Colletotrichum orbiculare* (Hammerschmidt et al. 1976; Moran 1998) or other phytopathogens. However, this increased peroxidase activity may also play a role in a plant's defense against insects, although the specific function of this enzyme in defense is unclear. We found that the elevated level of peroxidase activity in tomato after virus inoculation

may have a slight effect on an insect's food processing efficiencies, and this may be because of the toxicity of oxidized metabolites or free radicals (Duffey and Felton 1991).

In summary, this study compared the relative inducing effects of a phytopathogenic virus and herbivores on plants and on their subsequent herbivory. The result indicated that damage by insects had a stronger effect on plant chemistry and on performance of *H. armigera* than did virus infection. In addition, phytochemical analysis indicated that ToMV infection and damage by *H. armigera* can increase a host plant's level of peroxidase and polyphenol oxidase activity, respectively. In addition, *H. armigera* feeding can also reduce a host plant's carbohydrate concentration shortly after damage. A synthesis of these effects suggests that the increase in the level of polyphenol oxidase activity and the decrease in carbohydrate concentration may have some negative effects on the behavior and growth performance of *H. armigera*. Finally, although the literature concerning herbivores and pathogens has frequently shown that the effects of induced resistance are often not specific to any particular attacker (Paul et al. 2000), we found that the induced resistance did show a certain degree of specificity and that the resistance induced by a phytopathogen may not have a strong effect against subsequent herbivore attack.

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References

AYRES, P. G. 1992. Pests and Pathogens: Plant Responses to Foliar Attack. Bios Scientific, Oxford.

BALDWIN, I. T., and PRESTON, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137–145.

BEZEMER, T. M., and DAM, N. M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol* 20:617–624.

BI, J. L., MURPHY, J. B., and FELTON, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in corn to *Helicoverpa zea*. *J. Chem. Ecol* 23:97–117.

BOSTOCK, R. M., KARBAN, R., THALER, J. S., WEYMAN, P. D., and GILCHRIST, D. 2001. Signal interactions in induced resistance to pathogens and insect herbivores. *Eur. J. Plant Pathol* 107:103–111.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem* 72:284–285.

BREMAN, L. L. 1989. Tomato mosaic virus. *Plant Pathol. Circ.* 322:1–2.

CUNNINGHAM, J. P., ZALUCKI, M. P., and WEST, S. A. 1999. Learning in *Heliothis armigera*: a new look at the behaviour and control of a polyphagous pest. *Bull. Entomol. Res* 89:210–207.

DENNO, R. F., PETERSON, M. A., GRATTON, C., CHENG, J., LANGELLOTTO, G. A., HUBERTY, A. F., and FINKE, D. L. 2000. Feeding-induced changes in plant quality mediate interspecific competition between sap-feeding herbivores. *Ecology* 81:1814–1827.

DIONGUE, A., TALEKAR, N. S., CHANG, Y. F., and LAI, P. Y. 2004. Ovipositional responses of the tomato fruitworm (*Helicoverpa armigera*) to the presence of eggs and to different stages of the tomato plant. *Plant Prot. Bull* 46:303–314.

DOWD, P. F., and NORTON, R. A. 1995. Browning-associated mechanisms of resistance to insects in corn callus tissue. *J. Chem. Ecol* 21:583–600.

DUARTE, K. M. R., GOMES, L. H., GESZTESI, J. L., LOPES, J. D., and TAVARES, C. A. 2001. Monoclonal antibodies to identify tomato mosaic tobamovirus (ToMV). *Brazilian Journal of Microbiology* 32:240–242.

DUFFEY, S. S., and FELTON, G. W. 1991. Enzymatic antinutritive defenses of the tomato plant against insects, pp. 166–197, in P. A. Hedin (ed.). Naturally Occurring Pest BioregulatorsAmerican Chemistry Society, Washington, DC, USA.

FARRAR, R. R. Jr, BARBOUR, J. D., and KENNEDY, G. G. 1989. Quantifying food consumption and growth in insects. *Ann. Entomol. Soc. Am* 82:593–598.

FELTON, G. W., and EICHENSEER, H. 1999. Herbivore saliva and its effects on plant defense against herbivores and pathogens, pp. 19–36, in A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses against Pathogens and HerbivoresAPS, St. Paul, MN, USA.

FELTON, G. W., DONATO, K. K., VECCHIO, R. J., and DUFFEY, S. S. 1989. Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. *J. Chem. Ecol* 15:2667–2694.

FELTON, G. W., DONATO, K. K., BROADWAY, R. M., and DUFFEY, S. S. 1992. Impact of oxidized plant phenolics on the nutritional quality of dietary protein to a noctuid herbivore, *Spodoptera exigua*. *J. Insect Physiol* 38:277–285.

FITT, G. P. 1989. The ecology of *Heliothis* in relation to agro-ecosystems. *Annu. Rev. Entomol* 34:17–52.

GENOUD, T., and METRAUX, J. P. 1999. Crosstalk in plant cell signaling: structure function of the genetic network. *Trends Plant Sci* 4:503–507.

GREEN, S. K., HWANG, L. L., and YOU, Y. J. 1987. Epidemiology of tomato mosaic virus in Taiwan and identification of strains. *Journal of Plant Diseases and Protection* 4::86–387.

GUPTA, R., NARAYANA, B. V. L., REDDY, P. K., and RAO, G. V. R. 2003. Understanding *Helicoverpa armigera* pest population dynamics related to chicken crop using neural networks. pp. 723–726, in Proceedings of the Third IEEE International Conference on Data Mining, Nov.19–22, Melbourne, FL, USA.

HAMMERSCHMIDT, R. 1999. Induced disease resistance: how do induced plant stop pathogens? *Physiol. Mol. Plant Pathol* 55:77–84.

HAMMERSCHMIDT, R., ACRES, S., and KUC, J. 1976. Protection of cucumber against *Colletotrichum lagenarium* and *Cladosporium cucumerinum*. *Phytopathology* 66:790–793.

HATCHER, P. E. 1995. Three-way interactions between plant pathogenic fungi, herbivorous insects and their host plants. *Biol. Rev* 70:639–694.

HATCHER, P. E., and PAUL, N. D. 2000. Beetle grazing reduces natural infection of *Rumex obtusifolius* by fungal pathogens. *New Phytol* 146:325–333.

HATCHER, P. E., PAUL, N. D., AYRES, P. G., and WHITTAKER, J. B. 1994. Interactions between *Rumex* spp., herbivores and a rust fungus: *Gastrophysa viridula* grazing reduces subsequent infection by *Uromyces rumicis*. *Funct. Ecol* 8:265–272.

HATCHER, P. E., AYRES, P. G., and PAUL, N. D. 1995. The effect of natural and simulated insect herbivory, and leaf age, on the process of infection of *Rumex crispus* L. and *R. obtusifolius* L. by *Uromyces rumicis* (Schum.) Wint. *New Phytol* 130:239–249.

HAWKSWORTH, D. L. 1991. The fungal dimension of biodiversity: magnitude, significance, and conservation. *Mycol. Res* 95:641–655.

HAYNES, K. F., and MILLAR, J. G. 1998. Methods in Chemical Ecology, Vol. 2. Chapman and Hall, New York.

JOHNSON, S. N., DOUGLAS, A. E., WOODWARD, S., and HARTLEY, S. E. 2003. Microbial impacts on plant–herbivore interactions: the indirect effects of a birch pathogen on a birch aphid. *Oecologia* 134:388–396.

KARBAN, R., and MYERS, J. H. 1989. Induced plant responses to herbivory. *Annu. Rev. Ecol. Syst* 20:331–348.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, IL.

KARBAN, R., ADAMCHAK, R., and SCHNATHORST, W. C. 1987. Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. *Science* 235:678–679.

KRUESS, A. 2002. Indirect interaction between a fungal plant pathogen and a herbivorous beetle of the weed *Cirsium arvense*. *Oecologia* 130:563–569.

LEE, K. P., BEHMER, S. T., SIMPSON, S. J., and RAUBENHEIMER, D. 2002. A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval). *J. Insect Physiol* 48:655–665.

LIAO, L. H. 2003. Nutritional adaptation of galling insects investigated by globular galls of *Trioza shuiliensis* (Yang) on *Machilus japonica* var. *kusanoi* (Hyata) Liao. MS thesis. Department of Entomology, National Chung Hsing University, Taiwan.

MADSEN, J. D. 1997. Seasonal biomass and carbohydrate allocation in a southern population of *Eurasian watermilfoil*. *J. Aquat. Plant Manage* 35:15–21.

MALECK, K., and DIETRICH, R. A. 1999. Defense on multiple fronts: how do plants cope with diverse enemies? *Trends Plant Sci* 4:215–219.

MAYER, R. T., INBAR, M., MCKENZIE, C. L., SHATTERS, R., BOROWICZ, V., ALBRECHT, U., POWELL, C. A., and DOOSTDAR, H. 2002. Multitrophic interactions of the silverleaf whitefly, host plants, competing herbivores, and phytopathogens. *Arch. Insect Biochem. Physiol* 51:151–169.

MCKENZIE, C. L., SHATTERS, R. G. Jr., DOOSTDAR, H., LEE, S. D., INBAR, M., and MAYER, R. T. 2002. Effect of geminivirus infection and *Bemisia* infection on accumulation of pathogenesis-related proteins in tomato. *Arch. Insect Biochem. Physiol* 49:203–214.

MILLER, A. R., and KELLEY, T. J. 1989. Mechanical stress induces peroxidase activity in cucumber fruit. *HortScience* 24:650–652.

MORAN, P. J. 1998. Plant-mediated interactions between insects and a fungal plant pathogen and the role of plant chemical responses to infection. *Oecologia* 115:523–530.

PACKARD, G. C., and BOARDMAN, T. J. 1999. The use of percentages and size-specific indices to normalize physiological data for variation in body size: wasted time, wasted effort? *Comp. Biochem. Physiol* 122:37–44.

PAUL, N. D., HATCHER, P. E., and TAYLOR, J. E. 2000. Coping with multiple enemies: an integration of molecular and ecological perspectives. *Trends Plant Sci* 5:220–225.

RAUBENHEIMER, D. 1995. Problems with ratio analysis in nutritional studies. *Funct. Ecol* 9:21–29.

RAUBENHEIMER, D., and SIMPSON, S. J. 1992. Analysis of covariance: an alternative to nutritional indices. *Entomol. Exp. Appl* 62:221–231.

RAUBENHEIMER, D., and SIMPSON, S. J. 2003. Nutrient balancing in grasshoppers: behavioural and physiological correlates of dietary breadth. *J. Exp. Biol* 206:1669–1681.

REED, W., and PAWAR, C. S. 1982. Heliothis: a global problem, pp. 9–14, in W. Reed and V. Kumble (eds.). Proceedings of the International Workshop on Heliothis Management. Pantanchera, India, ICRISAT.

ROSTÁS, M., and HILKER, M. 2002. Asymmetric plant-mediated cross-effects between a herbivorous insect and a phytopathogenic fungus. *Agric. For. Entomol* 4:223–231.

ROSTÁS, M., and HILKER, M. 2003. Indirect interactions between a phytopathogenic and an entomopathogenic fungus. *Naturwissenschaften* 90:63–67.

ROSTÁS, M., BENNETT, R., and HILKER, M. 2002. Comparative physiological responses in Chinese cabbage induced by herbivory and fungal infection. *J. Chem. Ecol* 28:2449–2463.

ROSTÁS, M., SIMON, M., and HILKER, M. 2003. Ecological cross-effects of induced plant responses toward herbivores and phytopathogenic fungi. *Basic Appl. Ecol* 4:43–62.

RYAN, C. A., GREGORY, P., and TINGEY, W. 1982. Phenolic oxidase activities in glandular trichomes of *Solanum berthaultii*. *Phytochemistry* 21:1885–1887.

SAS Institute 1999. User's Manual, Version 8.0. SAS Institute, Cary, NC.

SCHOONHOVEN, L. M., JERMY, T., and LOON, J. J. A. 1998. Insect–Plant Biology. Chapman and Hall, New York.

SIEMENS, D. H., and MITCHELL-OLDS, T. 1996. Glucosinolates and herbivory by specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): Consequences of concentration and induced resistance. *Environ. Entomol* 25:2344–2353.

SIMPSON, S. J., and RAUBENHEIMER, D. 2001. The geometric analysis of nutrient–allelochemical interactions: a case study using locusts. *Ecology* 82:422–439.

STOUT, M. J., and DUFFEY, S. S. 1996. Characterization of induced resistance in tomato plants. *Entomol. Exp. Appl* 79:273–283.

STOUT, M. J., FIDANTSEF, A. L., and BOSTOCK, R. M. 1999. Signal interactions in pathogen and insect attack: systemic plant-mediated interactions between pathogens and herbivores of the tomato, *Lycopersicon esculentum*. *Physiol. Mol. Plant Pathol* 54:115–130.

STOUT, M. J., WORKMAN, K. V., BOSTOCK, R. M., and DUFFEY, S. S. 1998. Specificity of induced resistance in the tomato, *Lycopersicon esculentum*. *Oecologia* 113:74–81.

STOUT, M. J., THALER, J. S., and THOMMA, B. 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annu. Rev. Entomol* 51:1663–1689.

SVALHEIM, O., and ROBERTSEN, B. 1990. Induction of cucumber hypocotyls by wounding and fungal infection. *Physiol. Plant* 78:261–267.

THALER, J. S., FIDANTSEF, A. L., DUFFEY, S. S., and BOSTOCK, R. M. 1999. Trade-offs in plant defense against pathogens and herbivores: a field demonstration of chemical elicitors of induced resistance. *J. Chem. Ecol* 25:1597–1608.

THOMPSON, S. N., REDAK, R. A., and WANG, L. W. 2005. Nutrition interacts with parasitism to influence growth and physiology of the insect *Manduca sexta* L. *J. Exp. Biol* 208:611–623.

UNDERWOOD, N. 1999. The influence of induced plant resistance on herbivore population dynamics, pp. 211–229, in A. A. Agrawal, S. Tuzun, and E. Bent (eds.). Induced Plant Defenses against Pathogens and HerbivoresAPS, St. Paul, MN, USA.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insect. *Adv. Insect Physiol* 5:229–288.

WITTSTOCK, U., and GERSHENZON, J. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol* 5:300–307.

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Effect of a Rugulosin-producing Endophyte in *Picea glauca* on *Choristoneura fumiferana*

J. David Miller · Mark W. Sumarah · Gregory W. Adams

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Abstract Disease-free second instar *Choristoneura fumiferana* (eastern spruce budworm) were placed on trees infected with a rugulosin-producing needle endophyte in two experiments. They were allowed to grow to sixth instar when survivors were collected. First, by using 3-yr old trees, a comparison was made of budworm growth on infected and uninfected trees. A second experiment used 4-yr old trees to study the effect of rugulosin content in the needle on growth. This permitted an examination of a dose response in relation to growth, and allowed us to eliminate the potential for differences in environment or foliar chemistry affecting the results. At sixth instar, budworms feeding on infected trees that contained rugulosin were smaller than those on uninfected trees. At needle concentrations above the dietary low observed effect level of rugulosin for *C. fumerana* >0.5 μg g^{-1}, a dose response was seen. For the first time, this demonstrates an inverse effect in outdoor nursery experiments between budworm weight and rugulosin concentration.

Keywords Endophyte · Rugulosin · *Choristoneura fumiferana* · *Picea glauca*

J. D. Miller (✉) · M. W. Sumarah
Ottawa-Carleton Institute of Chemistry, Carleton University,
Ottawa, ON K1S 5B6, Canada
e-mail: david_miller@carleton.ca

G. W. Adams
J.D. Irving, Limited, Sussex Tree Nursery,
181 Aiton Road,
Sussex, NB E4G 2V5, Canada

Introduction

Toxic metabolites produced by endophytic fungi (*Epichloë* and *Neotyphodium* species) in fescue grasses greatly reduce the populations of associated herbivorous insects. This has a beneficial effect on plant fitness. These fungi produce various alkaloids that affect herbivore growth (insects, mammals; Clay and Schardl 2002), and they are found in plant tissues in the 1–30 μg g^{-1} range for positive samples (Rottinghaus et al. 1991; Spiering et al. 2005). There is some evidence for translocation of the toxins, as well as for a possible role of plant enzymes in changing their structures (Spiering et al. 2005). There is also one report that the presence of the endophyte directly affects the growth of a nematode that damages one grass species, regardless of the toxins (Panaccione et al. 2006).

Conifer needles are also infected by systemic fungal endophytes. Carroll and Carroll (1978) first proposed that these may play a role in limiting insect herbivory among other interactions (Carroll 1988; Ganley et al. 2004). We have been studying their role in *Picea glauca* (white spruce) in limiting conifer needle herbivory by *Choristoneura fumiferana* and some other insect species. *C. fumerana* is a major cyclical pest of spruce and fir trees, especially in the northeast US and Canada (Royama et al. 2005). Over the past two decades, we have collected foliar endophytes of conifers from the Acadian forest and studied them for their ability to produce compounds toxic to *C. fumerana* larvae (Findlay et al. 2003a,b; Sumarah, Punaiani, Blackwell, and Miller—unpublished).

Successful experimental inoculation of *P. glauca* seedlings with the needle endophyte 5WS22E1 (DAOM 229536, CBS 120377), which produces rugulosin, has been demonstrated in previous studies conducted in growth

chambers and under nursery conditions (Miller et al. 2002; Sumarah et al. 2005, 2008). Based on DNA sequence information, this fungus is a species of *Phialocephala* (unpublished data), related to strains previously reported as endophytic in Norway spruce (Grunig et al. 2002). Grown under nursery conditions, needle samples from 2-yr old infected trees contained 0.15 to 24.8 μg g^{-1} rugulosin with a geometric mean concentration of 1 μg g^{-1} (Sumarah et al. 2005). Young trees have a uniform distribution of toxin, so the variation in concentration is largely among individuals. As the trees age, there is interbranch variation in rugulosin concentration. Once inoculated as seedlings, trees in a test site maintained similar needle rugulosin concentrations 5 yr post inoculation (Sumarah et al. 2008).

In growth chamber studies, occurrence of the fungus and its toxin in needles reduced the growth rate of *C. fumerana*. The effect level was between 0.5 and 8 μg g^{-1} needle dry weight (0.7 and 10 μg g^{-1} adjusted for recovery). This approximated a best estimate of the effect level in diet based on the extent data (Miller et al. 2002). *In vitro*, rugulosin reduced body weight and/or affected instar development in *C. fumerana* (Calhoun et al. 1992; Sumarah et al. 2008), *Lambdina fiscellaria* and *Zeiraphera canadensis* (Sumarah et al. 2008) at dietary concentrations in the 10–50 μM range. The purpose of this experiment was to determine whether similar concentrations of rugulosin in needles reduced the growth of *C. fumerana* in nursery-grown trees.

Methods and Materials

Nursery Experiments Tests were conducted at the J.D. Irving, Limited Sussex Tree Nursery in New Brunswick, Canada (45°43′N, 65°31′W; elevation 21.30 m). In two successive years, several hundred seedlings grown as container stock in multipot 67 seedling containers were inoculated with the rugulosin-producing endophyte 5WS22E1 (DAOM 229536; CBS 120377). A description of the *P. glauca* populations, planting and inoculation methods used in these experiments is given in Sumarah et al. (2005). All trees were labeled with nine digit codes, and all measurements done with the samples blinded.

Three-year old Seedlings The first group was sown on June 6, 2003, and inoculated on August 1, 2003. After 4 mo in the greenhouse, they were tested for endophyte colonization by enzyme-linked immunosorbent assay (ELISA) (Sumarah et al. 2005) and segregated. Along with 5WS22E1-free seedlings from the same crop, the endophyte-positive group was maintained under commercial conditions. After 1 yr, all seedlings were transplanted into plastic pots and held at the nursery site for 1 more year post inoculation.

Four-year old Seedlings The second group was from a test sown on February 1, 2002 and inoculated on April 28, 2002. These had been tested for endophyte infection after 4 mo growth by ELISA. The seedlings were transferred to pots after 1 yr and held as above.

Experiment 1 with Younger Trees This comprised 100 trees, 50 of which were infected with the endophyte and a similar control group. On May 25, 2006, five second instars were placed on a first-year lateral branch on shoots with buds on each tree by using a fine artist's brush (nylon #5). The buds were swelling when the larvae were applied. Insects were obtained as second instars from Insect Production Services, Natural Resources Canada, Canadian Forest Service (Sault Ste. Marie, ON) and stored at 5°C. The whole tree (the experimental unit) was covered with a mesh screen bag (polyester drapery shear material) prepared after Parsons et al. (2005) for both endophyte-positive and negative trees.

Experiment 2 with Older Trees Two budworms were placed as above on each of three or four first-year branches on shoots with buds on each of 48 four-year old trees. Each branch (the experimental unit) was then covered with a mesh bag (Fig. 1). This experiment permitted a factorial

Fig. 1 Photograph of 4-yr old trees showing mesh coverings on individual branches

analysis of inter-tree (differences in needle chemistry due to shade history, needle class, etc.) and inter-branch variation in rugulosin concentration (Sumarah et al. 2008).

The use of too many insects would have defoliated the trees regardless of the presence of the toxin in most if not all treatments. The number of budworm on the two sizes of trees in the present study was determined by using data from Parsons et al. (2005). They showed that the lowest detectable damage on balsam fir of early instar sawfly larvae occurred at a density of 50 larvae/m^2 branch.

Temperature recorders (Hobo Dataloggers, Onset Computer Corporation, Pocasset, MA, USA) were placed in the holding area. The development of the insects was visually monitored until the budworms reached sixth instar. At termination, all insects that could be found were collected and frozen individually. These were weighed on a Mettler PJ360 analytical balance (± 0.02 mg). Previous studies demonstrated that the frozen wet weight was correlated with the freeze-dried weight (N=134 animals, Pearson correlation R=0.841, Bonferroni-adjusted P<0.001). Head capsule widths were determined by using a stereo microscope at ×40 with ocular and stage micrometers. After collecting the budworm, the trees were sprayed with insecticide to ensure that no larvae survived to escape.

On June 26, 2006, both experiments were terminated and five to six lateral branches were removed from the 3-yr old trees and frozen. The branches where budworms were collected from the 4-yr old trees were removed and frozen individually. Needles were removed from all branches individually, freeze-dried, and used to determine infection by ELISA and rugulosin after Sumarah et al. (2005, 2008). The method limit of detection (LOD) for cell mass was 60 ng g^{-1}, and the limit of quantification (LOQ), i.e., a positive, was 120 ng g^{-1} dry weight of needle. The LOD and LOQ for rugulosin were both 150 ng g^{-1} (Sumarah et al. 2005).

Analyses of variance (ANOVA) with the Tukey–Kramer test were performed on the weights and head capsule widths of the insects from the 3-yr old trees with NCSS 2004 software (Kaysville, UT). An equal variance t test was also done on the weights with the same software.

On the 4-yr old trees, insects were stratified into those collected from needles with toxin concentrations approximating the needle no observed effect level (>0.5 µg g^{-1}; Miller et al. 2002). As noted by the same authors, this is similar to that estimated from the low observed effect of rugulosin for *C. fumerana* concentrations in synthetic diet based on the extant data (10 µM; Calhoun et al. 1992; Sumarah et al. 2008 found the value to be 25 µM). This takes into consideration several factors that necessarily must be estimated. This includes the mean ratio of dry weight/wet weight for needles, reported summer water content of spruce needles (Gary 1971), and hydrated insoluble content of needles (Reina et al. 2001; Zwiazek 1991). The artificial spruce budworm diet used has a water activity (a_w) of ~0.99, i.e., is essentially aqueous.

For statistical purposes, values negative by ELISA and below the analytical detection limit were entered at half the detection limit for rugulosin (as is normal for exposure characterization). Needles that were negative for rugulosin but positive by ELISA were entered at the detection limit because there was positive evidence for the presence of the fungus in the needle. Routine statistical tests as well as the paired t-tests, Wilcoxon signed rank test, and ANOVA were performed on the data from the 4-yr old trees by using SYSTAT v 10.2 (San Jose, CA, USA).

Results

The mean temperature was 18.4±2.2°C starting at 15–17°C and rising to 23°C in the last few days. Virtually all the insects recovered were sixth instar.

Three-year old Trees ELISA analysis of the control group revealed that five of the endophyte negative trees were positive at 36 mo, i.e., were false negatives when previously tested. These were excluded from the analysis. The geometric mean toxin concentration corrected for recovery in the endophyte positive group was 0.85 µg g^{-1}. The number of budworms recovered per tree was 3.3 for the infected (161 total) and 3.5 for the uninfected trees (152 total). ANOVA indicated that the weights of treated and control budworms were significantly different (P=0.018; df=1; F ratio 5.64). The distribution of insect weights between endophyte-infected and uninfected 3-yr old trees is shown in Fig. 2. Mean weight of the controls at termination

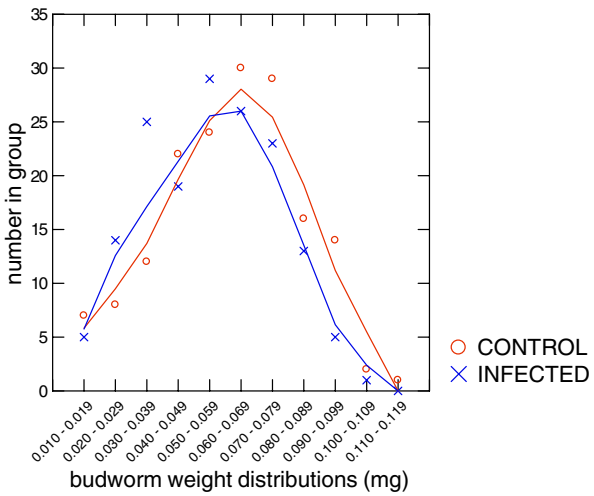

Fig. 2 Distribution of insect weights between endophyte-infected and uninfected 3-yr old trees; the weights between the two groups were significantly different by ANOVA, P=0.018)

was 0.061±0.02 mg and 0.055±0.02 mg for those collected on the infected trees. This difference was statistically different (P=0.009, equal-variance t test). Mean head capsule widths for the budworm on the control and infected trees were 1.89±0.21 mm and 1.90±0.18 mm, respectively. These were not different by ANOVA (P=0.646; df=1, F ratio 0.21).

Four-year old Trees The geometric mean rugulosin concentration corrected for recovery was 0.8 μg g^{-1} in all samples and the mean of the positives ≥0.5 μg g^{-1} was 1.3 μg g^{-1}. By ANOVA, there was no significant interaction between tree and insect weight, or tree and rugulosin concentration. The mean weight of the 166 insects recovered from the 4-yr old trees was 0.072± 0.02 mg. Considering the branches above 0.5 μg g^{-1}, there was an inverse relationship between rugulosin concentration and budworm weight (Pearson correlation −0.288; Bonferroni-adjusted P=0.023). Head capsule widths averaged 1.95±0.15 mg.

An average of 1.4 budworms were recovered from the original two placed on each branch. When the branches were stratified into quartiles of rugulosin concentration (40 each), the frequency of branches with two survivors was higher in the three quartiles with lower rugulosin concentrations compared to that with the highest concentration (ca. 1.6 versus 1.3; Wilcoxon signed rank test, P=0.025).

Disregarding a few trees where budworms were collected from only one branch, the trees fell into one of three distributions. A large percentage (38%) comprised the situation where all branches were above the threshold of rugulosin toxicity, and 16% where all were below the threshold. In neither case, would there be intra-tree variation in rugulosin concentration to study. The remainder, comprising the majority, had individual branches above and below the threshold. Typical data from trees with multiple branches analyzed are shown in Table 1. All three branches analyzed on tree 1034 had concentrations far in excess of the toxic threshold. Only one budworm per branch was recovered, and the animal from the branch with the lowest concentration had the highest weight, higher than the mean noted above. One branch from tree 1360 was far above the threshold. In this case, the weight of the insect collected was at the mean. Two insects were collected from the two remaining branches with a mean weight at the average. A similar pattern can be seen in tree 2022.

Discussion

In growth chamber studies, the occurrence of fungus and its toxin in needles reduced *C. fumerana* growth. These experiments were done by using detached needles from 4-mo old seedlings with an established method to screen for

Table 1 Budworm weights on different branches from 4-yr old trees and rugulosin concentration

Tree	Branch	Budworm	Weight (mg)	Rugulosin (μg g^{-1})
1034B	1	1	0.061	4.20
1034B	2	1	0.114	1.53
1034B	3	1	0.067	3.59
1040B	1	1	0.081	0.09[a]
1040B	2	2	0.045	0.39[a]
1040B	2	1	0.044	0.39[a]
1040B	3	1	0.038	0.87
1148B	1	1	0.116	0.09[a]
1148B	3	1	0.052	1.29
1148B	3	2	0.044	1.29
1171B	1	1	0.028	0.89
1171B	1	2	0.083	0.89
1171B	2	1	0.048	2.27
1360B	1	1	0.072	3.55
1360B	2	2	0.062	0.09[a]
1360B	2	1	0.077	0.09[a]
1360B	3	1	0.061	0.09[a]
1360B	3	2	0.080	0.09[a]
2022B	1	1	0.035	10.21
2022B	2	1	0.057	0.17[a]
2022B	3	2	0.072	0.17[a]
2022B	3	1	0.066	0.17[a]

[a] Below dietary concentration of threshold for toxicity of rugulosin *in vitro.*

variation in foliar resistance (Miller et al. 2002). The present experiments were conducted by placing budworm on trees that grow outdoors.

We have examined two aspects of the effect of rugulosin in needles on *C. fumerana* growth under outdoor nursery conditions. The first was a comparison of the impact on budworm growth on infected trees and uninfected trees. The second was to use a group of older trees such that needles from the infected tree served as a control. This strategy is typically used in toxicology to eliminate possible confounding variables that arise from intra-individual variance. The intention here was to look for evidence of a dose response to rugulosin. These might be anticipated to result from potential changes in needle chemistry because of variables that include needle age, shade, and the fungus itself. Shading (Lhotakova et al. 2007) and needle age as well as soil conditions are known to affect foliar composition, and these in turn can affect budworm growth (Carisey and Bauce 1997; Clancy et al. 2004; Nealis and Nault 2005). The trees used in the present studies represented a diverse genetic population used for reforestation in eastern Canada and Maine.

Although an attempt was made to terminate the experiments so that there was a distribution of instars, virtually all were sixth instar. This is probably because of higher temperatures in the final 24 hr of the experiments compared to the previous week. Mean head capsule widths were ~1.94 mm. This value is at the upper end of those found in nature. McGugan (1954) reported that the mean head capsule widths from a natural epidemic in northwestern Ontario were 1.63 mm for males and 1.79 mm for females. Field collections from New Brunswick were reported to be 1.66 mm without sex being specified (Anon. 1981, Data Fact Sheet, Determination of spruce budworm larval stage; CANUSA Spruce Budworm Program). This difference may relate to the fact that the animals used in this experiment were parasite- and disease-free versus the natural situation (see the following).

The distribution of animal weights collected on infected needles at termination was different from the respective controls (Fig. 2). There was also a statistically significant difference in budworm weights between the two groups. As in the growth chamber, the presence of the fungus and its toxin reduced *C. fumerana* growth. Similar experiments on infected and uninfected grass endophytes have resulted in similar findings and effects on development (e.g., Hardy et al. 1985).

The second aspect of the present experiments was the use of older trees from which the effect on budworm growth on multiple branches from the same infected tree could be observed. This was possible because variation in rugulosin concentration was observed among individual branches within a single tree. As was found previously, rugulosin concentrations were typically above those that affect the growth of *C. fumerana* in needles and *in vitro* (Miller et al. 2002; Sumarah et al. 2008) and *Lambdina fiscellaria* and *Zeiraphera canadensis in vitro* (Sumarah 2008). In this component of the study, two effects were seen. First, a comparison of those branches with concentrations above the threshold of rugulosin toxicity demonstrated there was an inverse relationship between budworm weight and rugulosin concentration (-0.288; $P=0.023$). A further support of the reliability of this conclusion comes from the fact that on the branches with the highest rugulosin concentrations, survival was significantly lower than on branches compared to those with lower concentrations. In addition, there was a small but significantly increased weight in the survivor population found on branches in the quartile of branches with the highest rugulosin concentration. It is known that when *C. fumerana* are under nutrient limitations, they will resort to cannibalism, which provides a plausible explanation of the latter observation. Nonetheless, there was evidence of a rugulosin dose response where single animals were recovered (e.g., tree 1034B).

In synthetic diet (Calhoun et al. 1992; Sumarah et al. 2008), the presence of ca. 10- to 25-μM rugulosin resulted in the reduction in *C. fumerana* growth rate (ranging to 50 μM for some other herbivorous insect species). In needles, the minimum lowest observed effect level of >0.5 μg g^{-1} appears close to this value (Miller et al. 2002; see above). Using the weight of evidence rule in toxicology, the effects seen in this study are largely explained by the toxin, and are consistent with other endophyte interactions that have been studied.

In a monograph on *C. fumerana* population dynamics in New Brunswick, Royama (1984) discusses the cyclical nature of epidemics to date. He postulated an unknown factor to modulate upward the observed population changes in relation to his model. The central characteristic of the unknown factor was that it increased the response of the insect populations to known factors such as predation and disease. As the primary effect of rugulosin *in vitro* and in the plant is to slow the growth rate, the insects would be exposed to these environmental and biotic factors for longer durations. We have also postulated that this effect might put cohorts of the insects out of reproductive synchrony.

Another hypothesis is that the presence of the toxins would result in immune modulation, which is a feature of mammalian exposure. Moth immunology remains incompletely understood but shares some features of the mammalian system with more primitive features. In both experiments, the insects were uniformly larger than expected from field data (McGugan 1954; Anon 1981), i.e., at the top of the normal range for sixth instar. As noted, these animals were disease-free and reared on trees grown under optimal conditions. When budworm populations are

expanding, the percentage of insects infested with parasitoids rises (e.g., Shepherd 1959; Quayle et al., 2003). In addition, there is a prevalence of pathogenic *Bacillus* species on budworm collected on balsam fir in New Brunswick (Strongman et al. 1997). Adventitious occurrence of the facultative fungal pathogen *Aspergillus fumigatus* is common in trees infested with *C. fumerana* (Miller et al. 1985; Sumarah et al. 2008). Had the tests been done in the wild and thereby including other mortality factors, fitness on rugulosin-containing branches might have been lower.

In summary, we have shown that the presence of a foliar endophyte of conifers and its toxin, rugulosin, in trees grown under production conditions resulted in reduced growth of *C. fumerana* in a dose-dependent manner. In addition, these experiments show that the effect is primarily explained by rugulosin. Further experiments are needed to examine the implications for growth and development under field conditions.

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References

CALHOUN, L. A., FINDALY, J. A., MILLER, J. D., and WHITNEY, N. J. 1992. Metabolites toxic to spruce budworm from balsam fir needle endophytes. *Mycol. Res.* 96:281–286.

CARISEY, N., and BAUCE, E. 1997. Balsam fir foliar chemistry in middle and lower crowns and spruce budworm growth, development, food and nitrogen utilization. *J. Chem. Ecol.* 23:1963–1978.

CARROLL, G. C. 1988. Fungal endophytes in stems and leaves: from latent pathogen to mutualistic symbiont. *Ecology* 69:2–9.

CARROLL, G. C., and CARROLL, F. E. 1978. Studies on the incidence of coniferous needle endophytes in the Pacific Northwest. *Can. J. Bot.* 56:3034–3043.

CLANCY, K. M., CHEN, Z., and KOLB, T. E. 2004. Foliar nutrients and induced susceptibility: genetic mechanisms of Douglas-fir resistance to western spruce budworm defoliation. *Can. J. For. Res.* 34:939–949.

CLAY, K., and SCHARDL, C. 2002. Evolutionary origins and ecological consequences of endophyte symbiosis with grasses. *Am. Nat.* 160:S99–S127.

FINDLAY, J. A., LI, G., MILLER, J. D., and WOMILOJU, T. 2003a. Insect toxins from conifer endophytes pp. 13–16, in NYaylı, and MKüçük (eds.). Proceedings of the 1st International Congress on the Chemistry of Natural Products (ICNP–2002)Karadeniz Technical University, Trabzon, Turkey.

FINDLAY, J. A., LI, G., MILLER, J. D., and WOMILOUJU, T. O. 2003b. Insect toxins from spruce endophytes. *Can. J. Chem.* 81:284–292.

GANLEY, R. J., BRUNSFELD, S. J., and NEWCOMBE, G. 2004. A community of unknown, endophytic fungi in Western White pine. *Proc. Natl. Acad. Sci. U. S. A.* 101:10107–10112.

GARY, H. L. 1971. Seasonal and diurnal changes in moisture contents and water deficits of Engelmann spruce needles. *Botanical Gazette* 132:327–332.

GRUNIG, C. R., SIEBER, T. N., OGERS, S. O., and HOLDRIEDER, O. 2002. Genetic variability among strains of *Phialocephala fortinii* and phylogenetic analysis of the genus *Phialocephala* based on rDNA ITS sequence comparisons. *Can. J. Bot.* 80:1239–1249.

HARDY, T. N., CLAY, K., and HAMMOND, A. M. 1985. Fall armyworm (Lepidoptera, Noctuidae)—a laboratory bioassay and larval preference study for the fungal endophyte of perennial ryegrass. *J. Econ. Ent.* 78:571–575.

LHOTAKOVA, Z., ALBRECHTOVA, J., MALENOVOSKY, Z., OCK, B. N., POLAKA, T., and CUDLIN, P. 2007. Does the azimuth orientation of Norway spruce (*Picea abies*/L./Karst.) branches within sunlit crown part influence the heterogeneity of biochemical, structural and spectral characteristics of needles? *Environ. Exp. Bot.* 59:283–292.

MCGUGAN, B. M. 1954. Needle-mining habits and larval instars of the spruce budworm. *Can. Entomol.* 36:439–454.

MILLER, J. D., STRONGMAN, D., and WHITNEY, N. J. 1985. Observations on fungi associated with spruce budworm infested balsam fir needles. *Can. J. For. Res.* 15:896–901.

MILLER, J. D., MACKENZIE, S., FOTO, M., ADAMS, G. W., and FINDLAY, J. A. 2002. Needles of white spruce inoculated with rugulosin-producing endophytes contain rugulosin reducing spruce budworm growth rate. *Mycol. Res.* 106:471–479.

NEALIS, V. G., and NAULT, J. R. 2005. Seasonal changes in foliar terpenes indicate suitability of Douglas-fir buds for western spruce budworm. *J. Chem. Ecol.* 31:683–696.

PANACCIONE, D. G., KOTOCON, J. B., SCHARDL, C. L., JOHNSON, R. D., and MORTON, J. B. 2006. Ergot alkaloids are not essential for endophytic fungus-associated population suppression of the lesion nematode, *Pratylenchus scribneri*, on perennial ryegrass. *Nematology* 8:583–590.

PARSONS, K., QUIRING, D., PIENE, H., and MOREAU, G. 2005. Relationship between balsam fir sawfly density and defoliation in balsam fir. *For. Ecol. Manag.* 205:325–331.

QUAYLE, D., EGNIERE, J., CAPPUCCINO, N., and DUPONT, A. 2003. Forest composition, host-population density and parasitism of *Choristoneura fumiferana* eggs by *Trichogramma minutum*. *Ent. Exp. Applic.* 107:215–227.

REINA, J. J., DOMINGGUEZ, E., and HEREDIA, A. 2001. Water sorption–desorption in conifer cuticles: The role of lignin. *Physiologia Plantarum* 112:372–378.

ROTTINGHAUSE, G. E., GARNER, G. B., CORNELL, C. N., and ELLIS, J. L. 1991. HPLC method for quantitating ergovaline in endophyte-infested tall fescue: seasonal variation of ergovaline levels in stems with leaf sheaths, leaf blades and seed heads. *J. Agric. Food Chem* 39:112–115.

ROYAMA, T. 1984. Population dynamics of the spruce budworm. *Ecol. Monogr.* 54:429–462.

ROYAMA, T., MACKINNON, W. E., KETTELA, E. G., CARTER, N. E., and HARTLING, L. K. 2005. Analysis of spruce budworm outbreak cycles in New Brunswick, Canada, since 1952. *Ecology* 86:1212–1224.

SHEPHERD, R. F. 1959. Phytosociological and environmental characteristics of outbreak and non-outbreak areas of the two-year cycle spruce budworm, *Choristoneura fumiferana*. *Ecology* 40:608–620.

SPIERINGS, M. J., LANE, G. A., CHRISTENSEN, M. J., and SCHMIDT, J. 2005. Distribution of the fungal endophyte *Neotyphodium lolii* is not a major determinant of the distribution of fungal alkaloids in *Lolium perenne* plants. *Phytochemistry* 66:195–202.

STRONGMAN, D. B., EVERLEIGH, E. S., VAN FRANKENHUYZEN, K., and OYAMA, T. 1997. The occurrence of two types of entomopathogenic bacilli in natural populations of the spruce budworm, *Choristoneura fumiferana*. *Can. J. For. Res.* 27:1922–1927.

SUMARAH, M. W., MILLER, J. D., and ADAMS, G. W. 2005. Measurement of a rugulosin-producing endophyte in white spruce seedlings. *Mycologia* 97:770–776.

SUMARAH, M. W., ADAMS, G. W., BERGHOUT, J., SLACK, G., WILSON, A. and MILLER, J. D. 2008. Spread and persistence of a rugulosin-producing endophyte in *Picea glauca* seedlings. *Mycological Res.* (in press).

ZWIAZEK, J. J. 1991. Cell wall changes in white spruce (*Picea glauca*) needles subjected to repeated drought stress. *Physiol. Planta.* 82:513–518.

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Altitudinal Variation of Phenolic Contents in Flowering Heads of *Arnica montana* cv. ARBO: a 3-Year Comparison

Renate Spitaler · Andrea Winkler · Isabella Lins · Sema Yanar · Hermann Stuppner · Christian Zidorn

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Abstract In continuation of our studies of altitudinal effects on secondary metabolite profile of flowering heads from taxa of the Asteraceae, we investigated phenolic contents and radical scavenging potential from cultivated plants of *Arnica montana* cv. ARBO during the growing seasons 2003, 2004, and 2005. By conducting experiments on potted plants, we excluded that differences in phenolic contents from plants grown at different altitudes were related primarily to differences in soil composition at these sites. To assess altitudinal and interseasonal variation, plants of *A. montana* cultivar ARBO were grown in nine experimental plots at altitudes between 590 and 2,230 m at Mount Patscherkofel near Innsbruck, Austria. In all growing seasons and regardless of the soil the plants were grown in, the proportion of flavonoids with vicinal-free hydroxy groups in ring B to flavonoids lacking this feature, and the total amount of caffeic acid derivatives, significantly increased with elevation. These increases of antioxidant phenolics corresponded to an increase of the radical scavenging potential of extracts from plants grown at different altitudes. The results are discussed in regard to previous studies that suggest that enhanced UV-B radiation and decreased temperatures trigger augmented biosynthesis of UV-absorbing and antioxidant phenolics in higher plants.

Keywords *Arnica montana* · Altitudinal effects · Chemical ecology · Phenolics · Caffeic acid esters · Flavonoids · Natural products · UV radiation · Radical scavenging activity · Temperature regime

R. Spitaler · A. Winkler · I. Lins · S. Yanar · H. Stuppner · C. Zidorn (✉)
Institut für Pharmazie, Abteilung Pharmakognosie, Universität Innsbruck, Innrain 52, Josef-Moeller-Haus, A-6020 Innsbruck, Austria
e-mail: Christian.H.Zidorn@uibk.ac.at

Introduction

Previous studies on altitudinal variation of phenolic compounds in different wild and cultivated taxa from the Asteraceae suggested that factors related to altitude of the growing site are causing significant shifts in the quantity of secondary metabolites in flowering heads (Zidorn and Stuppner 2001; Zidorn et al. 2005; Spitaler et al. 2006). Such effects were first observed in wild Asteraceae populations that grow in natural habitats, e.g., in populations from *Leontodon helveticus* Mérat emend. Widder growing in the Eastern Alps (Zidorn and Stuppner 2001). Subsequent studies confirmed that altitudinal effects also occur in populations from neophytic taxa in newly acquired distribution areas (Zidorn et al. 2005). Genetic differences as the only source of altitudinal variation were ruled out by cultivation experiments with *Arnica montana* L. cv. ARBO (Spitaler et al. 2006) in the Tyrolean Alps.

A multitude of environmental factors change with altitude of the growing site. These include precipitation, mean temperature, soil, wind speed, low and high temperature extremes, duration of snow cover, length of vegetation period, and intensity of radiation under clear sky conditions. Enhanced UV-B radiation and lower temperatures at high altitudes have been intensely discussed as having an impact on plant secondary metabolism (Körner 1999; Bilger et al. 2007). The increase of irradiance, especially UV-B radiation, is well documented for the Alps and the Innsbruck area, in particular (Blumthaler et al. 1997). Irradiance under clear sky conditions increases by 8% for total irradiance, 9% for UV-A radiation, and 18% for

erythemal effective radiation (mainly UV-B) per 1,000 m of altitude.

As a protective response against damage from excessive UV-B radiation, plants enhance the biosynthesis of UV-B-absorbing and antioxidant phenolic compounds (Markham et al. 1998b; Körner 1999). The induction of enzymes involved in flavonoid biosynthesis with experimentally supplemented UV radiation is well known (Wellmann 1975; Jaakola and Määttä-Riihinen 2004). The role of phenolics in protecting plants from peak UV-B exposure is not limited to UV-B screening (Markham et al. 1998b), and observed changes in ratios among different types of flavonoids (*ortho*-dihydroxylated vs others) indicate that the enhanced radical scavenging potentials of *ortho*-dihydroxylated flavonoids like luteolin and quercetin are also contributing to UV-B protective activity (Markham et al. 1998a; Spitaler et al. 2006).

Field experiments to assess the actual impact of the altitude of the growing site on plant secondary metabolism have been performed only for a limited number of species. The present study aimed to clarify three questions. These questions are based on an *Arnica* field trial (Spitaler et al. 2006): (1) Do (random) natural climate fluctuations cause the observed variation; (2) are altitudinal variations due to differences in soil characteristics; and (3) do differences in profiles of phenolics correlate with antioxidant capacity?

Methods and Materials

Plant Material Plantlets from *A. montana* cultivar ARBO were purchased from Saatzucht Steinach GmbH (Steinach/Germany) in May 2002 and planted at Mount Patscherkofel as described previously (Spitaler et al. 2006). Experimental plots were located at the following altitudes above mean sea level: site 1, 590 m; site 2, 870 m; site 3, 1,020 m; site 4, 1,260 m; site 5, 1,430 m; site 6, 1,620 m; site 7, 1,890 m; site 8, 1,960 m; site 9, 2,230 m. In 2004, a second batch of plantlets was planted in black plastic pots (23.5 cm diameter; depth 18.5 cm) filled with a mixture (1/1, *v*/*v*) of peat and sand. These pots were transferred to the experimental sites during the summer of 2004 and dug into the surrounding soil. To exclude ontogenetic differences, only flowering heads with at least two rows of flowering ray florets were collected (Douglas et al. 2004). Terminal flowering heads of each stem were collected separately from lateral capitula. Only terminal flowering heads were analyzed (Spitaler et al. 2006). Flowering heads were collected in batches of 12, air-dried, and afterwards kept at −20°C until analysis. For each site and harvest year, three batches of flowering heads were collected and analyzed separately. Flowering heads were pooled in batches of 12 to minimize the effects of chance factors due to variations among individual plants, without the necessity of an unreasonably high number of high-performance liquid chromatography (HPLC) analyses. Three batches per altitude and season (and soil treatment) were analyzed to gain some idea about variation among the populations during each season at each altitude.

Plants for phytochemical analyses were collected on the following dates (numbers in brackets refer to the nine experimental sites from 1, lowest altitude, to 9, highest altitude): 2003 growing season: see Spitaler et al. (2006); 2004 growing season: 07.06. (1); 17.06. (2, 3); 30.06. (4, 5, 6); 08.07. (6, 7); 21.07. (4, 6, 7); and 04.08. (7, 8); 2005 growing season natural soil: 31.05. (1); 06.06. (2); 10.06. (1); 22.06. (4, 5, 6); 30.06. (4, 5, 6, 7); 09.07. (7, 8); 14.07, (7, 8); 21.07. (8); 04.08. (9); and 12.08. (9); and 2005 growing season potted plants: 30.05. (2); 31.05. (1); 10.06. (3); 17.06. (2, 3): 22.06. (3, 4, 5, 6); 30.06. (4, 5); 09.07. (7, 8); 14.07. (8); 21.07. (7, 8); 28.07. (9); 04.08. (7, 9); and 12.08. (9).

Extract Preparation Each batch of 12 air-dried flowering heads was ground, and phenolics were analyzed by HPLC/DAD as described previously (Spitaler et al. 2006). In detail, after adding 2.50 mg of the internal standard compound cynarin as a stock solution in MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, *v*/*v*/*v*), ground flowering heads (500.0 mg) were sonicated twice for 30 min with a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, *v*/*v*/*v*), and once for 30 min with a mixture of MeOH and H_2O (1/1, *v*/*v*; total extraction volume 25 ml for each cycle). Extracts were filtered, the remaining plant material rinsed with 20 ml of a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, *v*/*v*/*v*), and the combined extracts were filled up to 100.0 ml with a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, *v*/*v*/*v*); 10.0 ml of this solution was brought to dryness *in vacuo* and redissolved in 2.00 ml of a mixture of MeOH, $(CH_3)_2CO$, and H_2O (3/1/1, *v*/*v*/*v*). After filtration, this solution was used for HPLC analysis. Comparative investigations that used different extraction media and longer times of sonication and a larger number of sonication cycles proved that the chosen procedure led to an exhaustive extraction. All quantitative analyses were run in triplicate. The extraction procedure for the 1,1-diphenyl-2-picrylhydrazyl (DPPH) assays was identical to the one described above, with the exception that no cynarin (a potent radical scavenger itself) was added before the first step of extraction.

HPLC Analyses HPLC analyses of phenolics were performed as described previously (Spitaler et al. 2006). In detail, HP-1090 and HP-1100 ChemStations equipped with DAD detectors were employed, and the following parameters were applied: column, Phenomenex Synergi Hydro-

Rp 80A 150×4.6 mm (4 μm material); guard column, Phenomenex Security Guard C18 (ODS, Octadecyl) 4.0 mm×3.0 mm; mobile phase A, H_2O/HCOOH/CH_3COOH (99/0.9/0.1, *v*/*v*/*v*); phase B, MeCN/MeOH/HCOOH/CH_3COOH (89/10/0.9/0.1, *v*/*v*/*v*/*v*); flow rate, 1.00 ml/min; injection volume 10 μl; detection wavelength 350 nm; oven temperature, ambient; linear gradient, 0 min 5% B, 5 min 15% B, 20 min 16% B, 35 min 18% B, 45 min 19% B, 55 min 27.5% B, 60 min 65% B, 65 min 98% B, 70 min stop; post time, 12 min. The amounts of phenolics were estimated by comparing the peak areas obtained for the particular flavonoids **F1**–**F6** and caffeic acid derivatives **P1**–**P9** with the peak area obtained for the internal standard cynarin (**CYN**).

Compound Identification Phenolics were grouped into flavonoids (**F**) and caffeic acid derivatives (phenolic acids, **P**) based on their characteristic UV spectra (**F**: a broad maximum at 350 nm, **P**: a maximum at 330 nm with a shoulder at 295 nm). Peaks assignable to flavonoids (**F1**–**F6**) and phenolic acids (**P1**–**P9**) were numbered consecutively with increasing HPLC retention times. Peak characterization and compound identification was performed as previously described (Spitaler et al. 2006). Peak identities are assigned in Table 1.

DPPH Assays DPPH assays were performed in 96-well-plates by using a Hidex Chameleon plate reader in the absorption mode at 515 nm. DPPH was purchased from Sigma-Aldrich, Steinheim, Germany. Ascorbic acid, which was used as a reference compound, was purchased from Merck, Darmstadt, Germany. The final concentration of DPPH in the test mixture was set to 40.0 mg/l. *Arnica* extracts were tested in final concentrations of 1.00, 0.50, 0.25, 0.125, and 0.0625 mg/ml. The reference compound ascorbic acid (Merck, Darmstadt, Germany) was measured in final concentrations of 20.0, 10.0, 6.00, 2.00, and 1.00 μg/ml. Each concentration was applied six times on one plate, and for each extracts, three plates were prepared. After the solutions were mixed, plates were shaken slightly and kept in the dark for 30 min. Then, plates were measured three times at 515 nm.

IC_{50} values for each replicate of the extracts and ascorbic acid solutions were calculated by using the following formula: $IC_{50}=[(50-LP)/(HP-LP)\times(HC-LC)]+LC$. LP = low percentage, i.e., highest percent inhibition less than 50%; HP = high percentage, i.e., lowest percent inhibition greater than 50%; HC = high concentration, i.e., concentration of test substance (or extract) at the high percentage, LC = low concentration, i.e., concentration of test substance (or extract) at the low percentage. To get standardized results

Table 1 Pearson correlation coefficients (*r*) and corresponding *P* values for the observed correlation between the contents of *Arnica montana* phenolics and the altitude of the growing site

Compound Common Name	Number	2003[a]		2004[b]		2005[c]		2005 Potted Plants[d]	
		r	*P*	*r*	*P*	*r*	*P*	*r*	*P*
Quercetin 3-*O*-β-d-glucoside	**F1**	0.247	0.224	0.216	0.374	**0.490**	**0.021**	0.281	0.184
Patuletin 3-*O*-β-d-glucoside	**F2**	0.162	0.430	−0.016	0.947	0.109	0.629	−0.215	0.313
Kaempferol 3-*O*-β-d-glucoside	**F3**	**−0.534**	**0.005**	−0.377	0.111	−0.205	0.360	−0.105	0.625
Kaempferol 3-*O*-β-d-glucuronide	**F4**	−0.219	0.282	−0.391	0.098	0.349	0.111	−0.059[e]	0.783[e]
6-Methoxykaempferol 3-*O*-β-d-glucoside	**F5**	−0.157	0.444	−0.159	0.516	−0.014	0.952	N.a.	N.a.
Hispidulin	**F6**	0.113	0.581	**−0.617**	**0.005**	**−0.718**	**0.000**	**−0.532**	**0.007**
Sum of flavonoids **F1–F6**	$\sum_{F1-F6}$	−0.067	0.746	−0.175	0.474	0.255	0.253	0.035	0.869
Quotient Q/K=(**F1**+**F2**)/(**F3**+**F4**+**F5**+**F6**)	Q/K	**0.607**	**0.001**	**0.738**	**0.000**	**0.658**	**0.001**	**0.484**	**0.017**
Chlorogenic acid	**P1**	−0.209	0.307	0.104	0.673	**0.772**	**0.000**	**0.825**	**0.000**
Unknown hydroxycinnamate ester	**P2**	−0.017	0.935	**−0.604**	**0.006**	N.d.	N.d.	0.312	0.138
Unknown hydroxycinnamate ester	**P3**	−0.218	0.284	0.413	0.079	**0.769**	**0.000**	**0.535**	**0.007**
3,5-Dicaffeoylquinic acid	**P4**	0.102	0.621	0.324	0.176	0.397	0.068	**0.619**	**0.001**
1-Methoxyoxaloyl-3,5-dicaffeoylquinic acid	**P5**	**0.705**	**0.000**	**0.524**	**0.021**	**0.484**	**0.022**	**0.607**	**0.002**
4,5-Dicaffeoylquinic acid	**P6**	0.338	0.091	−0.164	0.501	0.417	0.053	0.348	0.096
Unknown hydroxycinnamate ester	**P7**	**0.544**	**0.004**	**0.467**	**0.044**	**0.537**	**0.010**	**0.636**	**0.001**
Unknown Hydroxycinnamate ester	**P8**	**0.449**	**0.021**	**0.601**	**0.006**	**0.460**	**0.031**	**0.782**	**0.000**
Unknown hydroxycinnamate ester	**P9**	**0.628**	**0.001**	0.416	0.076	**0.544**	**0.009**	**0.464**	**0.022**
Sum of caffeic acid derivatives **P1–P9**	$\sum_{P1-P9}$	**0.422**	**0.032**	0.381	0.107	**0.670**	**0.001**	**0.696**	**0.000**

r Values with $P<0.05$ printed in bold face

F Flavonoids, ***P*** caffeic acid derivatives

[a] Detailed quantification data for each compound, sample, and site are available as supplementary material to the Spitaler et al. (2006) paper.

[b,c,d] Detailed quantification data are published in the diploma theses of Yanar (2006), Lins (2007), and Winkler (2006), respectively.

[e] Not separated from peak **F5** in this set of analyses

Table 2 Overview of total amounts (mg/g) of flavonoids ($\sum_{F1-F6}$) in flowering heads of *Arnica montana* cv. ARBO grown in different altitudes and harvested in different growing seasons

Altitude (m)	2003		2004		2005		2005 Potted Plants	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
590	19.88	3.51	17.06	3.22	14.00	0.67	15.86	0.75
870	22.42	0.82	16.27	N.a.[a]	21.47	0.79	18.31	2.19
1,020	17.80	3.23	14.78	N.a.[a]	N.a.[c]	N.a.[c]	19.13	1.40
1,260	21.30	2.34	16.38	5.23	19.70	0.85	21.06	1.11
1,430	23.45	1.21	20.37	3.48	21.05	2.45	21.25	0.32
1,620	20.70	0.27	16.60	0.83	21.02	2.65	21.73	2.50
1,890	21.53	1.58	17.13	1.98	22.17	2.78	19.89	3.39
1,960	18.26	1.48	13.18	3.48	18.21	2.66	16.22	6.41
2,230	17.39	1.40	N.a.[b]	N.a.[b]	15.62	0.44	17.21	4.15

[a] These sites yielded in the harvest year at hand only one charge of flowering heads for analysis and therefore SDs between charges could not be calculated.
[b] Due to adverse weather conditions, this site yielded no flowering heads in the 2004 growing season.
[c] Plants of site 3 in the natural soil had all died until 2005.

for the radical scavenging activity of the extracts, which can be compared to results from other studies, inhibitory concentrations were expressed as ascorbic acid equivalents per gram dried plant material by using the following formula: AAE (mg/g)=1,000×IC_{50AA}/IC_{50AMO}. AAE: ascorbic acid equivalents, IC_{50AA}: 50% inhibitory concentration of ascorbic acid (AA), $IC50_{AMO}$: 50% inhibitory concentration of *A. montana* (AMO) flowering heads.

Data Analysis Pearson correlation coefficients and linear regression equations were calculated by using the MINITAB 13.31 software package (State College, PA, USA).

Results

Variation of Phenolics in Relation to Altitude Analogous to a previous investigation, six flavonoids **F1-F6** and nine phenolic acids **P1-P9** were quantified by HPLC: quercetin 3-*O*-β-D-glucoside **F1**, patuletin 3-*O*-β-D-glucoside **F2**, kaempferol 3-*O*-β-D-glucoside **F3**, kaempferol 3-*O*-β-D-glucuronide **F4**, 6-methoxykaempferol 3-*O*-β-D-glucoside **F5**, hispidulin **F6**, chlorogenic acid **P1**, 3,5-dicaffeoylquinic acid **P4**, 1-methoxyoxaloyl-3,5-dicaffeoylquinic acid **P5**, 4,5-dicaffeoylquinic acid **P6**, and unknown hydroxycinnamate esters **P2**, **P3**, **P7**, **P8**, and **P9** (Spitaler et al. 2006 and Table 1). In Table 1, Pearson correlation coefficients for individual compounds and for the sums of compound groups (flavonoids and caffeic acid derivatives, and the ratio of *ortho*-dihydroxy vs other flavonoids) are summarized.

Quantification data for the total flavonoid contents are summarized in Table 2. Total flavonoid content showed no significant positive correlation with altitude of the growing site in any growing season (Table 1). Neither did most of the individual compounds show statistically significant correlation with altitude. In contrast, the ratio of flavonoids with a 3′,4′-dihydroxylation pattern in ring B to flavonoids lacking this feature [(**F1**+**F2**)/(**F3**+**F4**+**F5**+**F6**)] showed highly significant increases with altitude (ranging from r=0.484, P=0.017 to r=0.738, P<0.001). This correlation was stable over all growing seasons, including potted plants and plants grown in the natural soil. When all results for the quotient of *Q*/*K* from all growing seasons and soil treatments were combined (which might be a bit problematic from a purely statistical point of view), the Pearson correlation coefficient was r=0.528 (P< 0.001; Fig. 1).

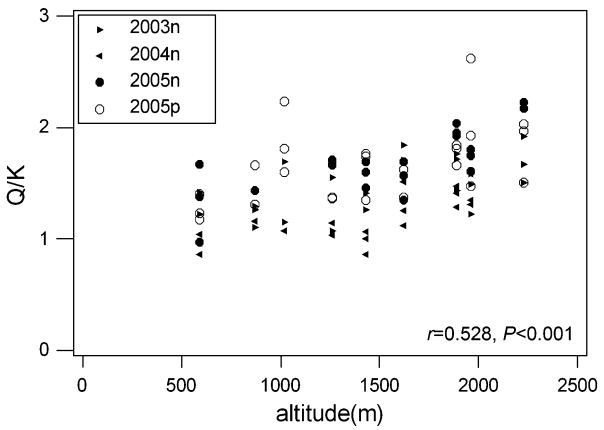

Fig. 1 Correlation of the altitude of the growing site and the quotient of *ortho*-dihydroxy-substituted flavonoids to other flavonoids {*Q*/*K*= [(F1+F2)/(F3+F4+F5+F6)]} in flowering heads of *A. montana* harvested in different years. Each symbol represents one analyzed batch. Plants grown in natural soil (n) and potted plants (p), respectively. Correlation coefficients and corresponding *P* values for each season are displayed in Table 1

Quantification of total caffeic acid derivatives (Table 3) revealed that they were positively correlated with altitude of the growing site in all investigated seasons, although the coefficients and their corresponding P values varied between $r=0.381$, $P=0.107$, and $r=0.696$, $P<0.001$ (Table 1).

The correlation of the quotient of the total caffeic acid derivatives to total flavonoids ($\sum_{P1-P9}/\sum_{F1-F6}$)positively correlated with altitude ($r=0.351$, $P=0.001$ for all investigated samples). However, it is hard to judge whether this result implies a significant shift in biosynthetic pathways or whether it is merely a mathematical artefact resulting from the fact that one variable (caffeic acid derivative contents) varies with altitude, whereas the second (total flavonoid contents) does not.

Variation of Antioxidant Activity in Relation to Altitude The radical scavenging activity of extracts obtained from flowering heads harvested at different altitudes was assessed for samples harvested in 2004 and 2005, but not for the 2003 harvest because the DPPH assaying system for crude plant extracts had not yet been established in our lab when the HPLC analyses of the 2003 samples were performed.

Results of the DPPH assays expressed as ascorbic acid equivalents (in mg) per gram dried plant material are summarized in Table 4. The corresponding Pearson correlation coefficients of altitude of the growing site and the DPPH radical scavenging potential were $r=0.606$ ($P=0.006$) for 2004, $r=0.395$ ($P=0.069$) for 2005 for plants in natural soil (n) and $r=0.752$ ($P<0.001$) for 2005 for potted plants (p). These coefficients were calculated from the mean values obtained for each batch, not from mean values for each site. The latter procedure would have resulted in even higher correlation coefficients [$r=0.787$, $P=0.020$ (2004), $r=0.491$, $P=0.217$ (2005n), $r=0.928$, $P<0.001$ (2005p)], although due to smaller sample sizes, there are also higher P-values.

Table 4 Overview of the radical scavenging activity (given as equivalents of ascorbic acid in mg per g dry plant material) of extracts from flowering heads of *A. montana* cv. ARBO grown in different altitudes and harvested in different growing seasons.[a,b]

Altitude (m)	2004		2005		2005 potted plants	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
590	45.34	3.29	25.78	2.63	32.09	4.06
870	47.86	N.a.[c]	31.62	0.30	37.99	8.44
1,020	44.83	N.a.[c]	N.a.	N.a.	41.99	0.23
1,260	51.63	3.93	27.58	4.69	37.86	3.41
1,430	54.52	1.43	31.48	0.33	44.22	1.66
1,620	58.72	5.62	30.16	1.23	42.53	3.99
1,890	51.46	4.19	28.98	2.76	46.02	1.03
1,960	56.01	7.18	31.03	1.98	46.94	6.56
2,230	N.a.[d]	N.a.[d]	30.94	1.08	49.89	8.04

[a] Data are means calculated from means of three analyzed batches. Each batch was measured in triplicate. However, only means for each batch were used to calculate the SD given here (Lombardi-Boccia, 2007).
[b] These data were not sampled from the 2003 harvest.
[c] These sites yielded in the harvest year at hand only one charge of flowering heads for analysis and therefore SDs between charges could not be calculated.
[d] Due to adverse weather conditions, this site yielded no flowering heads in the 2004 growing season.

Table 3 Overview of total amounts of caffeic acid derivatives ($\sum_{P1-P9}$) in flowering heads of *Arnica montana* cv. ARBO grown in different altitudes and harvested in different growing seasons

Altitude (m)	2003		2004		2005		2005 Potted Plants	
	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x	$\bar{x}$	s_x
590	28.10	1.52	27.88	0.61	24.03	5.93	21.57	1.88
870	33.55	2.38	31.41	N.a.[a]	31.66	1.24	25.93	10.01
1,020	31.92	0.32	27.39	N.a.[a]	N.a.[c]	N.a.[c]	32.26	3.52
1,260	33.74	4.78	28.41	1.40	29.88	1.18	32.47	0.05
1,430	30.41	0.79	34.23	2.97	32.04	2.02	27.98	1.13
1,620	38.01	5.01	35.50	1.01	30.79	1.32	30.71	2.38
1,890	32.81	4.76	30.88	1.04	30.49	3.33	32.22	1.56
1,960	31.33	2.73	26.06	0.86	35.89	3.46	42.69	8.10
2,230	38.03	3.77	N.a.[b]	N.a.[b]	41.35	0.20	42.09	11.70

[a] These sites yielded in the harvest year at hand only one charge of flowering heads for analysis and therefore SDs between charges could not be calculated.
[b] Due to adverse weather conditions, this site yielded no flowering heads in the 2004 growing season.
[c] Plants of site 3 in the natural soil had all died until 2005.

Discussion

The data confirm that climatic factors related to altitude of the growing site have profound and reproducible effects on the quantitative composition of profiles of certain secondary metabolites in flowering heads of genetically homogenous plants of *A. montana*. The results also confirm that changes in the quantity of phenolic compounds in flowering heads have an impact on the radical scavenging activity of extracts derived from these heads. Moreover, we demonstrated that altitudinal effects on the quantity and relative composition of phenolics are not caused primarily by changes in soil composition at the growing sites.

The most pronounced effect and the one least varying within the years of observation was the ratio of 3′,4′-dihydroxylated flavonoids to flavonoids without that substitution pattern (quercetin vs kaempferol derivatives). The data revealed that the DPPH radical scavenging potential of flowering heads from plants grown at higher altitudes was significantly higher than that of lowland plants. This result was not surprising as caffeic acid derivatives, whose amounts were also positively correlated with altitude, are potent radical scavengers (Rice-Evans et al. 1996). However, the result is nonetheless noteworthy as other compounds, which are present in plant extracts but which are not detected by the employed HPLC system (such as ascorbic acid), are also contributing to the overall antioxidant potential of the extracts. Moreover, the positive correlation of the ratio of quercetin derivatives to kaempferol derivatives to the altitude of the growing site is also a factor that contributes to the enhanced radical scavenging activity of flowering heads from higher altitudes because quercetin derivatives are three to four times more potent scavengers than kaempferol derivatives (Rice-Evans et al. 1996).

The results provide further proof for the assumption that factors related to altitude indeed have an effect on plant secondary metabolism. However, the data are not conclusive as to which factors are decisive in causing these changes. In addition to the enhanced UV-B radiation at higher altitudes, which was assumed to be the main factor responsible for these differences in previous studies (Spitaler et al. 2006; Alonso-Amelot et al. 2007), the decreased temperature at higher altitudes might play a more important role than previously assumed. Bilger et al. (2007) demonstrated that decreased temperatures during the growth period trigger increased biosynthesis rates of phenolics in a variety of plant species even in the absence of UV-B radiation. Phenolic compounds play a vital role in the hydrogen peroxide scavenging system of plants, which besides phenolics comprises peroxidase, ascorbic acid, and glutathione (Takahama and Oniki 1997). This system functions less efficiently at low temperatures, and more phenolics have to be produced to prevent damage to plants grown at lower temperatures (Bilger et al. 2007). Even if the later harvest dates of the plants grown at higher altitudes are taken into account, the mean temperature during the growing and flowering phase of the high altitude plants was significantly lower than that of the lowland plants. Thus, it may be assumed that not only the increase of UV-B at higher altitude sites but also the lower temperatures at these sites are contributing to the observed altitudinal variations of phenolics.

As reported previously (Spitaler et al. 2006), factors other than temperature and UV-B radiation, which usually change with altitude in mountain ranges of Central Europe and which also might have an impact on plant secondary metabolism, are nearly unaffected by altitude in the Tyrolean Central Alps due to their continental climate. These other factors are, therefore, to be excluded as potential factors that contribute to the altitudinal variation observed in the secondary metabolite profiles of *A. montana* cv. ARBO in the Tyrolean Alps.

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References

ALONSO-AMELOT, M. E., OLIVEROS-BASTIDAS, A., and CALCAGNO-PISARELLI, M. P. 2007. Phenolics and condensed tannins of high altitude *Pteridium arachnoideum* in relation to sunlight exposure, elevation, and rain regime. *Biochem. Syst. Ecol*. 35:1–10.

BILGER, W., ROLLAND, M., and NYBAKKEN, L. 2007. UV screening in higher plants induced by low temperature in the absence of UV-B radiation. *Photochem. Photobiol. Sci* 6:190–195.

BLUMTHALER, M., AMBACH, W., and ELLINGER, R. 1997. Increase in solar UV radiation with altitude. *J. Photochem. Photobiol. B* 39:130–134.

DOUGLAS, J. A., SMALLFIELD, B. M., BURGESS, E. J., PERRY, N. B., ANDERSON, R. E., DOUGLAS, M. H., and GLENNIE, V. L. 2004. Sesquiterpene lactones in *Arnica montana*: A rapid analytical method and the effects of flower maturity and simulated mechanical harvesting on quality and yield. *Planta Med*. 70:166–170.

JAAKOLA, L. and MÄÄTTÄ-RIIHINEN, K. 2004. Activation of flavonoid biosynthesis by solar radiation in bilberry (*Vaccinium myrtillus* L.) leaves. *Planta* 218:721–728.

KÖRNER, C. 1999. Alpine Plant Life. Functional Plant Ecology of High Mountain Ecosystems., Springer, Berlin.

LINS, I. 2007. Quantitative Variationen im Inhaltsstoffspektrum von *Arnica montana* in Abhängigkeit von der Höhenlage und chemosystematische Untersuchungen innerhalb der Gattung *Leontodon*. Diploma Thesis University of Innsbruck, Innsbruck.

LOMBARDI-BOCCIA, G. 2007. Rebuttal to comment on nutrients and antioxidant molecules in yellow plums (*Prunus domestica* L.) form conventional and organic productions: a comparative study. *J. Agric. Food Chem*. 55:4279–4280.

MARKHAM, K. R., RYAN, K. G., BLOOR, S. J., and MITCHELL, K. A. 1998a. An increase in the luteolin: apigenin ratio in *Marchantia polymorpha* on UV-B enhancement. *Phytochemistry* 48:791–794.

MARKHAM, K. R., TANNER, G. J., CAASI-LIT, M., WHITECROSS, M. I., NAYUDU, M., and MITCHELL, K. A. 1998b. Possible protective role for 3′,4′-dihydroxyflavones induced by enhanced UV-B in a UV-tolerant rice cultivar. *Phytochemistry* 49:1913–1919.

RICE-EVANS, C. A., MILLER, N. J., and PAGANGA, G. 1996. Structure-antioxidant activity relationships of flavonoids and phenolic acids. *Free Rad. Biol. Med*. 20:933–956.

SPITALER, R., SCHLORHAUFER, P. D., ELLMERER, E. P., MERFORT, I., BORTENSCHLAGER, S., STUPPNER, H., and ZIDORN, C. 2006. Altitudinal variation of secondary metabolite profiles in flowering heads of *Arnica montana* cv. ARBO. *Phytochemistry* 67:409–417.

TAKAHAMA, U. and ONIKI, T. 1997. A peroxidase/phenolics/ascorbate system can scavenge hydrogen peroxide in plant cells. *Physiol. Plant*. 101:845–852.

WELLMANN, E. 1975. UV Dose-dependent induction of enzymes related to flavonoid biosynthesis in cell suspension cultures of parsley. *FEBS Lett*. 51:105–107.

WINKLER, A. 2006. Quantitative Variationen im Inhaltsstoffspektrum von *Arnica montana* in Abhängigkeit von Höhenlage, Substrat und Herkunft. Diploma Thesis University of Innsbruck, Innsbruck.

YANAR, S. 2006. *Arnica montana* - Quantifizierung der phenolischen Inhaltsstoffe und Bestimmung der Radikalfängeraktivität der Blütenkopfextrakte. Diploma Thesis University of Innsbruck, Innsbruck.

ZAMG 2002. Klimadaten von Österreich. CD-ROM. Zentralanstalt für Metereologie und Geodynamik Wien.

ZIDORN, C., SCHUBERT, B., and STUPPNER, H. 2005. Altitudinal differences in the contents of phenolics in flowering heads of three members of the tribe Lactuceae (Asteraceae) occurring as introduced species in New Zealand. *Biochem. Syst. Ecol*. 33:855–872.

ZIDORN, C. and STUPPNER, H. 2001. Evaluation of chemosystematic characters in the genus *Leontodon*. *Taxon* 50:115–133.

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Salivary Amylase Induction by Tannin-Enriched Diets as a Possible Countermeasure Against Tannins

G. da Costa · E. Lamy · F. Capela e Silva · J. Andersen · E. Sales Baptista · A. V. Coelho

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Abstract Tannins are characterized by protein-binding affinity. They have astringent/bitter properties that act as deterrents, affecting diet selection. Two groups of salivary proteins, proline-rich proteins and histatins, are effective precipitators of tannin, decreasing levels of available tannins. The possibility of other salivary proteins having a co-adjuvant role on host defense mechanisms against tannins is unknown. In this work, we characterized and compared the protein profile of mice whole saliva from animals fed on three experimental diets: tannin-free diet, diet with the incorporation of 5% hydrolyzable tannins (tannic acid), or diet with 5% condensed tannins (*quebracho).* Protein analysis was performed by one-dimensional gel electrophoresis combined with Matrix-Assisted Laser Desorption Ionization-Time of Flight mass spectrometry to allow the dynamic study of interactions between diet and saliva. Since abundant salivary proteins obscure the purification and identification of medium and low expressed salivary proteins, we used centrifugation to obtain saliva samples free from proteins that precipitate after tannin binding. Data from Peptide Mass Fingerprinting allowed us to identify ten different proteins, some of them showing more than one isoform. Tannin-enriched diets were observed to change the salivary protein profile. One isoform of α-amylase was overexpressed with both types of tannins. Aldehyde reductase was only identified in saliva of the quebracho group. Additionally, a hypertrophy of parotid salivary gland acini was observed by histology, along with a decrease in body mass in the first 4 days of the experimental period.

Keywords Salivary proteins · Amylase · SDS-PAGE · Mass spectrometry · Defense mechanisms against tannins · Taste

Introduction

Tannins are plant secondary metabolites (PSMs) with an enormous structural diversity and are found worldwide in many families of higher plants. One of the characteristic properties of these natural high-molecular-mass polyphenols is their high capacity to bind proteins, forming

G. da Costa and E. Lamy have contributed equally to this work.

G. da Costa · A. V. Coelho (✉)
ITQB-Instituto de Tecnologia Química e Biológica,
Oeiras, Portugal
e-mail: varela@itqb.unl.pt

E. Lamy · E. Sales Baptista
ICAM-Instituto de Ciências Agrárias Mediterrânicas,
Universidade de Évora,
Évora, Portugal

E. Lamy · E. Sales Baptista
Departamento de Zootecnia, Universidade de Évora,
Évora, Portugal

F. Capela e Silva
Departamento de Biologia, Universidade de Évora,
Évora, Portugal

F. Capela e Silva
Centro de Investigação em Ciências e Tecnologias da Saúde,
Universidade de Évora,
Évora, Portugal

J. Andersen
Department of Biochemistry and Molecular Biology, University of Southern Denmark,
Odense, Denmark

A. V. Coelho
Departamernto de Química Universidade de Évora,
Évora, Portugal

complexes that tend to precipitate (Haslam 1998). In the mouth, the precipitates are perceived via mechanoreceptors as rough, puckering, or drying oral sensations characteristic of astringency (Breslin et al. 1993; Prinz and Lucas 2000). The astringent properties, together with the frequently associated bitter taste (Lesschaeve and Noble 2005), may result in the avoidance of some plants or plant parts by herbivores. Taste perception is generally considered to be an adaptive response in assessing nutritional value and/or coping with toxicity in potential foods (Le Magnen 1986). Animals with a relatively high occurrence of bitter and potentially toxic compounds in their diet (e.g., browsing herbivores) are believed to have developed a high bitter-taste threshold and tolerance (Glendinning 1994). The cause for this tolerance can be rooted in physiological postingestive adaptative mechanisms, namely, modifications of saliva composition or flow rate, which can contribute to mitigate the negative biological effects of PSMs.

Saliva contains a complex mixture of proteins with different biological roles in digestion, host defense, and lubrication (Humphrey and Williamson 2001). Its composition is diverse among animal species and changes with circadian rhythm (Hardt et al. 2005a) and diet (Katsukawa and Ninomiya 1999; Neyraud et al. 2006), among other factors. As the secretion of salivary fluid and proteins is controlled by autonomic nerves (Proctor and Carpenter 2007), saliva plasticity represents a rapid mechanism that allows animals to adapt to random dietary changes, thus conferring an important advantage. Rats and mice that consumed tannin-rich diets showed both parotid gland hypertrophy and increased synthesis and accumulation of salivary proline-rich proteins (PRPs; Mehansho et al. 1983, 1985, 1987; Jansman et al. 1994). These molecular and cellular effects produced by high-tannin diets, both on the salivary gland tissue and on the saliva of mice and rats, are indistinguishable from those produced by recurrent stimulation with the beta-adrenergic agonist isoproterenol (Ann et al. 1987, 1997). Recently, Gho et al. (2007) observed the induction of a group of salivary polypeptides (designated by isoproterenol-induced polypeptides) both by tannins and isoproterenol. Mice salivary PRP induction was suggested as acting as a countermeasure against tannins through the formation of tannin–protein complexes that remain insoluble in the conditions found in the digestive compartments (Hagerman et al. 1998; Lu and Bennick 1998). Apart from these protein species, little is known about possible changes in other salivary proteins that are induced by tannins, namely proteins that do not form insoluble complexes with these PSMs. Besides PRPs, isoproterenol also induces changes in the expression levels of salivary proteins, such as cystatins (Shaw and Yu 2000) and amylase (Gallacher and Petersen 1983), among others. Therefore, the possibility of changes in relative amounts of other proteins cannot be excluded. The characterization of such modifications may be useful for better understanding mammalian physiological countermeasures. Rodents have been traditionally used as an animal model for studies of the anatomy of the salivary glands and physiology, and several proteins have been reported as constituents of their saliva: PRPs (Lin and Ann 1991), namely the parotid salivary protein, (Madsen and Hjorth 1985) and amylase (Hagenbüchle et al. 1980). However, to our knowledge, a systematic characterization of rodent saliva proteome has not been reported. The most complete rodent salivary protein profile reported to date is a two-dimensional map of rat parotid saliva, in which the identification of detected proteins was inferred from determined molecular masses in the gel (Williams et al. 1999).

Peptide mass fingerprinting (PMF) that uses matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectra is a current strategy for the identification of proteins expressed in certain physiological conditions, allowing comparisons of different treatments. Here, our first objective was to identify salivary proteins from mice whole saliva by using MALDI-TOF mass spectrometry after separation by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequently to evaluate if ingestion of tannins induced changes in the expression of these proteins. We tested the influence of hydrolyzable (tannic acid) and condensed (*quebracho*) tannins on saliva samples from which insoluble tannin–protein complexes had been removed before the analysis, to better assess proteins expressed in lower concentrations. Light microscopy was also used to study morphological changes in parotid glands.

Methods and Materials

Animals Thirty-one inbred male Balb/c mice, 4-week-old, were obtained from the licensed bioterium of Instituto Gulbenkian de Ciência (Oeiras, Portugal). The animals were housed in mice cages, type IV (Techniplast; (10 to 11 mice per cage), according to European Union (EU) recommendations and revision of Appendix A of European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (ETS No. 123) and maintained on a 12:12 h, light/dark cycle at a constant temperature of 22°C with ad libitum access to water and to a standard diet with 21.86% crude protein (dry basis) in the form of pellets (RM3A-P; Dietex International, UK). Animals were individually marked and submitted to a 7-day acclimation period to minimize stress associated with transportation. This period was followed by a 7-day pretrial period to allow adaptation to the ground diet used during the feeding trials. The standard pellet diet was ground daily

with a blender to obtain a meal with visibly homogeneous fine-sized particles. Before the feeding-trial period, animals were individually weighed and allocated to three experimental groups, each group having no significant differences in body mass (24.5±3 g).

All procedures involving the animals were approved by the scientific committee, supervised by a Federation of European Laboratory Animal Science Associations (FELASA)-trained scientist and conforming to the regulations of the Portuguese law (*Portaria 1005/92*), following European Union Laboratory Animal Experimentation Regulations.

Feeding Trials Immediately after the pretrial period, we started the 10-day experimental period, the first day of which we consider day 1. The control group (n=10) received a tannin-free diet, the same standard ground diet as in the pretrial period. The tannic acid group (TA; n=10) and the quebracho group (Q; n=11) received the standard ground diet plus tannic acid (Merck Ref 1.00773.100; hydrolyzable tannin) or quebracho extract (Tupafin-Ato, SilvaChimica SRL; 72%±1.5 of condensed tannins), respectively, added to obtain a mixture with 5 g tannin/100 g wet weight of the standard diet. Food and water were provided ad libitum, and the diets were prepared daily with a blender, as described for the pretrial period. Body mass changes during the first 8 days were determined daily.

Saliva and Salivary Gland Collection and Sample Preparation On day 11, individual mice whole saliva was collected. The mice remained in the cages, with food and water available, until the time of collection. Saliva production was induced with an intraperitoneal injection of pilocarpine (Sigma; 5 mg kg^{-1} body weight), dissolved in 1 ml 9% (*m*/*v*) sodium chloride, and prepared immediately before use, as described by Muenzer et al. (1979). Saliva was individually collected by aspiration from all the mice, directly from their mouths with a micropipette. Saliva samples were frozen immediately in liquid nitrogen and stored at −80°C until required. Before protein quantification, samples were centrifuged at 16,000×*g* for 5 min at 4°C to remove particulate matter and salivary proteins that could be precipitated. Only the soluble fraction was used for further analyses. All animals were injected intraperitoneally with anesthetic (xylazine hydrochloride combined with ketamine hydrochloride) and euthanized with an overdose. Both parotid salivary glands were dissected, washed briefly with phosphate buffer 0.1 M, pH 7.4, and fixed in 10% neutral buffered formalin, to carry out further routine histological procedures.

Protein Quantification and Gel Electrophoresis Total protein concentration was determined by the bicinchoninic acid (BCA) protein assay method (Pierce) by using a microplate reader (SpectroMAX 340, Molecular Devices, Union City, CA, USA). One-dimension SDS-PAGE was run with 20 μg of saliva total protein after reduction with dithiothreitol (USB) and alkylation with iodoacetamide (Sigma). Denatured protein samples were loaded on bis-tris polyacrilamide 4–12% gradient precast gels (100×100×1 mm; Nupage Invitrogen) with MES SDS (Nupage Invitrogen) used as a running buffer. Molecular mass markers (BioRad, Ref 161-0317) were run simultaneously with the samples in each gel to calibrate molecular masses of the protein sample bands. Protein bands were stained with colloidal Coomassie blue (Neuhoff et al. 1988) and with Comassie blue R-250 following Beeley et al (1991). Digital images of the gels were acquired with a densitometer with internal calibration (Molecular Dynamics, Amersham Biosciences Europe GmbH, Freiburg, Germany), and gels were subjected to linescan analysis by using Imagequant Software 5.0 (Amersham Biosciences Europe GmbH, Freiburg, Germany). Sensitivity 9.0 and kernel 4.0 were the software parameters used to assign the detected significant bands in the protein profiles obtained. Only bands present in at least 50% of individuals were considered.

Protein Identification Protein bands were excised from all gels and the polypeptides subjected to digestion with a sequence grade modified trypsin (Promega) according to Pandey et al. (2000). Sample peptides were assayed for peptide mass fingerprinting in a Voyager-DE STR (Applied Biosystems, Foster City, CA, USA) MALDI-TOF mass spectrometer. Peptide co-crystallization was achieved by applying 0.5 μl of the peptide digest on the MALDI plate and adding an equal volume of recrystallized matrix α-cyano-4-hydroxycinnamic acid 10 mg/ml prepared in acetonitrile 70% (*v*/*v*) with 0,1% trifluoroacetic acid (*v*/*v*). The mixture was allowed to air dry (dried droplet method). Average spectra were obtained in the mass range between 800 and 4,000 Da, using three spectra acquired with 500 laser shots in the positive-ion reflectron mode. Spectra were processed and analyzed by the Data Explorer (version 4.0, Applied Biosystems, Foster City, CA, USA). Monoisotopic peptide masses were used to search for protein identification with Mascot software (Matrix Science, UK; Perkins et al. 1999). Searches were performed in the National Center for Biotechnology Information nonredundant protein-sequence database. A minimum mass accuracy of 100 ppm, one missed cleavage in peptide masses, and carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively, were considered. Criteria used to accept the identification were significant homology scores achieved in Mascot, significant sequence-coverage values, and similarity between the protein molecular mass calculated from the gel and for the identified protein (Roxo-Rosa et al. 2006).

Table 1 Mean body masses in grams of mice fed with different diets through the first 8 days of the experiment (mean±SD)

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8
Control	24.90± 2.14^{a,A}	24.93±2.22^{a,A}	25.58± 2.15^{b,A}	25.65± 2.04^{b,A}	25.56±2.05^{b,A}	25.64 ±1.99^{b,A}	25.70± 2.13^{b,A}	25.75±1.95^{b,A}
Tannic acid	24.24± 2.29^{a,A}	23.38±2.31^{b,c,B}	23.12± 2.14^{b,B}	23.27± 2.23^{b,c,B}	23.32±2.22^{b,c,B}	23.69±2.20^{c,d,B}	24.06±2.15^{a,d,B}	24.02±2.18^{a,d,A}
Quebracho	25.04± 1.36^{a,A}	25.38±1.34^{a,b,A}	25.20±1.32^{a,b,C}	24.98±1.31^{a,C}	25.71±1.40^{b,d,e,A}	25.97±1.32^{c,d,e,A}	26.40±1.37^{c,e,A}	26.14±1.29^{c,e,A}

Same lowercase letters indicate no differences among the values of the columns in the same line (Tukey–Hsu test; $P<0.05$). Same uppercase letters indicate no differences among the values of the lines in the same column (Tukey–Hsu test; $P<0.05$).

Histology To confirm that the doses of tannins used were enough to induce significant changes in glandular morphology, salivary glands were observed through light microscopy with a Nikon Eclipse 600 microscope (Kanagawa, Japan). After embedding the fixed parotid glands in paraffin wax, using routine procedures, a series of sections of 5 mm thick were cut with a microtome, and the slides were stained with hematoxylin and eosin. Three animals from each group were used, and for each animal, ten pictures from random parotid areas were collected with a Nikon DN100 camera (Kanagawa, Japan) at ×200 magnification. For each animal, the area and perimeter of 150 parotid acini were randomly chosen and measured by using SigmaScan Pro5.0 software (SPSS, Chicago, IL, USA).

Statistical Analysis Body mass data were tested for normality and homocedasticity by Kolmogorov–Smirnov and Levene tests, respectively. Body mass was analyzed according to a general linear model procedure with two fixed factors and one nested factor:

$$Y_{ijkl} = \mu + T_i + W_k + T(A)_{i(j)} + T \times W_k + \varepsilon_{ijkl}$$

where Y_{ijkl} are the observed values for body mass, μ is the observed mean value, T_i is the fixed effect of treatment, W_k is the fixed effect of weighing day, $T(A)_{i(j)}$ is the nested effect, and ε_{ijkl} is the random error or residual effect.

Means significantly different were submitted to post hoc comparisons of means (Tukey–Hsu test) and regarded as significantly different when $P<0.05$.

All statistical analysis procedures were performed by NCSS 2001 software package (Kaysville, UT, USA).

Results

Body Mass Animals were weighed daily to search for changes in body masses that reflect physiological adaptation of the animals to tannin diets, including the induction of PRPs (Mehansho et al. 1983; Skopec et al. 2004). We observed that tannic acid and quebracho groups lost weight during the first 3–4 days, respectively, after which they recovered, whereas the control group increased in body mass in the first 2 days and remained nearly stable after that. Statistically significant differences were observed on days 3 and 4, with the animals from the tannin groups presenting lower mean body masses than animals from control group (Table 1). Despite the increases in body masses after day 4, for animals from either tannic acid or quebracho groups, that increase was less pronounced in the tannic acid group, with animals remaining with lower body masses until the end of the trial.

Histology A dramatic increase in the acinar area and the perimeter of the parotid salivary gland was observed in the animals that received tannin-enriched diets over a period of 10 days (Fig. 1). Levels of 5 g of hydrolyzable or condensed tannin per 100 g wet weight in the diet seem to produce a hypertrophy of the secretor tissue (Table 2).

Saliva Protein Concentration Total protein saliva concentration, after the 10 days of the feeding trial, was significantly higher in the control group than in the quebracho and in tannic acid groups (Table 3).

Saliva Protein Profile We characterized the saliva profile of the control group by using the Imagequant 5.0 software to assign significative bands in the profiles obtained. These results are in agreement with visual observation of the gel pattern. Despite slight interindividual variations in whole saliva composition, 21 protein bands (from a to u) were present consistently in all animal saliva protein profiles ($n=10$). Fig. 2a shows a typical 1-DE pattern of the control group saliva and also an overlay of the ten linescans resulting from the analysis of the control group saliva protein profiles. The molecular masses of the proteins were calculated by gel analysis software, after superimposing the linescan of the molecular mass markers on each sample linescan.

With regard to the control group, interindividual protein profile variability was assessed for the TA ($n=10$) and

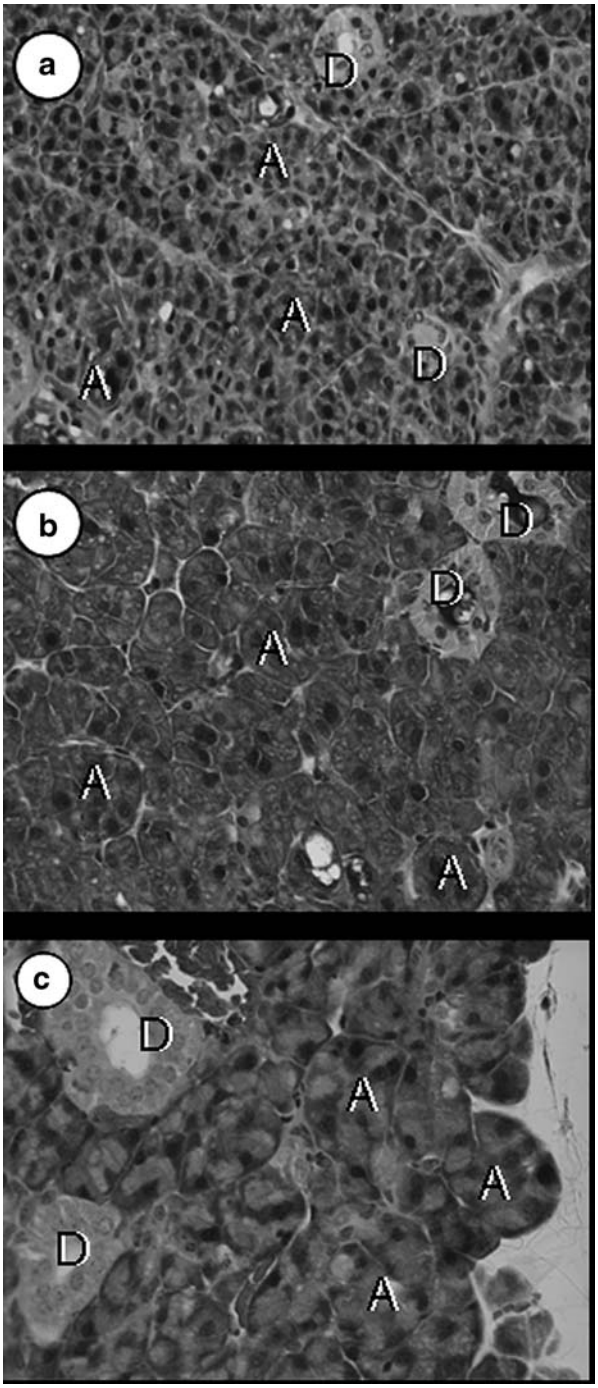

Fig. 1 Microscopic appearance of parotid glands (×200) from **a** control, **b** tannic acid, and **c** quebracho groups. Acini from control group are significantly smaller that acini from the two tannin enriched diet groups. *A* Acinus; *D* salivary duct

Q (n=11) groups. Only slight variation was found among the animals on the tannin-fed diets. By comparing the saliva protein profile of the control group with the TA group, we identified one additional band (band β, Fig. 2b) around molecular mass 51 kDa that was not visible in the control saliva protein profile. The saliva protein profile of the Q group, when compared with the control saliva protein profile, presented two additional bands (Fig. 2c). There was an intense protein band presented in the saliva protein profile of the TA group around 51 kDa (band β), and an additional band (band γ) of reduced intensity and molecular mass around 20 kDa.

Protein Identification Identification of all protein bands excised from the control group can be seen in Table 4. The only band whose identification was not possible was band *p*, probably due to the presence of a low amount of protein. From the 21 protein bands excised, we identified ten different polypeptides. The other ten bands were assigned to four of the already identified polypeptides: four additional bands to salivary amylase 1, one to carbonic anhydrase VI, four to parotid secretory protein (PSP), and one to androgen-binding protein. This was attributed to the existence of post-translational modifications and/or protein fragments. Band *u* was assigned to a mixture of β and γ subunits of the androgen-binding protein.

The two protein bands that were detected additionally in the saliva profile of the animals submitted to a tannin-enriched diet (β and γ) were also used for protein identification following the PMF approach. As seen in Table 4, these two bands were identified as amylase 1 and aldehyde reductase, respectively. Aldehyde reductase was observed only in the Q group.

Discussion

Tannins act as feeding deterrents, due both to their aversive chemosensory properties (such as bitter taste and astringency) and the negative postingestive effects they exert (Glendinning 1994). A reduction in body mass in the first days of tannin administration has been reported previously (Mehansho et al. 1983, 1985; Jansman et al. 1994; Shimada et al. 2004, 2006). In mice and rats, this effect is reversed after 2–3 days, when a hypertrophy of parotid glands occurs, coincident with a dramatic increase in salivary PRPs production (Mehansho et al. 1983). In the present work, tannin-fed mice decreased in body mass in the first days (Table 1). This effect ceased after 3–4 days. Despite the body mass recovery of tannin-fed mice, animals from the tannic acid group remained with lower body masses until the end of the trial, which suggests a greater capacity to adapt to quebracho tannin. The parotid hypertrophy usually associated with tannin consumption was also observed in this experiment. This effect in parotid glands is associated with the expression of a number of isoproterenol-induced salivary proline-rich polypeptides (Lopez-Solis and Kemmerling 2005; Gho et al. 2007), which seems to be related to β1 adrenergic activity (Gonzalez et al 2000). We

Table 2 Comparison of acinar areas and perimeters between control and tannin-enriched dietary groups (mean±SD)

	Diet							
	Control		Tannic acid		Quebracho		*t* (C, TA)	*t* (C, Q)
	X	SD	*X*	SD	*X*	SD		
Area (pixel)	15,674.9	5,787.0	28,021.5	10,729.3	33,303.8	13,696.1	−12.4[a]	−14.5[a]
Perimeter (pixel)	504.6	96.9	676.0	129.2	739.2	153.3	−13.0[a]	−15.8[a]

C Control; *TA* tannic acid; *Q* quebracho

[a] Differences are significant for $P<0.05$. Independent sample *t* test for equality of means was used to test differences in acinar area and perimeter between each diet group.

thus found those changes relevant, demonstrating that the tannin levels used were enough to induce an effect at glandular level.

This study helps explain changes in whole saliva protein composition that occur when mice consume tannin-enriched diets. Most studies suggest a defense response associated with the induction of salivary proteins (mainly PRPs) that precipitate PSMs, preventing them from exerting negative effects (Bennick 2002). We predicted that tannin consumption would also influence other salivary proteins. To ensure that the minor expressed-salivary proteins would not be masked by overexpression of the tannin-binding proteins, we removed the insoluble fraction that is expected to contain the tannin–protein complexes. We assumed that this was achieved since total protein concentration from the tannin-fed groups was lower compared with the control group. The lack of observation of pink bands in the Coomassie stained gels (according to Beeley et al. 1991) supports the idea that we efficiently removed the majority of PRPs.

Although a great number of salivary protein studies have been performed on mice, a global perspective that relates whole saliva protein profile to protein identification is presently unknown. Williams et al. (1999) provide a 2-DE protein profile, referring to the presence of PRPs, amylase, acidic epididymal glycoprotein, deoxyribonuclease, parotid secretory protein, and common salivary protein, although a protein identification was not performed but rather inferred from the position of protein spots. Our results showed the presence of ten different salivary proteins, some showing more than one isoform. This situation is documented in human saliva (Hirtz et al. 2005b), where a simultaneous low diversity in terms of variety of accessions and a high complexity in terms of number of protein bands identified in the same accession are reported. This degree of redundancy displayed by extensive salivary protein polymorphisms seems to be important in saliva plasticity. Moreover, the presence of several families of structurally and functionally closely related molecules indicates that these proteins have been subjected to evolutionary pressures that may reflect selection for improved function (Oppenheim at al. 2007).

The identified proteins have been described previously in saliva: acidic chitinase, mucin apoprotein, amylase 1, carbonic anhydrase VI, apolipoprotein A-I, parotid secretory protein, immunoglobulin heavy chain, submandibular gland 15 kDa protein, androgen-binding protein, and glandular kallikrein (Table 4). The first seven have multiple functions in saliva and are involved in the formation of enamel pellicle, have hydrolytic activity, and act as a first line of defense against microorganisms (Yao et al. 2003; Huang 2004; Vitorino et al. 2004; Hardt et al. 2005b). The submandibular gland 15 kDa protein is known as a gross cystic disease fluid protein that was initially described in humans as a protein secreted by the T47D human breast cancer cell line. This protein is produced in many exocrine glands such as sweat, salivary and lachrymal; however, its function remains unknown (Myal et al. 1998). Androgen-binding protein

Table 3 Whole saliva protein concentration (mean±SD)

	Diet							
	Control		Tannic acid		Quebracho		*t* (C, TA)	*t* (C, Q)
	X	SD	*X*	SD	*X*	SD		
Protein concentration (μg/ml)	2,522.20	601.99	1,889.63	377.65	1,652.61	303.56	2.82[a]	4.12[a]

C Control; *TA* tannic acid; *Q* quebracho

[a] Differences are significant for $P<0.05$. Independent sample *t* test for equality of means was used to test differences in protein concentration between each diet group.

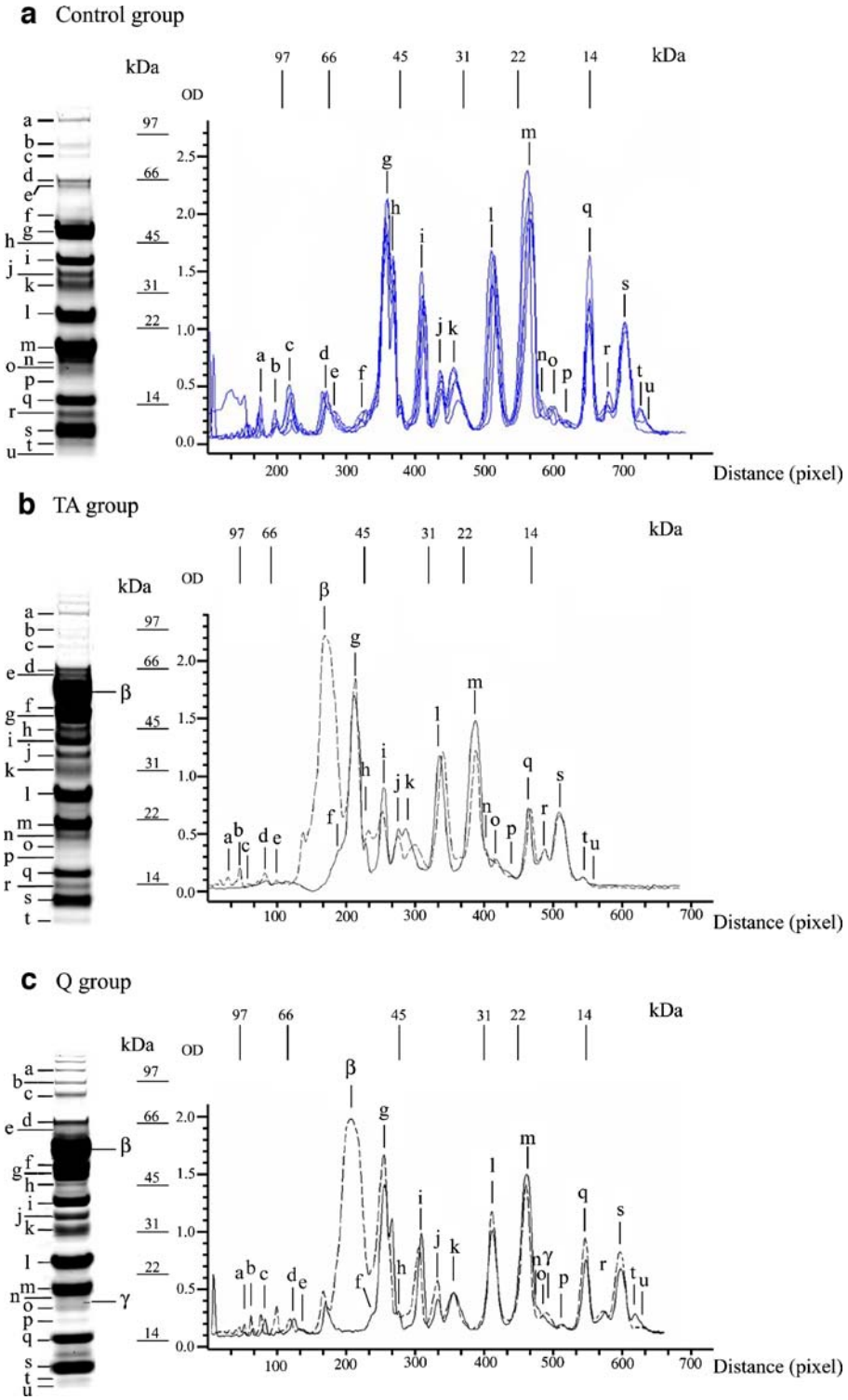

Fig. 2 For each dietary group, a typical one-dimensional (1-D) gel pattern and a linescan analysis of salivary proteins are represented on the *left hand side* and on the *right side*, respectively. Molecular markers are represented on the *right side of the 1-D gel* and on the *top on the linescan*. The letters *a-u,* β and γ represent the protein bands and the individual peaks assigned by the software. The graphics represent an overlay of **a** ten gel linescan analysis of the control group; **b** a linescan analysis of the control group and of the TA group; **c** a linescan analysis of the control group and of the Q group

Table 4 Proteins identified in saliva from animals of each dietary group

Band ID	Dietary group	Protein name	Estimated[a] MW (kDa)	Theoretical[b] MW (kDa)	Score	Coverage (%)	Peptides matched/ peptides submitted	Swiss-Prot Code	Reference
a	C, TA, Q	Acidic chitinase	99	52	119	35	15/42	Q91XA9	(1)
b	C, TA, Q	Muc10 protein	78	29	77	31	15/79	Q80XS5	(2)
c	C, TA, Q	Salivary amylase 1	66	58	81	34	21/72	Q921Y7	(3–6)
d	C, TA, Q	Salivary amylase 1	59	58	74	25	16/69	Q921Y7	(3–6)
e	C, TA, Q	Salivary amylase 1	54	58	136	39	22/78	Q921Y7	(3–6)
f	C, TA, Q	Salivary amylase 1	51	58	179	49	23/45	Q921Y7	(3–6)
β	TA, Q	Salivary amylase 1	51	57	115	31	15/32	Q921Y7	(3–6)
g	C, TA, Q	Salivary amylase 1	48	58	339	59	24/55	Q921Y7	(3–6)
h	C, TA, Q	Glandular kallikrein	45	29	103	39	22/83	P36368	(7)
i	C, TA, Q	Similar to carbonic anhydrase 6	40	37	92	39	18/45	Q80YB7	(5,6)
j	C, TA, Q	Similar to carbonic anhydrase 6	37	37	91	39	18/44	Q80YB7	(5,6)
k	C, TA, Q	Apolipoprotein A-I	33	31	71	20	7/23	Q00623	(3,5)
l	C, TA, Q	Parotid secretory protein	26	25	78	37	67/45	P07743	(5)
m	C, TA, Q	Parotid secretory protein	20	25	83	42	7/42	P07743	(5)
n	C, TA, Q	Parotid secretory protein	18	25	71	42	7/53	P07743	(5)
γ	Q	Aldehyde reductase	18	37	86	27	7/18	Q80XJ7	
o	C, TA, Q	Parotid secretory protein	16	25	63	37	5/50	P07743	(5)
p	C, TA, Q	n.d.	15						
q	C, TA, Q	Parotid secretory protein	14	25	83	42	7/43	P07743	(5)
r	C, TA, Q	Immunoglobulin heavy chain variable region	12	13	65	52	6/42	Q683Y7	(3,5,6)
s	C, TA, Q	14 kDa submandibular gland protein	9	17	94	58	6/35	P02816	(8)
t	C, TA, Q	Androgen binding protein β subunit	9	10	104	54	7/41	Q7TNM9	(9)
u	C, Q	Androgen binding protein γ subunit	7	10	210	54	7/38	Q8JZX1	(9)
	C, Q	Androgen binding protein β subunit	7	13		48	9/38	Q7TNM9	

The nonidentified proteins are labeled as n.d.; articles reporting the presence of the correspondent protein in saliva: (1) Stjein et al. 1999; (2) Denny et al. 1996; (3) Huang 2004; (4) Yao et al. 2003; (5) Vitorino et al. 2004; (6) Hardt et al. 2005a, b; (7) Kim et al. 1991; (8) Myal et al. 1998; (9) Karn and Laukaitis 2003

C Control; *TA* tannic acid; *Q* quebracho

[a] Molecular mass measured based on electrophoretic mobility

[b] Determined molecular weight for identified protein.

secreted by mice submandibular salivary glands has been hypothesized as having a major role in mate selection. This member of the secretoglobins family is secreted into saliva in two different dimer forms: an α subunit disulfide bridged to either a β or a γ subunit (Karn and Laukaitis 2003). However, cellular site(s) of synthesis, mode of function, and evolution patterns of this biologically important protein are otherwise unknown (Dlouhy et al. 1987). Glandular kallikrein, mainly secreted by submandibular glands, is a peptidase that belongs to the serine proteases family, which brings about maturation of growth factors and polypeptide hormones, by conversion of inactive precursors to biologically active peptides (Kim et al. 1991). Parasympatic stimulation that causes an increase in saliva flow will promote low outputs of this protein without degranulation of acinar or granular tubules cells (Shori and Asking 2001).

The control group electrophoresis gel pattern presents five different bands identified as salivary amylase 1, between masses 48 and 66 kDa—one intense band at a lower molecular mass (band *g*), and four weak or medium intense bands at higher molecular mass values. Different bands may correspond to different isoforms. Glycosylation and deamidation of amylase 1 have been described (Bank et al. 1991; Hirtz et al. 2005a). Glycosylation could be an explanation for the higher molecular mass of the amylase bands observed in the gel, compared to the amylase native form. Knowing that glycosylated asparagine residues almost always occur in the sequence Asn-X Ser/Thr, two potential *N*-glycosylation sites for mice α-amylase are 412–414 and 461–463 (peptide residue numbered taking into account that the 15 amino acid peptide signal is lost during the secretion process). These two sites were also referred for human α-amylase (Bank et al. 1991; Hirtz et al. 2005a). The peptide that contains the first site is detected for all our amylase bands, so a glycosylation should not occur in that position. On the other hand, *N*-glycosylation can occur in the second potential site, since the peptide that contains it systematically fails to be detected in mass spectra. Although some (Bank et al., 1991) have considered that *N*-glycosylation is more prone to occur in the first potential site, Hirtz et al. (2005a) found results similar to ours for human α-amylase. The lack of the specific detection of glycoproteins does not allow the confirmation of this hypothesis since the absence of peptide 458–466 can also be explained based on experimental limitations. Bank et al. (1991) proposed that amylase-deaminated isoforms correspond to the lower molecular-mass bands observed in the saliva SDS-PAGE profile. By using the software Findmod (http://www.expasy.ch/tools/findmod/), it is possible to assign two peaks in the salivary amylase mass spectra of band *g* as deaminated peptides, both in the control group and in the Q and AT groups. These post-translational modifications can occur at one of two residues of peptide 46–76 and at one of three residues of peptide 414–436. These modified peptides were also observed for band β, present only in the animal groups that were submitted to the tannin-enriched diet. The deamination of these residues does not seem to be characteristic of any of the amylase isoforms observed.

Although salivary proteins can bind tannins as a mode of defensive action, other physiological mechanisms, including enzymatic or immune responses, can not be excluded. The only effect common to both types of tannins in the salivary profile of proteins was an increase in amylase. The overexpression of amylase 1 induced in both groups (band *β*, Fig. 2) overlaps the medium molecular mass and less intense band of this protein in the control group (band *f*, Fig. 2). This suggests that only the medium molecular mass isoform of amylase 1 is overexpressed in the tannin-rich diet fed groups or that a new amylase isoform is induced in these groups. Furthermore, protein band *g* (Fig. 2), the most intense band in the control group, identified as amylase 1, did not show any significant expression level change with tannin treatment. Several differences can be observed between the tryptic peptide mass spectra of band *β* and of band *g*. We could not assign these peaks to described modifications of salivary amylase 1, but it suggests that a different isoenzyme of salivary amylase 1 is being expressed in the Q and AT groups.

The increase in salivary α-amylase levels may be a consequence of tannin stimulation of sympathetic pathways. Isoproterenol and tannins lead to similar changes in salivary glands, and it has been suggested that these changes are due to stimulation of the β1 adrenergic receptors (Waters et al. 1998). Beta adrenergic agonists are capable of stimulating salivary α-amylase release (Gallacher and Petersen 1983). There is a recurrent pattern across studies that shows that salivary α-amylase levels increase in response to physical and psychological stress (Chatterton et al. 1996; for a comprehensive review see Granger et al. 2007). As a result, salivary α-amylase has been used as a surrogate marker of the autonomic/sympathetic nervous system component of stress in humans. Other oral stimulators that influence texture and flavor perception have also been observed to increase α-amylase expression. Amylase increased with taste stimulation in rabbits (Gjorstrup, 1980) and humans (Neyraud et al. 2006), and a strong correlation between α-amylase and texture perception was observed by Engelen et al. (2007).

We suggest that, despite the primary biological function of salivary α-amylase being the digestion of polysaccharides, the increase of its expression is not a direct consequence of dietary carbohydrates but a result of adrenergic stimulation. Indirectly, this increase can also represent a co-adjuvant for the inhibition of tannin biological activity. Tannins are potent inhibitors of salivary amylase 1 (Kandra et al. 2004; McDougall et al. 2005), although affinity of α-amylase for tannins is not as high as PRPs affinity (De Freitas and Mateus 2001). Tannins have been described as responsible for an enlarged pancreas (Ahmed et al. 1991; Mahmood and Smithard. 1993), stimulation and secretion of an increased amount of amylase, which, to some extent, may counteract the inhibition of this enzyme during gut digestion. Although α-amylase in saliva and the pancreas are produced by independent sources, we believe that a similar function could be involved, and amylase content may increase when animals are fed with tannins, thus counteracting the inhibition of this enzyme in the mouth.

Salivary amylase is stored in large dense-core secretory granules that undergo stimulated secretion in response to extracellular stimulation. (Gorr et al. 2005). The α-amylase

increase observed in the present study could result from the exocytosis of acinar secretory mature granules, as usually occurs in the first hours after isoproterenol administration (Henriksson 1982). Moreover, chronic stimulation with isoproterenol is reported to decrease (Madsen and Hjorth 1985; Ann et al. 1987) or maintain (Bedi 1993) amylase levels. To the best of our knowledge, this is the first reference of overexpression of salivary amylase 1 after the ingestion of polyphenols and, surprisingly, not all the amylase isoforms changed with tannins but only one. Band ß could be an induction of a newly produced isoform, or could represent an overexpression of one of the previously visible isoforms.

Another detected difference was the expression of protein band γ (n=8) in the quebracho tannin-fed diet group. This protein was identified as aldehyde reductase. Aldehyde reductase belongs to the aldo-keto reductases (AKR) superfamily. They are carbonyl-reducing enzymes, along with the short-chain dehydrogenases/reductases, and are responsible for the reduction of aldehydes, ketones, and quinones to their corresponding hydroxyl derivatives (Hoffmann and Maser 2007). Although salivary levels of enzymes that belong to the human aldo-keto reductase superfamily were found to be high in humans subjected to a continuous intake of coffee, which has a high content of polyphenols (Sladeck 2003), we interpret this result with caution. First, this enzyme, although ubiquitous (present in plants, fungi, insects, fish, bacteria), has been found mainly in the intracellular media and in tissues. In mice, it is found in several tissues, such as the liver (Ahmed et al. 1978), lung (Nakayama et al. 1986), and ovary (Iwata et al. 1990). A search in the University of California at Los Angeles Human Salivary Proteome Project database (http://www.hspp.ucla.edu) found only two salivary proteins from the AKR family 1 in saliva (Aldose reductase-like and *Trans*-1,2-dihydrobenzene-1,2-diol dehydrogenase). Secondly, quebracho used in this study is a plant extract that contains only 72% tannins. The presence of carbonyl-group-bearing substances, such as small phenolics, other PSMs, or even quinones, resulting from oxidation during the extraction process, is not to be overlooked. The presence of aldehyde reductase could be a consequence of chemical species other than tannin.

We conclude that amylase up-regulation is an unspecific adaptation of saliva to dietary tannins that could be a consequence of the stimulation of sympathetic pathways and of amylase inhibition. Indirectly, and through these mechanisms, tannin adverse effects could be reduced, probably due to preferential involvement of one amylase isoform more prone for this activity. Despite the similarities of results obtained for tannic acid and quebracho, we think that further studies with other forms of tannins would help to clarify whether a specific protein defense response exists for the type of tannin.

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References

AHMED, N. K., FELSTED, R. L., BACHUR, and N. R. 1978. Heterogeneity of anthracycline antibiotic carbonyl reductases in mammalian livers. *Biochem. Pharmacol.* 27:2713–2719.

AHMED, A. E., SMITHARD, R., and ELLIS, M. 1991. Activities of enzymes of the pancreas, and the lumen and mucosa of the small intestine in growing broiler cockerels fed on tannin-containing diets. *Br. J. Nutr.* 65:189–197.

ANN, D. K., CLEMENTS, S., JOHNSTONE, E. M., and CARLSON, D. M. 1987. Induction of tissue-specific proline-rich protein multigene families in rat and mouse parotid glands by isoproterenol. Unusual strain differences of proline-rich protein mRNAs. *J. Biol. Chem.* 262:899–904.

ANN, D. K., LIN, H. H., and KOUSVELARI, E. 1997. Regulation of salivary gland specific gene expression. *Crit. Rev. Oral Biol. Med.* 8:244–252.

BANK, R. A., HETTEMA, H. E., ARWERT, F., AMERONGEN, A. V., and PRONK, J. C. 1991. Electrophoretic characterization of post-translational modifications of human parotid salivary alpha-amylase. *Electrophoresis* 12:1032–1041.

BEDI, G. S. 1993. The effect of adrenergic agonists and antagonists on the expression of proteins in rat submandibular and parotid glands. *Crit. Rev. Oral Biol. Med.* 4:565–571.

BEELEY, J. A., SWEENEY, D., LINDSAY, J. C., BUCHANAN, M. L., SARNA, L., and KHOO, K. S. 1991. Sodium dodecyl sulphate-polyacrylamide gel electrophoresis of human parotid salivary proteins. *Electrophoresis* 12:1032–1041.

BENNICK, A. 2002. Interaction of plant polyphenols with salivary proteins. *Crit. Rev. Oral Biol. Med.* 13:184–196.

BRESLIN, P. S. A., GILMORE, M. M., BEAUCHAMP, G. K., and GREEN, B. G. 1993. Psychophysical evidence that oral astringency is a tactil sensation. *Chem. Senses.* 18:405–415.

CHATTERTON Jr, R. T., VOGELSONG, K. M., LU, Y. C., ELLMAN, A. B., and HUDGENSG, A. 1996. Salivary alpha-amylase as a measure of endogenous adrenergic activity. *Clin. Physiol.* 16:433–448.

DE FREITAS, V., and MATEUS, N. 2001. Structural features of procyanidin interactions with salivary proteins. *J. Agric. Food Chem.* 49:940–945.

DENNY, P. C., MIRELS, L., and DENNY, P. A. 1996. Mouse submandibular gland salivary apomucin contains repeated *N*-glycosylation sites. *Glycobiology* 6:43–50.

DLOUHY, S. R., TAYLOR, B. A., and KARN, R. C. 1987. The genes for mouse salivary androgen-binding protein (ABP) subunits alpha and gamma are located on chromosome 7. *Genetics* 115:535–543.

ENGELEN, L., VAN DEN KEYBUS, P. A., DE WIJK, R. A., VEERMAN, E. C., AMERONGEN, A. V., BOSMAN, F., PRINZ, J. F., and VAN DER BILT, A. 2007. The effect of saliva composition on texture perception of semi-solids. *Arch. Oral Biol.* 52:518–525.

GALLACHER, D. V., and PETERSEN, O. H. 1983. Stimulus-secretion coupling in mammalian salivary glands. *Int. Rev. Physiol.* 28:1–52.

GHO, F., PENA-NEIRA, A., and LOPEZ-SOLIS, R. O. 2007. Induction of salivary polypeptides associated with parotid hypertrophy by gallotannins administered topically into the mouse mouth. *J. Cell Biochem.* 100:487–498.

GJORSTRUP, P. 1980. Taste and chewing as stimuli for the secretion of amylase from the parotid gland of the rabbit. *Acta Physiol. Scand.* 110:295–301.

GLENDINNING, J. I. 1994. Is the bitter rejection response always adaptive? *Physiol. Behav.* 56:1217–1227.

GONZALEZ, M. J., PEÑA, Y., LILLO, S., ALLIENDE, C., and LOPEZ-SOLIS, R. O. 2000. Cell-enlargement-related polypeptides are induced via beta(1)-adrenoceptors in mouse parotids. *Exp. Mol. Pathol.* 69:91–101.

GORR, S.-U., VENKATESH, S. G., and DARLING, D. S. 2005. Parotid secretory granules: crossroads of secretory pathways and protein storage. *J. Dent. Res.* 84:500–509.

GRANGER, D. A., KIVLIGHAN, K. T., EL-SHEIKH, M., GODIS, E. B., and STROUD, L. R. 2007. Salivary alpha-amylase in biobehavioral research: recent developments and applications. *Ann. N Y Acad. Sci.* 1098:122–144.

HAGERMAN, A. E., ICE, M. E., and ITCHARD, N. T. 1998. Mechanisms of protein precipitation for two tannins, pentagalloyl glucose and epicatechin (4-8) catechin (procyanidin). *J. Agric. Food Chem.* 46:2590–2595.

HAGENBÜCHLE, O., BOVEY, R., and YOUNG, R. A. 1980. Tissue-specific expression of mouse α-amylase genes: Nucleotide sequence isoenzyme mRNAs from pancreas and salivary gland. *Cell* 21:179–187.

HARDT, M., WITKOWSKA, H. E., WEBB, S., THOMAS, L. R., DIXON, S. E., HALL, S. C., and FISHER, S. J. 2005a. Assessing the effects of diurnal variation in the composition of human parotid saliva: quantitative analysis of native peptides using iTRAQ reagents. *Anal Chem.* 77:4947–4954.

HARDT, M., THOMAS, L. R., DIXON, S. E., NEWPORT, G., AGABIAN, N., PRAKOBPHOL, A., HALL, S. C., WITKOWSKA, H. E., and FISHER, S. J. 2005b. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry* 44:2885–2899.

HASLAM, E. 1998. Practical Polyphenols: From Structure to Molecular Recognition and Physiological Action. Cambridge University Press, Cambridge.

HENRIKSSON, R. 1982. Beta 1- and beta 2-adrenoceptor agonists have different effects on rat parotid acinar cells. *Am. J. Physiol.* 242: G481–G482.

HIRTZ, C., CHEVALIER, F., CENTENO, D., OFIDAL, V., EGEA, J. C., OSSIGNOL, M., SOMMERER, N., and PERIERE, D. D. 2005a. MS characterization of multiple forms of alpha-amylase in human saliva. *Proteomics* 5:4597–4607.

HIRTZ, C., CHEVALIER, F., CENTENO, D., EGEA, J. C., OSSIGNOL, M., SOMMERER, N., and PÉRIÈRE, D. D. 2005b. Complexity of the human whole saliva proteome. *J. Physiol. Biochem.* 61:469–80.

HOFFMANN, F., and MASER, E. 2007. Carbonyl reductases and pluripotent hydroxysteroid dehydrogenases of the short-chain dehydrogenase/reductase superfamily. *Drug Metab. Rev.* 39:87–144.

HUANG, C. M. 2004. Comparative proteomic analysis of human whole saliva. *Arch. Oral Biol.* 49:951–962.

HUMPHREY, S. P., and WILLIAMSON, R. T. 2001. A review of saliva normal composition, flow and function. *J. Prosthet. Dent.* 85:162–169.

IWATA, N., INAZU, N., and SATOH, T. 1990. Immunological and enzymological localization of carbonyl reductase in ovary and liver of various species. *J. Biochem. (Tokyo)* 107:209–212.

JANSMAN, A. J., FROHLICH, A. A., and MARQUARDT, R. R. 1994. Production of proline-rich proteins by the parotid glands of rats is enhanced by feeding diets containing tannins from faba beans (*Vicia faba* L.). *J. Nutr.* 124:249–258.

KANDRA, L., GYÉMÁNT, G., ZAJACZ, A., and BATTA, G. 2004. Inhibitory effects of tannin on human salivary alpha amylase. *Biochem. Biophy. Res. Comm.* 319:1265–1271.

KARN, R. C., and LAUKAITIS, C. M. 2003. Characterization of two forms of mouse salivary androgen-binding protein (ABP): Implications for evolutionary relationships and ligand-binding function. *Biochemistry* 42:7162–7170.

KATSUKAWA, H., and NINOMIYA, Y. 1999. Capsaicin induces S-like substances in submandibular saliva of the rat. *J. Dent. Res. 78*:1609–1616.

KIM, W. S., NAKAYAMA, K., NAKAGAWA, T., KAWAMURA, Y., HARAGUCHI, K., and MURAKAMI, K. 1991. Mouse submandibular gland prorenin-converting enzyme is a member of glandular kallikrein family. *J. Biol. Chem.* 266:19283–19287.

LE MAGNEN, J. 1986. Hunger. Problems in the Behavioural Sciences. Cambridge University Press, Cambridge.

LESSCHAEVE, I., and NOBLE, A. C. 2005. Polyphenols: factors influencing their sensory properties and their effects on food and beverage preferences. *Am. J. Clin. Nutr.* 81:330S–335S.

LIN, H. H., and ANN, D. K. 1991. Molecular characterization of rat multigene family encoding proline-rich proteins. *Genomics* 10:102–113.

LOPEZ-SOLIS, R. O., and KEMMERLING, U. 2005. Codominant expression of genes coding for different sets of inducible salivary polypeptides associated with parotid hypertrophy in two inbred mouse strains. *J. Cell Biochem.* 95:99–107.

LU, Y., and BENNICK, A. 1998. Interaction of tannin with human salivary proline-rich proteins. *Arch. Oral Biol.* 43:717–728.

MADSEN, H. O., and HJORTH, J. P. 1985. Molecular cloning of mouse PSP mRNA. *Nuc. Acids Res.* 13:1–13.

MAHMOOD, S., and SMITHARD, R. 1993. A comparison of effects of body weight and feed intake on digestion in broiler cockrels with effects of tannins. *Br. J. Nutr.* 70:701–709.

MCDOUGALL, G. J., SHPIRO, F., DOBSON, P., SMITH, P., BLAKE, A., and STEWART, D. 2005. Different polyphenolic components of soft fruits inhibit alpha-amylase and alpha-glucosidase. *J. Agric. Food Chem.* 53:2760–2766.

MEHANSHO, H., HAGERMAN, A. E., CLEMENTS, S., BUTLER, L. G., OGLER, J., and CARLSON, D. M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghum with high tannin levels. *Proc. Natl. Acad. Sci. USA.* 80:3948–3952.

MEHANSHO, H., HAGERMAN, A. E., CEMENTS, S., BUTLER, L., OGLER, J., and CARLSON, D. M. 1985. Indution of proline rich glycoproteins synthesis in mouse salivary glands by isopretrenol and by tannins. *J. Biol. Chem.* 260:4418–4423.

MEHANSHO, H., OGLER, J., and CARLSON, D. M. 1987. Dietary tannins and salivary proline rich peroteins: interation, inductions and defense mechanisms. *Annu. Rev. Nutr.* 7:423–440.

MUENZER, J., BILDSTEIN, C., GLEASON, M., and CARLSON, D. M. 1979. Purification of proline-rich proteins from parotid glands of isoproterenol-treated rats. *J Biol. Chem.* 254:5623–5628.

MYAL, Y., IWASIOW, B., COSBY, H., YARMILL, A., BLANCHARD, A., TSUYUKI, D., FRESNOZA, A., DUCKWORTH, M. L., and SHIU, R. P. C. 1998. Analysis of tissue- and hormone-specific regulation of the human prolactin-induceble protein/gross cystic disease fluid proein-15 gene in transgenic mice. *J. Mol. Endocrinol.* 21:217–223.

NAKAYAMA, T., YASHIRO, K., INOUE, Y., MATSUURA, K., ICHIKAWA, H., HARA, A., and SAWADA, H. 1986. Characterization of pulmonary carbonyl reductase of mouse and guinea pig. *Biochim. Biophys. Acta* 882:220–227.

NEUHOFF, V., AROLD, N., TAUBE, D., and EHRHARDT, W. 1988. Improved staining of proteins in polyacrilamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brillant Blue G-250 and R-250. *Electrophoresis* 9:255–262.

NEYRAUD, E., SAYD, T., MORZEL, M., and DRANSFIELD, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. *J. Proteome Res.* 5:2474–2480.

OPPENHEIM, F. G., SALIH, E., SIQUEIRA, W. L., ZHANG, W., and HELMERHORST, E. J. 2007. Salivary proteome and its genetic polymorphisms. *Ann N Y Acad. Sci.* 1098:22–50.

PANDEY, A., ANDERSEN, J. S., and MANN, M. 2000. Use of mass spectrometry to study signaling pathways. *Sci STKE* 37:PL1.

PERKINS, D. N., PAPPIN, D. J., CREASY, D. M., and COTTRELL, J. S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20:3551–3567.

PRINZ, J. F., and LUCAS, P. W. 2000. Saliva tannin interactions. *J. Oral Rehabil.* 27:991–994.

PROCTOR, G. B., and CARPENTER, G. H. 2007. Regulation of salivary gland function by autonomic nerves. *Auton. Neurosci.* 133: 3–18.

ROXO-ROSA, M., DA COSTA, G., LUIDER, T. M., SCHOLTE, B. J., COELHO, A. V., AMARAL, D. M., and PENQUE, D. 2006. Proteomic analysis of nasal cells from cystic fibrosis (CF) patients and non-CF control individuals: search for novel biomarkers of CF lung disease. *Proteomics* 6:2314–25.

SHAW, P. A., and YU, W. A. 2000. Autonomic regulation of cystatin S gene expression in rat submandibular glands. *Auton. Neurosci.* 83:49–57.

SHIMADA, T., SAITOH, T., and MATSUI, T. 2004. Does acclimation reduce the negative effects of acorn tannins in the wood mouse *Apodemus speciosus?*. *Acta Theriol.* 49:203–214.

SHIMADA, T., SAITOH, T., SASAKI, E., NISHITANI, Y., and OSAWA, R. 2006. Role of tannin-binding salivary proteins and tannase-producing bacteria in the acclimation of the Japanese wood mouse to acorn tannins. *J. Chem Ecol.* 32:1165–1180.

SHORI, D. K., and ASKING, B. 2001. Dynamics of protein and fluid secretion from the major salivary glands of rat: relevance of research findings to clinically observed defective secretion in cystic fibrosis. *Pflugers Arch.* 443:Suppl 1535–543.

SKOPEC, M. M., HAGERMAN, A. E., and KARASOV, W. H. 2004. Do salivary proline-rich proteins counteract dietary hyrdolyzable tannin in laboratory rats. *J. Chem. Ecol.* 30:1679–1692.

SLADECK, N. 2003. Human aldehyde dehydrogenase: Potential pathological, pharmacological and toxicological impact. *J. Biochem. Mol. Toxicol.* 17:7–23.

STJEIN, V., AMEROGEN, A.V., VEERMAN, E.C.I., KASANMOENTALIB, S., and OVERDUK, B., 1999. Chitinase in whole human saliva and in whole saliva of patients with periodontal inflammation. *Eur. J. Oral Sci.* 107:328–337.

VITORINO, R., LOBO, M. J., FERRER-CORREIRA, A. J., DUBIN, J. R., TOMER, K. B., DOMINGUES, P. M., and AMADO, F. M. 2004. Identification of human whole saliva protein components using proteomics. *Proteomics* 4:951–962.

WATERS, C. A., MORAND, J. N., SCHATZMAN, R. C., and CARLSON, D. M. 1998. Induction of p34cdc2 in mouse parotid glands upon activation of beta 1-adrenergic receptors. *Cell Mol. Biol. (Noisy-le-grand)* 44:333–342.

WILLIAMS, K. M., EKSTROM, J., and MARSHAL, T. 1999. High resolution electrophoretic analysis of rat parotid salivary proteins. *Electrophoresis* 20:1373–1381.

YAO, Y., BERG, E., COSTELLO, C. E., TROXLER, R. F., and OPPENHEIM, F. G. 2003. Identification of proteins components in human acquired enamel pellicle and whole saliva using novel proteomics approaches. *J. Biol. Chem.* 278:5300–5308.

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Comparison of Electrophoretic Protein Profiles from Sheep and Goat Parotid Saliva

Elsa Lamy · Gonçalo da Costa · Fernando Capela e Silva · José Potes · Ana Varela Coelho · Elvira Sales Baptista

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Abstract Saliva provides a medium for short-term adaptation to changes in diet composition, namely, the presence of plant secondary metabolites. Salivary proteins have biological functions that have particular influence on oral homeostasis, taste, and digestive function. Some salivary proteins, such as proline-rich proteins, are present in browsers but absent in grazers. Despite the significance of salivary proteins, their expression patterns in many herbivores are unknown. We investigated the sodium dodecyl sulfate-polyacrylamide gel electrophoresis profile of parotid salivary proteins from two domesticated species, one a grazer, the sheep, *Ovis aries*, and the other a mixed feeder, the goat, *Capra hircus*, both fed on the same conventional diet. With 12.5% polyacrylamide linear gels, we observed uniform patterns of salivary proteins within the two species. In the goat profile, 21 major bands were observed, and 19 in the sheep profile. Each band was subjected to peptide mass fingerprinting for purposes of identification, allowing for 16 successful protein identifications. Marked differences were observed between the species in the region of 25–35 kDa molecular weights: one band was present in significantly different intensities; three bands were present only in goats; and one band was present only in sheep. This is the first report of a comparison of the protein salivary composition of sheep and goats and suggests that future research should be conducted to reveal a physiological function for salivary proteins related to the differences in feeding behavior of these species.

Keywords *Capra hircus* · Feeding behavior · MALDI-TOF MS · *Ovis aries* · Parotid saliva · Protein identification · Salivary proteins · SDS-PAGE

Introduction

Salivary function is closely related to oral health and digestion. Humphrey and Williamson (2001) organized the functions of saliva into five major categories: (1) lubrication

E. Lamy · E. S. Baptista
ICAM-Instituto de Ciências Agrárias Mediterrânicas,
Universidade de Évora,
Evora, Portugal

G. da Costa · A. V. Coelho
ITQB-Instituto de Tecnologia Química e Biológica,
Oeiras, Portugal

F. C. e Silva
Departamento de Biologia, Universidade de Évora,
Evora, Portugal

F. C. e Silva · J. Potes
Centro de Investigação em Ciências e Tecnologias da Saúde,
Evora, Portugal

J. Potes
Departamento de Medicina Veterinária, Universidade de Évora,
Evora, Portugal

A. V. Coelho
Departamento de Química, Universidade de Évora,
Evora, Portugal

E. S. Baptista (✉)
Departamento de Zootecnia, Universidade de Évora,
Evora, Portugal
e-mail: elsaba@uevora.pt

and protection, (2) buffering action and clearance, (3) maintenance of tooth integrity, (4) antibacterial activity, and (5) taste and digestion. Saliva modulates taste perception through the transportation of taste substances and the protection of taste receptors, as well as through the chemical interaction of salivary constituents with taste substances. Salivary flow rate and composition are influenced by the quality of taste stimuli (Spielman 1990), drugs and physiological factors (Aps and Martens 2005), and, at the same time, salivary flow rate and composition affect taste perception (Matsuo 2000). Some salivary proteins are involved in feeding behavior, namely von Ebner's gland salivary protein (Kock et al. 1994), salivary cystatins (Katsukawa et al. 2002), and salivary kallikreins (Yamada et al. 2006). Levels of tannin-binding salivary proteins (TBSPs) in animal saliva are associated with tannin levels in the diet (Mehansho et al. 1983, 1987, 1992; Austin et al. 1989; Hagerman and Robbins 1993; Fickel et al. 1998; Makkar and Becker 1998; Clauss et al. 2003a, b). A recent review by Shimada (2006) stresses the importance of gathering basic information on salivary proteins as a way of understanding the relationships between feeding niches and saliva composition.

Sheep and goats are both generalist herbivores. They have similar body sizes and frequently graze together in major farming systems (Bartolome et al. 1998; El Aich and Waterhouse 1999). Although they are competing species that co-exist in the same niche and have access to the same forage items, they often show different feeding behavior, selecting and ingesting diets that overlap to variable degrees (Ngwa et al. 2000; Pande et al. 2002). In the context of the three feeding types proposed by Hofmann (1989), sheep are considered grazers, whereas goats are viewed as intermediate feeders, capable of dealing with large amounts of browse in their diets. There are several possible explanations for these differences in feeding behavior. According to the detoxification limitation hypothesis (Freeland and Janzen 1974, recently reviewed by Marsh et al. 2006), goats could have a greater ability to eliminate plant secondary metabolites (PSMs), when compared to sheep. A second explanation is based on one of the deductive generalizations of Hofmann's morphophysiological hypothesis, which suggests that goats may have large salivary glands that produce large amounts of fluid, which helps to digest browse and provides a medium of defense against PSMs.

To our knowledge, salivary protein expression patterns have not been reported from sheep and goats, and only a limited number have been identified for these two species. Austin et al. (1989) used electrophoretic approaches to search for TBSPs in the whole saliva of sheep, cattle, and deer but did not characterize the entire protein profile in the saliva. The aim of the present study was to gain a better understanding of the parotid saliva protein composition of the domestic sheep, *Ovis aries*, and of the goat, *Capra hircus*. To this end, we used one-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) for protein identification by peptide mass fingerprinting (PMF).

Methods and Materials

Animals To obtain saliva samples, we used adult females that had been reared in separate sheep and goat flocks and had grazed on Mediterranean rangeland. Collections were made during six different periods, over the course of 1 yr. In each period, five Merino sheep, *O. aries*, and five Serpentina goats, *C. hircus*, were used and kept in the same location in separated crates for 15 d preceding the saliva collection. During this period, all animals were fed with vetch-oat hay, *Vicia sativa*×*Avena sativa*, and had water and food available *ad libitum*. The objective of the pre-trial period was to keep sheep and goats in similar conditions so as to minimize diet effects when comparing them. Before each saliva collection period, polyethylene urinary cat stylet catheters (1.0 or 1.3×130 mm) were introduced into one of the parotid ducts of each animal, which had previously been anaesthetized intravenously with Xylazine/Ketamine (0.1/5.0 mg/kg). To facilitate the positioning of the catheter, an intravenous 14G cannula was previously inserted into the masseter muscle from the inside to the outside. The catheters were then introduced into the parotid papilla, from the exterior to the interior of the mouth (Fickel et al. 1998), by using guide wires. The free end of the catheter, which protruded 1 cm, was fixed to the cheek skin by transfixation knots. To avoid any possible effect of the anesthetics on the saliva composition (Edwards and Titchen 2003; Edwards et al. 2003), sample collection was initiated 1 d after surgery. In the morning and before food distribution, samples were collected during each of the following 3 d. At least 2 ml of parotid saliva were collected from each animal by aspiration with a syringe.

Saliva Collection and Preparation for Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Each saliva sample was collected into capped polypropylene sample tubes. All samples were frozen immediately in liquid nitrogen and stored at −70°C. Prior to protein quantification, samples were centrifuged at 16,000×*g* for 5 min at 4°C to remove particulate matter. Protein concentration of the parotid saliva was determined by the bicinchoninic acid (BCA) method (Pierce, Rockford, IL, USA), in which bovine serum albumin (BSA) is used as the standard. For

the analysis, 10 μl of either BSA (0–2.0 mg/ml) or saliva were mixed with 200 μl of the BCA reagent and incubated for 30 min at 37°C. Absorbance was measured at 565 nm by using a microtiter plate reader (SpectroMAX 340, Molecular Devices, Union City, CA, USA). Before SDS-PAGE separation, salivary proteins were concentrated with a 5-kDa cut-off ultra-filtration microfuge tube (Millipore, Eschborn, Germany; Ref: UFV5BCC00).

Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis Individual samples of parotid saliva from sheep and goats were run simultaneously in each gel for comparison. Proteins were separated with 12.5% SDS-PAGE (200× 200×1 mm) in a Protean II xi slab gel apparatus (BioRad, CA, USA). Saliva samples with 70 μg of protein were mixed with 4× concentrated SDS sample buffer (0.125 M Tris–HCl pH 6.8, 2% SDS, 5% 2-mercaptoethanol, 20% glycerol with traces of bromophenol blue). The mixture was heated at 90°C for 5 min and immediately cooled on ice until gel application. Electrophoresis was carried out by using a running buffer [0.025 M Tris, 0.192 M glycine, 1% (*w*/*v*) SDS] at pH 8.3, with 100 V constant current. After the sample entered the separation gel, the voltage was changed to 250 V. Molecular mass protein standards (from 15 to 200 kDa; PageRuler Protein Ladder, SM0661, Fermentas, Ontario, CA, USA) were also included in each gel for reference.

Gel Staining and Densitometry Gels were fixed and stained overnight in a solution of 0.1% Coomassie brilliant blue R-250 in 50% (*v*/*v*) methanol and 10% (*v*/*v*) acetic acid and destained with several changes of 10% (*v*/*v*) acetic acid, following the protocol of Beeley et al. (1991) for proline-rich proteins. Digital images of the gels were obtained by using a densitometer (Molecular Dynamics, Amersham Biosciences Europe GmbH, Freiburg, Germany), and the gels were subjected to linescan analysis by using ImageQuant 5.0 software with parameter sensitivity 9.0 and kernel 4.0 (Amersham Biosciences Europe GmbH) to assign the significant bands in the protein profiles.

Protein Identification For protein identification, the PMF approach was used. Stained bands were excised, washed, reduced with dithiothreitol, alkylated with iodoacetamide, and dried in a speedvac. Gel pieces were rehydrated with digestion buffer (50 mM NH_4HCO_3) containing trypsin (Promega, Madison, WI, USA) and incubated overnight at 37°C. The buffered peptides were acidified with formic acid, desalted, and concentrated with C8 microcolumns (POROS R2, Applied Biosystems, Foster City, CA, USA). The peptides were eluted with matrix solution that contained 10 mg/ml α-cyano-4-hydroxycinnamic acid dissolved in 70% (*v*/*v*) acetonitrile/0.1% (*v*/*v*) trifluoroacetic acid. The mixture was allowed to air-dry (dried droplet method). Mass spectra were obtained with a Voyager-DE STR (Applied Biosystems) MALDI-TOF mass spectrometer in the positive ion reflectron mode. External calibration was made by using a mixture of standard peptides (Pepmix 1, LaserBiolabs, Sophia-Antipolis, France). Spectra were processed and analyzed with MoverZ software (Genomic Solutions Bioinformatics, Ann Arbor, MI, USA). Peakerazor software (GPMAW, General Protein/Mass Analysis for Windows, Lighthouse Data, Odense, Denmark; http://www.gpmaw.com) was used to remove contaminant *m*/*z* peaks and for internal calibration. Monoisotopic peptide masses were used to search for protein identification by using Mascot software (Matrix Science, London, UK; http://www.matrixscience.com). Database searches were performed against MSDB (a non-identical protein sequence database maintained by the Proteomics Department at the Hammersmith Campus of Imperial College, London; http://csc-fserve.hh.med.ic.ac.uk/msdb.html) and SwissProt. The following criteria were used to perform the search: (1) mass accuracy of 50–100 ppm; (2) one missed cleavage in peptide masses; and (3) carbamidomethylation of Cys and oxidation of Met as fixed and variable amino acid modifications, respectively. Criteria used for protein identification in the Mascot software were (1) significant homology scores achieved in Mascot; (2) significant sequence coverage values; and (3) similarity between the protein molecular mass calculated from the gel and for the identified protein.

Results

Salivary Protein Profile Salivary protein concentrations in both animals ranged from 30 to 2,000 μg/ml. Twenty-one and 19 protein bands (Fig. 1) were reproducibly displayed in goat and sheep parotid saliva protein profiles, respectively. There was a similar pattern to the parotid saliva profiles for the two species, except for the 25 to 35 kDa molecular mass range. In this range, the parotid saliva electrophoretic profile from sheep had two visible bands (o and s, corresponding to approximately 32 and 26 kDa, respectively), whereas the profile from goat had four bands (p, q, r, and s, corresponding to approximately 30, 28, 27, and 26 kDa, respectively). Band o, which was an intense band in the sheep profile, was not present in goat saliva. Bands p, q, and r, observed in goats, were absent from sheep. Moreover, the protein band s, common to both species, was more intense in sheep. The parotid salivary protein profiles from individual goats (N=4) and sheep (N=5; Fig. 2) revealed patterns that were similar among individuals of each species but different between the species.

Salivary Protein Identification Mass spectra from a total of 21 and 19 peptide digests of well-resolved bands from

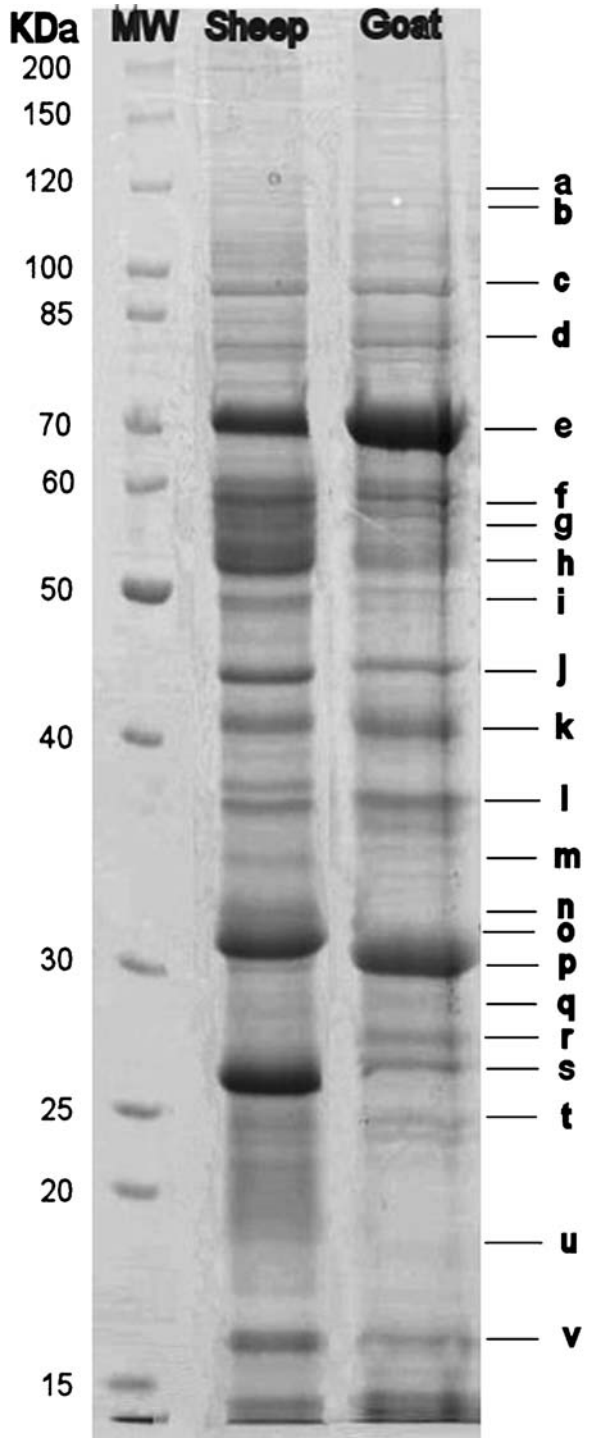

Fig. 1 Typical profiles of sheep and goat parotid salivary proteins in a 12.5% linear gel. Each lane represents the profile from an individual saliva sample. Molecular markers (*MW*) are represented on the *left side* of the figure. The protein bands are identified by letters *a* to *v*

goats and sheep, respectively, were analyzed. The MSDB database was searched by using a taxonomic restriction for "other mammals," and 16 different proteins were identified (Table 1). In most instances, the same proteins were identified in both species. However, the identification of the bands h, n, o, q, r, u, and v were not possible due perhaps to a low amount of protein in some bands or to the existence of several different proteins in the same band or even to a lack of homologous proteins in the searched protein sequence databases. Peptide map comparison for goat and sheep band f shows the presence of some peaks that correspond to the catalase predicted tryptic peptide masses; however, more peaks with relevant intensities were observed. This suggests that catalase is also present in goat f band, probably mixed with other unidentified proteins. Similar results were obtained when peptide maps for band s were compared between goat and sheep. Apolipoprotein A-I was likely present in sheep band s, but the band may also contain other unidentified proteins. This interpretation was supported both by the higher intensity of this band in sheep when compared with the corresponding one from goat and by the presence of mass peaks not observed in goat band s peptide map.

Discussion

Electrophoretic profiles of salivary proteins have been reported for several species, such as rats (Ekström et al. 1996; Williams et al. 1999a), ferrets (Williams et al. 1999b), and cats (Marshall et al. 1993), but the bulk of the studies on salivary profiles have been performed on humans (Ghafouri et al. 2003; Vitorino et al. 2004; Wilmarth et al. 2004; Hardt et al. 2005; Hirtz et al. 2005; Hu et al. 2005; Guo et al. 2006; Walz et al. 2006). According to our knowledge, this is the first study in which the SDS-PAGE electrophoretic profiles of parotid salivary proteins from sheep and goats have been characterized, with MALDI-TOF MS used to identify the more representative proteins.

The proteins identified in the present study (Table 1) can be sorted into three main functional categories. The largest group includes salivary proteins that exhibit immune response or oral protection functions: complement C3 precursor, gelsolin precursor, serotransferrin precursor, catalase, immunoglobulin, annexin A1, cathepsin H precursor, and glutathione S-transferase P. Among these, catalase and glutathione S-transferase P have a more specific role in detoxification. They are associated with feeding behavior because their presence has been associated with plant consumption (Felton and Duffey 1991; Rodman and Miller 1992; Sreerama et al. 1995; Lampe et al. 2000). Annexin A1 has also been related to taste perception (Neyraud et al. 2006).

A second functional category includes proteins involved in protein biosynthesis: elongation factor 2, heat shock protein HSP 90-beta, and protein disulfide-isomerase A3

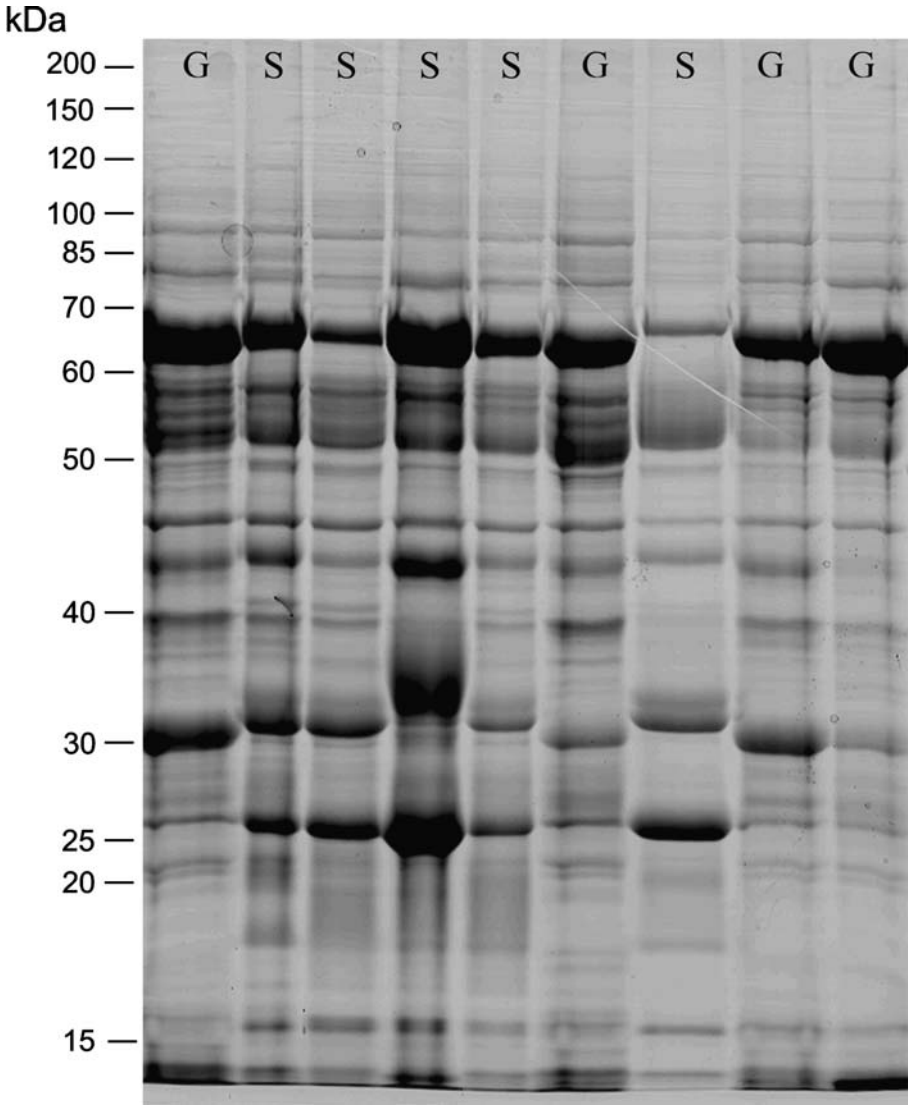

Fig. 2 SDS-PAGE of goat and sheep parotid salivary proteins. Samples were obtained from four different goat (*G*) and five different sheep (*S*) under the same dietary conditions. Similarities among individuals from the same species and differences between species are evident

precursor. The third functional category includes typical serum proteins that, among other functions, are concerned with transport: serotransferrin precursor, serum albumin precursor, and apolipoprotein A-I precursor. The functions of actin and deoxyribonuclease 1 in saliva are not well understood. Some authors have considered deoxyribonuclease 1 as a digestive enzyme (Takeshita et al. 2000), despite others having previously suggested that deoxyribonuclease 1 activity in human parotid saliva is insufficient to fulfill any digestive function (Yaegaki et al. 1982). The presence in saliva of cytoplasmatic proteins, such as actin, may be a consequence of the apocrine-like type of secretion reported for ruminant parotid glands (Stolte and Ito 1996).

Carbonic anhydrase VI is the only protein that has been previously reported from sheep parotid glands, and this is the only sheep salivary protein sequence deposited in databases (Fernley et al. 1988a, b). Carbonic anhydrase VI has a role in electrolytic equilibrium and in the buffer properties of saliva (Kimoto et al. 2006). Its presence in saliva has also been associated with the development of adequate taste function (Henkin et al. 1999).

Our results suggest a strong similarity between the electrophoretic profiles of sheep and goat salivary proteins. From the 16 proteins identified, only one, band p, is not common to both species (Table 1). The similarity likely reflects the phylogenetic proximity of the two species and the consumption of an equal diet during the study. Not surprisingly, we found more pronounced differences when we compared our results with the salivary protein composition of carnivores (Marshall et al. 1993; Williams et al. 1999b) and omnivores (Beeley et al. 1991; Williams et al. 1999a; Hardt et al. 2005). In the dietary habits ranging from carnivores through omnivores to those animals that are exclusively herbivores, plant allelochemical levels increase progressively. It has been suggested (McArthur et al. 1995) that during the evolution from meat to plant eater, selective

Table 1 Proteins identified from SDS-PAGE analysis of parotid saliva of goat, *Capra hircus*, and sheep, *Ovis aries*

Band ID	Animal species	Protein name	Score[a]	Coverage[b] (%)	Pep[c]	MSDB Accession number	MW a[d] (kDa)	MW t[e] (kDa)	Ref.[f]
a	Goat	Complement C3 precursor (fragment)	68	22	9/38	O46544_SHEEP	121	40	Andoh et al. 1997
	Sheep	Complement C3 precursor (fragment)	128	33	12/28				
b	Goat	Elongation factor 2	85	21	13/35	EF2_BOVIN	117	96	Xie et al. 2005
	Sheep	Elongation factor 2	86	24	16/53				
c	Goat	1) Gelsolin precursor +	1) 149	1) 32	1) 20/53				Xie et al. 2005
		2) Heat shock protein HSP 90-beta	2) 60	2) 19	2) 12/53	1)Q3SX14_BOVIN	100	1) 81	
	Sheep	1) Gelsolin precursor +	1) 102	1) 27	1) 16/52	2)HS90B_BOVIN		2) 84	
		2) Heat shock protein HSP 90-beta	2) 109	2) 25	2) 18/52				
d	Goat	Serotransferrin precursor	62	16	10/36	AAA96735	90	80	Xie et al. 2005; Huang 2004;
	Sheep	Serotransferrin precursor	78	17	12/37				Wilmarth et al. 2004
e	Goat	Serum albumin precursor	75	16	7/14	ABSHS	77	71	Xie et al. 2005; Huang 2004;
	Sheep	Serum albumin precursor	81	17	8/13				Wilmarth et al. 2004; Hardt et al. 2005; Ghafouri et al. 2003; Vitorino et al. 2004; Hu et al. 2005
f	Goat	Unidentified	128	32	16/50	CATA_BOVIN	70	60	Xie et al. 2005; Huang 2004
	Sheep	Catalase							
g	Goat	Protein disulfide-isomerase A3 precursor	94	23	13/36	JC2385	67	55	Xie et al. 2005
	Sheep	Protein disulfide-isomerase A3 precursor	73	19	9/32				
h	Goat	Unidentified							
	Sheep	Unidentified							
i	Goat	Ig heavy chain C region	88	39	8/30	C30554	58	52	Xie et al. 2005; Huang 2004;
	Sheep	Ig heavy chain C region	60	31	7/45				Wilmarth et al. 2004
j	Goat	Actin cytoplasmic 1 (Beta-actin)	90	33	10/30	ATBOB	51	42	Hu et al. 2005; Walz et al. 2006
	Sheep	Actin cytoplasmic 1 (Beta-actin)	98	42	14/47				
k	Goat	Carbonic anhydrase VI	63	38	9/41	CAH6_SHEEP	45	36	Xie et al. 2005; Wilmarth et al. 2004;
	Sheep	Carbonic anhydrase VI	133	58	12/32				Hardt et al. 2005; Ghafouri et al. 2003; Vitorino et al. 2004; Hu et al. 2005; Fernley et al. 1988a

Table 1 (continued)

Band ID	Animal species	Protein name	Score[a]	Coverage[b] (%)	Pep[c]	MSDB Accession number	MW a[d] (kDa)	MW t[e] (kDa)	Ref.[f]
l	Goat	Annexin A1	76	36	9/38	S28228	40	40	Xie et al. 2005;
	Sheep	Annexin A1	109	41	11/36				Wilmarth et al. 2004; Hu et al. 2005; Neyraud et al. 2006
m	Goat	Deoxyribonuclease-1	62	30	5/17	B26325	37	29	Tenjo et al. 1993;
	Sheep	Deoxyribonuclease-1	60	38	6/34				Nadano et al. 1993; Williams et al., 1999a
n	Goat	Unidentified							
	Sheep	Unidentified							
o	Goat	Not present							
	Sheep	Unidentified							
p	Goat	Cathepsin H precursor	55[g]	23	7/41	Q3T0I2[h]	30	38	Saliva Proteome Project, http://fields.scripps.edu/public/project/saliva/
	Sheep	Not present							
q	Goat	Unidentified							
	Sheep	Not present							
r	Goat	Unidentified							
	Sheep	Not present							
s	Goat	Apolipoprotein A-I precursor	174	44	17/51	AAI02942	25	30	Xie et al. 2005; Huang 2004; Wilmarth et al. 2004; Ghafouri et al. 2003
	Sheep	Unidentified							
t	Goat	Glutathione S-transferase Pi	96	64	10/47	AF186248	22	24	Xie et al. 2005; Wilmarth et al. 2004; Ghafouri et al. 2003; Vitorino et al. 2004; Hu et al. 2005
	Sheep	Unidentified	100	64	10/43				
u	Goat	Unidentified							
	Sheep	Unidentified							
v	Goat	Unidentified							
	Sheep	Unidentified							

[a] The minimum Mascot score for a probability less than 5% for the match to be a random event is 59.
[b] Percentage of identified protein sequence covered by matched peptides
[c] Number of peptides from experimental PMF whose masses match those from a theoretical PMF determined from a known sequence/Number of peptides from experimental PMF submitted for Mascot search
[d] Molecular weight apparent
[e] Molecular weight theoretical
[f] Articles reporting the presence of identified proteins in saliva
[g] For Swiss-Prot database searches the minimum score for a probability less than 5% for the match to be a random event is 53.
[h] Swiss-Prot accession number

pressure encouraged salivary proteins with defense functions against anti-nutritive and/or toxic substances present in plants. Saliva is one of the behavioral and physiological mechanisms that mammals have evolved for coping with hazards related to feeding. For herbivores, this can mean having to deal with toxic and anti-nutritive substances, whereas for omnivores, the major risk faced is that of foodborne illness. The tradeoff between costs/nutritional benefits could be reflected in the salivary profiles of different trophic groups, with differences in the proportion of proteins. In humans and rodents, the proportion of serum proteins, relative to total salivary proteins, is lower than the proportion observed in the present study for sheep and goats. Saliva with a composition similar to serum can be more useful for ruminants than for humans or rodents. The lack of digestive enzymes in ruminant saliva has been widely reported and probably reflects digestive characteristics, such as the low levels of starch in the diet and the

importance of ruminal fermentation of structural carbohydrates. An adequate digestion is achieved by the rhythms of salivary secretion and by a more marked role of saliva in providing and maintaining a buffered environment for ruminal fermentation, contributing to half of the bicarbonate entering the rumen (Owens et al. 1998). The digestive differences between ruminants and omnivores, such as humans and rodents referred to above, can also explain why the latter possess other salivary proteins, which we have not found in sheep and goats.

Despite the similarities, the differences found between sheep and goats parotid salivary protein profiles are also meaningful. From the bands common to both profiles, differences in intensity were observed only for band s, identified as apolipoprotein A-I, which was more intense in the sheep profile. The large number of peaks in the peptide map for band s suggests additional unidentified proteins of similar mass in the same gel band. A more pronounced difference was observed in the region of 25–35 kDa. Band p, which was only observed in the goat profile, contained cathepsin H. This protein is involved in the degradation of proteins in lysosomes, and no role in digestion has been attributed to it. As previously discussed, the presence of this cytoplasmic protein in saliva may result from the apocrine-like type of secretion characteristic of ruminants (Stolte and Ito 1996). In addition to the cathepsin peaks, a large number of other peaks were present in the peptide map, suggesting the presence of unidentified proteins in this intense gel band as well. It is possible that cathepsin is also present in sheep parotid saliva in low concentrations, which were insufficient to allow a band observation in Coomassie stained gels. For band o, which was only observed in the sheep profile, and bands q and r, only observed in the goat profile, we were unable to obtain identification.

Some authors (Austin et al. 1989; Fickel et al. 1998) refer to the presence of salivary PRPs in browser ruminant species and to their absence in sheep saliva. As sheep are grazers and goats are intermediate feeders, one possibility is that goats could have salivary PRPs. Human basic PRPs, which are the group of PRPs with a higher affinity for tannins, have molecular masses between 14 and 45 kDa (Bedi and Bedi 1995), which correspond to mass values of the unidentified bands. We tested the presence of PRPs by staining the gels with Coomassie brilliant blue R-250, following the protocol of Beeley et al. (1991), but were unable to observe the characteristic pink bands. The absence of salivary PRPs in sheep and goat parotid saliva may reflect the low tannin diet consumed by the two species during the experiment. Further studies with the incorporation of high levels of tannins into the diet may be useful in assessing the induction of this particular group of salivary proteins.

This study provides a first step to the full characterization of the goat and sheep parotid saliva protein profile, and it provides useful information that can be used to study further the immediate oral adaptation to the diet. Based on the differences between the species, even when fed under a similar feeding situation, we suspect that salivary protein composition can play an important role in feeding choices. The complexity of parotid saliva is evident from the great number of protein bands, the lack of identification of some of them, and the large number of tryptic peptides obtained for each one. This highlights the importance of the use of more powerful separation techniques. Moreover, we think that more dynamic information can be obtained by studying these two species subjected to different diets. We intend to use two-dimensional electrophoresis coupled to MS and MS/MS to study potential changes in the parotid saliva proteome caused by the consumption of tannin-enriched diets.

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References

ANDOH, A., FUJIYAMA, Y., KIMURA, T., UCHIHARA, H., SAKUMOTO, H., OKABE, H., and BAMBA, T. 1997. Molecular characterization of complement components (C3, C4 and factor B) in human saliva. *J. Clin. Immunol* 17:404–407.

APS, J. K., and MARTENS, L. C. 2005. The physiology of saliva and transfer of drugs into saliva. *Forensic Sci. Int* 150:119–131.

AUSTIN, P. J., SUCHAR, L. A., OBBINS, C. T., and HAGERMAN, A. E. 1989. Tannin binding proteins in the saliva of deer and their absence in the saliva of sheep and cattle. *J. Chem. Ecol* 15:1335–1347.

BEDI, G. S., and BEDI, S. K. 1995. Purification and characterization of rat parotid glycosylated basic and acidic proline rich proteins. *Prep. Biochem* 25:119–132.

BEELEY, J. A., SWEENEY, D., LINDSAY, J. C., BUCHANAN, M. L., SARNA, L., and KHOO, K. S. 1991. Sodium dodecyl sulphate polyacrylamide gel electrophoresis of human parotid salivary proteins. *Electrophoresis* 12:1032–1041.

BARTOLOME, J., FRANCH, J., PLAIXATS, J., and SELIGMAN, N. G. 1998. Diet selection by sheep and goats on Mediterranean heath-woodland range. *J. Range Manage* 51:383–391.

CLAUSS, M., LASON, K., GEHRKE, J., LECHNER-DOLL, M., FICKEL, J., GRÜNE, T., and JÜRGEN STREICH, W. 2003a. Captive roe deer (*Capreolus capreolus*) select for low amounts of tannic acid but not quebracho: fluctuation of preferences and potential benefits. *Comp. Biochem. Physiol. B Biochem. Mol. Biol* 136:369–382.

CLAUSS, M., LECHNER-DOLL, M., and STREICH, W. J. 2003b. Ruminant diversification as an adaptation to the physicomechanical characteristics of forage. A reevaluation of an old debate and a new hypothesis. *Oikos* 102:253–262.

EDWARDS, A. V., and TITCHEN, D. A. 2003. Autonomic control of protein production by the parotid gland of sheep. *Auton. Neurosci* 103:38–39.

EDWARDS, C. M., CORKERY, P. P., and EDWARDS, A. V. 2003. Submandibular responses to stimulation of the parasympathetic innervation in anesthetized sheep. *J. Appl. Physiol* 95:1598–1605.

EKSTRÖM, J., MARSHALL, T., TOBIN, G., and WILLIAMS, K. M. 1996. Electrophoretic analysis of rat parotid salivary protein composition:

investigation of the parasympathetic atropine resistant secretion. *Acta Physiol. Scand* 156:75–79.

EL AICH, A., and WATERHOUSE, A. 1999. Small ruminants in environmental conservation. *Small Rum. Res* 34:271–287.

FELTON, G. W., and DUFFEY, S. S. 1991. Protective action of midgut catalase in lepidopteran larvae against oxidative plant defenses. *J. Chem. Ecol* 17:1715–1732.

FERNLEY, R. T., WRIGHT, R. D., and COGHLAN, J. P. 1988a. Complete amino acid sequence of ovine salivary carbonic anhydrase. *Biochemistry* 27:2815–2820.

FERNLEY, R. T., COGHLAN, J. P., and WRIGHT, R. D. 1988b. Purification and characterization of a high-Mr carbonic anhydrase from sheep parotid gland. *Biochem. J* 249:201–207.

FICKEL, J., GORITZ, F., JOEST, B. A., HILDEBRANDT, T., HOFMANN, R. R., and BREVES, G. 1998. Analysis of parotid and mixed saliva in roe deer (*Capreolus capreolus* L.). *J. Comp. Physiol. [B]* 168:257–264.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals: the role of plant secondary compounds. *Am. Nat* 108:269–289.

GHAFOURI, B., TAGESSON, C., and LINDHAL, M. 2003. Mapping of proteins in human saliva using two-dimensional gel electrophoresis and peptide mass fingerprinting. *Proteomics* 3:1003–1015.

GUO, T., UDNICK, P. A., WANG, W., LEE, C. S., DEVOE, D. L., and BALGLEY, B. M. 2006. Characterization of the human salivary proteome by capillary isoelectric focusing/nanoreversed-phase liquid chromatography coupled with ESI-tandem MS. *J. Proteome Res* 5:1469–1478.

HAGERMAN, A. E., and OBBINS, C. T. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. *Can. J. Zool* 71:628–633.

HARDT, M., THOMAS, L. R., DIXON, S. E., NEWPORT, G., AGABIAN, N., PRAKOBPHOL, A., HALL, S. C., WITKOWSKA, H. E., and FISHER, S. J. 2005. Toward defining the human parotid gland salivary proteome and peptidome: identification and characterization using 2D SDS-PAGE, ultrafiltration, HPLC, and mass spectrometry. *Biochemistry* 44:2885–2899.

HENKIN, R. I., MARTIN, B. M., and AGARWAL, R. P. 1999. Decreased parotid saliva gustin/carbonic anhydrase VI secretion: an enzyme disorder manifested by gustatory and olfactory dysfunction. *Am. J. Med. Sci* 318:380–391.

HIRTZ, C., CHEVALIER, F., CENTENO, D., EGEA, J. C., OSSIGNOL, M., SOMMERER, N., and DE PERIERE, D. 2005. Complexity of the human whole saliva proteome. *J. Physiol. Biochem* 61:469–480.

HOFMANN, R. R. 1989. Evolutionary steps of ecophysiological adaptation and diversification of ruminants: a comparative view of their digestive system. *Oecologia* 78:443–457.

HU, S., XIE, Y. M., AMACHANDRAN, P., LOO, R. R. O., LI, Y., LOO, J. A., and WONG, D. T. 2005. Large-scale identification of proteins in human salivary proteome by liquid chromatography/mass spectrometry and two-dimensional gel electrophoresis-mass spectrometry. *Proteomics* 5:1714–1728.

HUANG, C. M 2004. Comparative proteomic analysis of human whole saliva. *Arch. Oral Biol* 49:951–962.

HUMPHREY, S. P., and WILLIAMSON, R. T. 2001. A review of saliva normal composition, flow and function. *J. Prosthet. Dent* 85:162–169.

KATSUKAWA, H., SHANG, Y., NAKASHIMAM, K., YANG, K. H., OHASHI, R., SUGITA, D., MISHIMA, K., NAKATA, M., NINOMIYA, Y., and SUGIMURA, T. 2002. Salivary cystatins influence ingestion of capsaicin-containing diets in the rat. *Life Sci* 71:457–467.

KIMOTO, M., KISHINO, M., YURA, Y., and OGAWA, Y. 2006. A role of salivary carbonic anhydrase VI in dental plaque. *Arch. Oral Biol* 51:117–122.

KOCK, K., ORLEY, S. D., MULLINS, J. J., and SCHMALE, H. 1994. Denatonium bitter tasting among transgenic mice expressing rat von Ebner's gland protein. *Physiol. Behav* 56:1173–1177.

LAMPE, J. W., CHEN, C., LI, S., PRUNTY, J., GRATE, M. T., MEEHAN, D. E., BARALE, K. V., DIGHTMAN, D. A., FENG, Z., and POTTER, J. D. 2000. Modulation of human glutathione S-transferases by botanically defined vegetable diets. *Cancer Epidemiol. Biomarkers Prev* 9:787–793.

MAKKAR, H. P. S., and BECKER, K. 1998. Adaptation of cattle to tannins: role of proline-rich proteins in oak-fed cattle. *Animal Sci* 67:277–281.

MARSH, K. J., WALLIS, I. R., ANDREW, R. L., and FOLEY, W. J. 2006. The detoxification limitation hypothesis: where did it come from and where is it going? *J. Chem. Ecol* 32:1274–1266.

MARSHALL, T., WILLIAMS, K. M., EKSTRÖM, J., TOBIN, G., BAYARD, C., and VESTERBERG, O. 1993. Electrophoretic analysis of stimulated cat parotid saliva. *Electrophoresis* 14:1328–1332.

MATSUO, R. 2000. Role of saliva in the maintenance of taste sensitivity. *Critical Reviews Oral Biol. Med* 11:216–229.

MCARTHUR, C., SANSON, G. D., and BEAL, A. M. 1995. Salivary proline-rich proteins in mammals: roles in oral homeostasis and counteracting dietary tannin. *J. Chem. Ecol* 21:663–691.

MEHANSHO, H., HAGERMAN, A., CLEMENTS, S., BUTLER, L. G., OGLER, J. C., and CARLSON, D. M. 1983. Modulation of proline-rich protein biosynthesis in rat parotid glands by sorghums with high tannin levels. *Proc. Natl. Acad. Sci. U.S.A* 80:3948–3952.

MEHANSHO, H., BUTLER, L. G., and CARLSON, D. M. 1987. Dietary tannins and salivary proline-rich proteins: interactions, induction and defense mechanism. *Annu. Rev. Nutr* 7:423–440.

MEHANSHO, H., ASQUITH, T. N., BUTLER, L. G., OGLER, J. C., and CARLSON, D. M. 1992. Tannin-mediated induction of proline-rich protein synthesis. *J. Agric. Food Chem* 40:93–97.

NADANO, D., YASUDA, T., and KISHI, K. 1993. Measurement of deoxyribonuclease I activity in human tissues and body fluids by a single radial enzyme-diffusion method. *Clin. Chem* 39:448–452.

NEYRAUD, E., SAYD, T., MORZEL, M., and DRANSFIELD, E. 2006. Proteomic analysis of human whole and parotid salivas following stimulation by different tastes. *J. Proteome Res* 5:2474–2480.

NGWA, A. T., PNE, D. K., and MAFENI, J. M. 2000. Feed selection and dietary preferences of forage by small ruminants grazing natural pastures in the Sahelian zone of Carmeroon. *Anim. Feed Sci. Technol* 88:253–266.

OWENS, F. N., SECRIST, D. S., HILL, W. J., and GILL, D. R. 1998. Acidosis in cattle: a review. *J. Anim. Sci* 76:275–286.

PANDE, R. S., KEMP, P. D., and HODGSON, J. 2002. Preference of goats and sheep for browse species under field conditions. *N. Z. J. Agric. Res* 45:97–102.

RODMAN, D. H., and MILLER, D. J. 1992. Enzyme activities associated with salivary glands of the froghopper *Eoscarta carnifex* (F.) (Homoptera, Cercopoidae): possible role of salivary catalase in phytotoxicity. *Aust. J. Zool* 40:365–370.

SHIMADA, T. 2006. Salivary proteins as a defense against dietary tannins. *J. Chem. Ecol* 32:1149–1163.

SPIELMAN, A. I. 1990. Interaction of saliva and taste. *J. Dent. Res* 69:838–843.

SREERAMA, L., HEDGE, M. W., and SLADEK, N. E. 1995. Identification of a class 3 aldehyde dehydrogenase in human saliva and increased levels of this enzyme, glutathione S-transferases, and DT-diaphorase in the saliva of subjects who continually ingest large quantities of coffee or broccoli. *Clin. Cancer Res* 1:1153–1163.

STOLTE, M., and ITO, S. 1996. A comparative ultrastructural study of the parotid gland acinar cells of nine wild ruminant species (Mammalia, Artiodactyla). *Eur. J. Morphol* 34:79–85.

TAKESHITA, H., MOGI, K., YASUDA, T., NAKAJIMA, T., NAKASHIMA, Y., MOI, S., HOSHINO, T., and KISHI, K. 2000. Mammalian deoxyribonucleases I are classified into three types: pancreas, parotid,

and pancreas-parotid (mixed), based on differences in their tissue concentrations. *Biochem. Biophys. Res. Commun* 269:481–484.

TENJO, E., SAWAZAKI, K., YASUDA, T., NADANO, D., TAKESHITA, H., IIDA, R., and KISHI, K. 1993. Salivary deoxyribonuclease I polymorphism separated by polyacrylamide gel-isoelectric focusing and detected by the dried agarose film overlay method. *Electrophoresis* 14:1042–1044.

VITORINO, R., LOBO, M. J., FERRER-CORREIRA, A. J., DUBIN, J. R., TOMER, K. B., DOMINGUES, P. M., and AMADO, F. M. 2004. Identification of human whole saliva components using proteomics. *Proteomics* 4:1109–1115.

WALZ, A., STUHLER, K., WATTENBERG, A., HAWRANKE, E., MEYER, H. E., SCHMALZ, G., BLUGGEL, M., and UHL, S. 2006. Proteome analysis of glandular parotid and submandibular-sublingual saliva in comparison to whole human saliva by two-dimensional gel electrophoresis. *Proteomics* 6:1631–1639.

WILLIAMS, K. M., EKSTRÖM, J., and MARSHALL, T. 1999a. High-resolution electrophoretic analysis of rat parotid salivary proteins. *Electrophoresis* 20:1373–1381.

WILLIAMS, K. M., EKSTRÖM, J., and MARSHALL, T. 1999b. The protein composition of ferret parotid saliva as revealed by high-resolution electrophoretic methods. *Electrophoresis* 20:2818–2823.

WILMARTH, P. A., IVIERE, M. A., USTVOLD, D. L., LAUTEN, J. D., MADEN, T. E., and DAVID, L. L. 2004. Two-dimensional liquid chromatography study of the human whole saliva proteome. *J. Proteome Res* 3:1017–1023.

XIE, H., HODUS, N. L., GRIFFIN, R. J., CARLIS, J. V., and GRIFFIN, T. J. 2005. A catalogue of human saliva proteins identified by free flow electrophoresis-based peptide separation and tandem mass spectrometry. *Mol. Cell Proteomics* 4:1826–1830.

YAEGAKI, K., SAKATA, T., OGURA, R., KAMEYAMA, T., and SUJAKU, C. 1982. Influence of aging on DNase activity in human parotid saliva. *J. Dent. Res* 61:1222–1224.

YAMADA, A., NAKAMURA, Y., SUGITA, D., SHIROSAKI, S., OHKURI, T., KATSUKAWA, H., NONAKA, K., IMOTO, T., and NINOMIYA, Y. 2006. Induction of salivary kallikreins by the diet containing a sweet suppressive peptide, gurmarin, in the rat. *Biochem. Biophys. Res* 346:386–392.

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Potential Chemosignals in the Anogenital Gland Secretion of Giant Pandas, *Ailuropoda melanoleuca*, Associated with Sex and Individual Identity

Jian-Xu Zhang · Dingzhen Liu · Lixing Sun · Rongping Wei · Guiquan Zhang · Honglin Wu · Hemin Zhang · Chenghua Zhao

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Abstract With a combination of dichloromethane extraction and analysis by gas chromatography–mass spectrometry (GC-MS), we found 39 compounds (corresponding to 38 GC peaks) in the anogenital gland secretion (AGS) of captive adult giant pandas, *Ailuropoda melanoleuca*, during the non-mating season. In addition to indole, squalene, and some of the straight-chain fatty acids that had been characterized previously from the AGS of giant pandas, we identified several new compounds such as decenal, two isomers of decadienal, phenylacetic acid, 5-methylhydantoin, hydroquinone, phenylpropanoic acid, and erucic acid. Quantitative comparison of the relative abundances of the 20 main GC peaks revealed that 5-methylhydantoin, indole, and erucic acid are putative female pheromones, whereas squalene and hydroquinone are putative male pheromones. In addition to the presence of a few individual-specific compounds, the relative abundances of most of the 21 constituents varied more among individuals than within individuals. This suggests that individual identity might be coded in both digital and analog form. The chemical composition of different AGS samples from the same pandas consistently displayed a minimum cluster distance, much smaller than that between samples from different individuals in a hierarchical linkage cluster (average linkage) dendrogram. Our results indicate that the AGS might contain an "odor fingerprint." Although putative sex pheromones such as squalene and erucic acid should be assessed further by bioassay, our study suggests that synthetic chemosignals might be useful in modulating the behavior and physiology of giant pandas.

Keywords *Ailuropoda melanoleuca* · Anogenital gland secretions (AGS) · Giant panda · Individuality · Pheromone · Sex · Sex specificity

J.-X. Zhang · C. Zhao
State Key Laboratory of Integrated Management of Pest Insects and Rodents in Agriculture, Institute of Zoology, Chinese Academy of Sciences, Beijing 100080, China

D. Liu (✉)
MOE, Key Laboratory of Biodiversity Science and Ecological Engineering, Institute of Ecology, Beijing Normal University, Beijing 100875, China
e-mail: dzliu@bnu.edu.cn

L. Sun
Department of Biological Sciences, Central Washington University, Ellensburg, WA 98926-7537, USA

R. Wei · G. Zhang · H. Wu · H. Zhang
Key Laboratory for Reproduction and Conservation Genetics of Endangered Wildlife of Sichuan Province, China Conservation and Research Center for the Giant Panda, Wolong 623006, China

Introduction

Pheromones play crucial roles in modulating sexual and social behavior in mammals (Brown and MacDonald 1985). However, compared with our knowledge of pheromonal compounds of insects, mammalian chemical signals have seldom been characterized structurally, partially owing to their complex composition. Nonetheless, a large amount of information has been amassed about pheromonal functions of whole odorant secretion/excretion in mammals (Brown and MacDonald 1985; Novotny et al. 1999). For a comprehensive understanding of mammalian chemical communication, it is necessary to combine bioassay and

chemical analysis to identify the pheromonal components involved (Singer et al. 1997; Novotny et al. 1999). Fortunately, a systematic approach to screening for pheromonal compounds from numerous scent sources by gas chromatography (GC) has been established in mice, whereby odorant components that vary with biological characters can be considered as pheromone candidates for further verification by bioassay (Singer et al. 1997; Novotny et al. 1999). By using this approach, we have recently characterized some new pheromone components from the preputial glands of the house mouse, *Mus musculus*, and Brandt's vole, *Lasiopodomys brandtii*, and from the flank glands of the golden hamster, *Mesocricetus auratus* (Zhang et al. 2007a, b; J.X. Zhang, unpublished data). These new pheromone components include high boiling compounds such as hexadecanol, hexadecyl acetate, farnesyl acetate, tetradecanoic acid, and hexadecanoic acid (Zhang et al. 2007a, b; J.X. Zhang, unpublished data). The discovery of these higher boiling compounds has extended the spectrum and concept of mammalian pheromones.

The giant panda, *Ailuropoda melanoleuca*, is an endangered species that inhabits fragmented mountainous areas of Sichuan, Shaanxi, and Gansu provinces in China. Poor in eyesight and hearing ability, pandas mainly use their anogenital gland secretions (AGS) and urine to communicate with each other and mediate their social interactions (Schaller et al. 1985; Swaisgood et al. 1999; Liu et al. 2002). Behavioral tests with giant pandas have revealed that the AGS carries a wealth of information about sex and individuality (Swaisgood et al. 2000; White et al. 2002, 2003; Liu et al. 2005; Tian et al. 2007). Male and female pandas show different behavioral responses to AGS samples from conspecific donors of the same and opposite sex. The chemosensory response in the panda differs between the sexes and is age-dependent (Swaisgood et al. 2000; White et al. 2002, 2003; Liu et al. 2005; Tian et al. 2007). Chemical analysis of the AGS should provide further information about pheromone-based behavioral discrimination in the panda. So far, however, sex-and age-dependent pheromonal components have not been identified due largely to the distractive presence of numerous inactive alkanes (e.g., Yuan et al. 2004; Liu et al. 2006) and to inadequacies in the analytical system, which is often capable of detecting only a small portion in the repertoire of odorant compounds (Hagey and MacDonald 2003).

In the current study, we improved our analytical system and used the relative abundances of the principal GC peaks (Sun and Müller-Schwarze 1998a, b) to compare qualitatively and quantitatively male and female AGS samples and to search for components that differ between the sexes in giant pandas during the non-mating season. With the relative standard deviation (RSD) and hierarchical cluster analysis of the relative abundances of the main GC peaks, we compared AGS samples among individuals. This method has been successfully used in similar studies in several *Mustela* species, the house mouse, the Brandt's vole, and the golden hamster (Zhang et al. 2003, 2005, 2007a, b; J.X. Zhang, unpublished data).

Methods and Materials

Subjects Sixteen adult giant pandas (M/F = 1:1, age >5yr) housed at the Bifengxia Panda Base ($N = 10$) and Wolong Breeding Center ($N = 6$), China Conservation and Research Center for the Giant Panda, Wolong, China were used as AGS donors. Six subjects were kept in traditional enclosures and ten in semi-natural enclosures (Table 1). Each traditional enclosure contained a night pen (5.8 × 2.3m) and an outdoor yard (5.8 × 13m) with grass, some climbing apparatuses, and a small pond as a water source. Each outdoor yard adjoined two others via a cement wall in which there was a small wire mesh fence door (1 × 10m). Each semi-natural enclosure contained a night pen (4 × 3m)

Table 1 Backgrounds and rearing details of individual giant pandas, *A. melanoleuca*, used for analysis of anogenital gland secretions

Number	Traditional Enclosures				Semi-natural Enclosures			
	Studbook No.	Name	Year of Birth	Sex	Studbook No.	Name	Year of Birth	Sex
1	474	Youyou[a]	1998	Female	357	Zhuangzhuang[a]	1989	Male
2	495	Yeye[a]	1999	Female	413	Didi	1994	Male
3	502	Wugang[a]	1999	Male	479	Qingqing	1999	Male
4	503	Lulu[a]	1999	Male	512	Lesheng	2000	Female
5	511	Ximei[a]	2000	Female	518	Longsheng	2000	Male
6	594	Qiangqiang	1987	Male	525	Longfei	2000	Male
7					547	Meiqing	2002	Female
8					549	Zhuyun	2002	Female
9					581	Caocao	2002	Female
10					656	Zizhu	1999	Female

[a] Indicates pandas housed at Wolong Breeding Center; others were housed at the Bifengxia Base.

and an outdoor yard (25 × 60m) that stretched along a 35 to 40° mountain slope with pine trees, shrubs, herbs, some bamboo, *Fargesia robusta*, and a small pond as a water source. Concrete walls separated these adjoining outdoor yards as well. Each subject could hear and see neighboring animals over the walls in certain areas of the yards. All subjects were provided with a daily routine of food consisting of steamed buns (five to six times per day), as well as apples and carrots (three to four times per day). Bamboo was available *ad libitum*. Additional details of housing, management, and diet are available elsewhere (Liu et al. 1998, 2003).

Scent Sample Collection and Extraction Scent samples were collected in 2006 during the non-mating season. The pandas used in our study were docile after several years in captivity, and hence, it was easy for us to collect their scent marks without any anesthesia. On 23 and 27 December 2006, we collected AGS samples with clean and sterilized cotton swabs (Chengdu Medical Inc., Chengdu, Sichuan, China) by rubbing each swab directly on the surfaces of panda anogenital glands while the subjects were induced with an apple or a carrot to sit by a zookeeper. All swabs used were previously treated with alcohol (99%) overnight and air-dried before use for sample collection. The swabs were only handled with gloved, not bare, hands. Odor samples of some pandas were collected twice (one collection on each of the two collection dates). We sealed all samples individually in clean glass vials with lids lined with Teflon and immediately stored them at −20°C in Bifengxia and Wolong, respectively, for 1wk. Later, the samples were packed on ice and air-shipped to the laboratory in Beijing (about 2-hr flight) and stored at −20°C for less than 1wk until they were extracted for analysis by GC–mass spectrometry (GC-MS).

We used dichloromethane (purity >99.5%, Beijing Fine Chemical Company, Ltd., Beijing, China) to extract the compounds from the AGS samples. To do so, we used a clean pair of scissors to cut away the outer 1mm layer of cotton that contained the AGS secretion and then put the resulting cotton pieces into a vial containing dichloromethane. The cotton was extracted in a volume of dichloromethane that reflected an extract concentration of approximately 1mg/10μl solvent. After 12hr, we removed the cotton pieces and stored the remaining solution at −20°C for less than 2d until they were analyzed by GC-MS. We wore PE disposable plastic gloves (Shanghai Dudeli Plastic Inc., Shanghai) when we handled the materials and never touched the swabs, cotton pieces, or instruments with bare hands.

GC-MS Analysis Analytical GC-MS was performed on an Agilent Technologies Network 6890N GC system coupled with 5973 Mass Selective Detector with the NIST/EPA/NIH Mass Spectral Library (2002 version; Agilent Technologies 2002). Chemstation Software (Windows 2000) was used for data acquisition and processing. The GC was equipped with a 30m HP5-MS glass capillary column (0.25mm i.d. × 0.25-μm film thickness). Helium was used as the carrier gas at the flow rate of 1.0ml/min. The temperature of the injector was set at 280°C. The oven temperature was programmed as follows: 100°C as the initial temperature, which was increased by 5°C/min up to 260°C and held for 10min. Finally, the temperature was increased to 280°C and held for 10min for a post-run cleaning of the column. Unknowns were identified by matching their retention times and mass spectra with authentic analogs after separation with the non-polar column (HP5-MS) and confirmed in some cases by using a polar column (DB-WAX, 30m long, 0.25mm i.d. × 0.25-μm film thickness) for separation and matching of retention times and mass spectra with standards. For the analysis with the polar column, GC injector temperature was set at 270°C; the oven temperature was initially set at 100°C, then increased by 5°C/min to 250°C and held for 20min at the flow rate of 1.0ml/min. Electron impact ionization was used at 70eV. Transfer line temperature was 280°C. Scanning mass ranged from 30 to 450amu. One microliter of sample was injected in the splitless mode.

Tentative identifications were made by matching the mass spectra of the GC peaks with those in the MS library (Agilent Technologies 2002). Thirteen (corresponding to 13 GC peaks) of the 39 tentatively identified compounds were verified by matching retention times and mass spectra with those of the authentic standards of *E*2-decenal (peak 1), 5-methylhydantoin (peak 2), hydroquinone (latter portion of peak 4), indole (peak 5), *E*2,*E*4-decadienal (peak 6), phenylpropanoic acid (peak 7), tetradecanoic acid (peak 13), hexadecanoic acid (peak 16), heptadecanoic acid (20), *Z*9-octadecenoic acid (peak 22), octadecanoic acid (peak 23), erucic acid (peak 31), and squalene (peak 38; Table 2). Although the chromatographic and spectral data for *Z*9-octadecenoic acid matched that of the authentic standard, we did not analyze this compound by dimethyldisulfide derivatization to unambiguously establish the position of the double bond. Thus, we take the more conservative approach and designate it as "an octadecenoic acid."

Phenylpropanoic acid (97%), tetradecanoic acid (99.5%), hexadecanoic acid (98%), heptadecanoic acid (95%), octadecanoic acid (97%), *Z*9-octadecenoic acid (97%), *E*-2-decenal (95%), *E*2,*E*4-decadienal (95%), indole (99%), and hydroquinone (99%) were purchased from ACROS Organics, Geel, Belgium. 5-Methylhydantoin (97%) and squalene (98%) were purchased from Sigma-Aldrich Inc., St. Louis, MO, USA. Erucic acid (96.5%) was purchased from Tokyo Kasei Kogyo Co. LTD., Tokyo, Japan.

Table 2 GC-MS data and identified compounds in anogenital gland secretion of giant pandas, *A. melanoleuca*

Peak No.	Retention Time (min)	Compounds	Diagnostic Ions [*m/z* (%relative intensity)]
1	4.79	*E*2-decenal[a]	83(100),70(81),43(40),55(40),110(32),69(29),57(27),67(23),*154*[b](0)
		Phenylacetic acid	91(100),*136*(57),92(30),65(21),39(12)
2	5.01	5-methylhydantoin[a]	42(100),*114*(83),43(70),57(7),86(7),70(5)
3	5.52	A decadienal	81(100),41(42),39(29),67(19),53(16),*152*(9)
4	5.59	Hydroquinone[a]	*110*(100),81(43),53(21),55(15),82(13),54(12),39(11),111(8),51(58)
5	5.70	Indole[a]	*117*(100),90(45),89(40),63(14)
6	5.93	*E*2,*E*4-decadienal[a]	81(100),41(19),67(17),67(17),57(15),55(13),55(13),95(12),39(11)
7	6.44	Phenylpropanoic acid[a]	91(100),104(58),*150*(47),77(17),78(16),65(15),51(14),39(9)
8	7.69	A decenoic acid	73(100),43(90),55(65),69(52),86(43),98(42),81(40),60(15),*170*(0)
9	9.50	Pentadecane	57(100),43(81),71(44),85(38),99(10),*212*(5)
10	11.62	Hexadecane	57(100),43(85),71(82),85(47),99(18),115(10),*226*(10)
11	13.01	Tridecanoic acid	73(100),60(85),43(67),55(62),129(57),171(47),57(45),*214*(25)
12	13.72	Heptadecane	57(100),71(75),43 (61),85(48),41(35), 55(23), 99(18)*240*(10)
13	15.16	Tetradecanoic acid[a]	73(100),60(90),43(72),55(71),129(51),185(30),*228*(17)
14	17.17	Pentadecanoic acid	73(100),60(87),43(87),55(85),57(81),129(48),199(40),*242*(40)
15	18.43	Hexadecanoic acid (branched)	73(100),43(98),57(80),60(79),129(52),213(50),*256*(45)
16	19.14	Hexadecanoic acid[a]	73(100),60(80),43(75),57(70),129(60),*256*(60),213(50),227(8)
17	20.33	Heptadecanoic acid (branched)	43(100),73(90),55(85),57(85),60(83),129(65),227(65),*270*(55)
18	20.46	Heptadecanoic acid (branched)	57(100),55(86),43(75),73(61),69(55),60(51),71(49),129(43),*270*(32)
19	20.59	Heptadecenoic acid	55(100),69(75),83(58),97(54),84(46),43(41),98(33),250(24),*268*(6)
20	20.98	Heptadecanoic acid[a]	73(100),43(86),60(84),57(68),*270*(63),55(60),129(34),227(30)
21	22.19	Octadecanoic acid (branched)	43(100),73(80),57(78),60(70),241(62),129(58),*284*(56), 185(34)
22	22.36	An octadecenoic acid[a]	55(100),69(75),83(63),97(58),43(58),264(23),60(15),*282*(4)
23	22.79	Octadecanoic acid[a]	73(100),43(90),*284*(83),55(82),60(80),57(78),129(72),185(40),241(49)
24	23.98	Unknown compound	174(100),328(15),175(4),224(4),343(3)
25	24.1	A nonadecenoic acid	55(100),69(79),83(64),97(54),43(48),67(39),57(36),60(12), *296*(2)
26	24.42	Nonadecanoic acid	73(100),43(94),*298*(94),55(84),57(84),60(80),129(56),71(47)
27	25.73	An eicosenoic acid	55(100),69(75),83(61),97(58),43(49),84(32),292(25),60(16),*310*(3)
28	26.12	Eicosanoic acid	73(100),43(96),55(91),*312*(89),57(87),60(71),69(58),129(57),84(45)
29	27.39	A heneicosenoic acid	55(100),69(95),83(88),97(72),43(52),306(42),98(41),60(16),*324*(1)
30	27.81	Heneicosanoic acid	43(100),57(88),73(84),*326*(84),69(82),60(67),84(61),97(55)
31	28.89	Erucic acid[a]	55(100),69(74),83(65),97(58),43(54),96(30), 320(28), 60(19),*338*(3)
32	29.35	Docosanoic acid	43(100),55(83),57(82),*340*(80),73(75),60(59),69(56),97(46)
33	30.54	A tricosenoic acid	55(100),83(76),69(71),97(54),43(50),57(37),334(32),60(22),*352*(2)
34	30.89	Tricosanoic acid	55(100),57(86),43(85),*354*(83),73(76),60(55),83(48),97(47),69(45)
35	31.87	A tetracosenoic acid	55(100),69(74),83(65),97(62),43(60),96(34),348(26),60(24), *366*(2)
36	32.34	Tetracosanoic acid	55(100),43(96),*368*(90),57(85),73(67),60(60),83(59),71(59)
37	32.67	A cholestatriene	349(100),*364*(66),141(33),350(28),251(20),365(18),237(18)
38	33.01	Squalene[a]	69(100),81(55),41(22),95(15),137(14),136(13),121(13),341(3),*410*(2)

[a] Compounds verified with authentic standards; other components were identified by comparison with spectra listed in the NIST (Agilent Technologies 2002) mass spectral library and analogous data.
[b] Italicized ions indicate the molecular ion

We converted the peak area of a particular compound into a percentage of the summed peak areas from the 20 main GC peaks (peaks 1–7, 11–14, 16, 20, 22, 23, 26–28, 31, 35, and 38) as a measure of the relative abundance of the relevant compound. If a given GC peak was too small to display the diagnostic MS ions of the corresponding compound, its area was taken as zero.

Statistical Analysis To test the hypothesis that there was sexual dimorphism in the relative abundances of the 20 main GC peaks in the crude extract of the AGS, we first examined the distribution of the raw data by the Kolmogorov–Smirnov test in SPSS for Windows (SPSS Inc. 1999). Then, we used either parametric tests (if the data were normally distributed) or non-parametric tests (if the data were not normally distributed) to analyze the relative abundances of the compounds. We used an independent two-tailed *t* test to analyze for sexual differences in the relative abundances of the compounds represented by peaks 13, 16, 22, 23, 26–28, and 38 (which had normally distributed raw data) and the Mann–Whitney *U* test for the remainder of the 20 main GC peaks (which did not have

normally distributed raw data). All statistical analyses were conducted by using SPSS for Windows (version 10.0; SPSS Inc. 1999). The critical values were set at $\alpha = 0.1$ (used commonly in similar studies, e.g., Gassett et al. 1996) except the intra-individual comparisons where α was set at 0.05.

Hierarchical cluster analysis is a statistical method for finding relatively homogenous clusters of cases based on measured characteristics. We used this analysis (average linkage) with Pearson's correlation coefficients to test the similarity of inter- and intra-individual AGS constituents (based on the 20 main GC peaks in the samples; Zhang et al. 2005).

To determine the variability of the AGS composition between individuals, RSD was used and calculated with the formula:

$$\mathrm{RSD} = (\mathrm{SD}/\mathrm{mean}) \times 100$$

where mean and SD are the average of each volatile peak-area percentage for all same-sex individuals and their standard deviation, respectively (Zhang et al. 2003).

Results

Anogenital Gland Secretion Constituents We tentatively identified 39 compounds from eight male and eight female giant pandas (Fig. 1). Chromatographic and spectral data were used to make these preliminary identifications (Table 2). By matching GC retention times and MS spectra with those of authentic standards, we identified peaks 1, 2 (latter portion of peak 2), 4, 5, 6, 7, 13, 16, 20, 22 (see note in "Methods and Materials"), 23, 31, and 38. The identities of these compounds were verified by a second analysis of the extracts and standards with the DB-WAX polar column.

Compounds in GC peak 2 were not completely resolved when analyzed under our conditions on the HP5-MS capillary column, but the peak included phenylacetic acid [as implied by *m/z* 136 (40 = relative intensity) and *m/z* 91 (100)] and 5-methylhydantoin (5-methyl-2,4-imidazolidinedione) [as indicated by *m/z* 114(94), *m/z* 43(58), and *m/z* 42(100)] (Table 2). GC peak 3 with similar MS data to *E*2, *E*4-decadienal (peak 6) might imply another decadienal (Table 2). Some peaks (e.g., peak 15, 17, 18, and 21) eluted earlier than hexadecanoic acid (peak 16), heptadecanoic acid (peak 20), or octadecanoic acid (peak 23), but showed similar MS data. They may be branched isomers of the corresponding acids. However, their identities were not verified by analysis with authentic standards (Table 2).

GC peaks 9, 10, and 12 were likely alkanes (Table 2). GC peaks 8, 15, 17–19, 21, 24, 25, 29, 30, 32–34, and 36 were tentatively identified as fatty acids and were seldom present in all samples. GC peak 37 might be cholestatriene. These trace GC peaks were excluded from the quantitative analysis (Fig. 1, Table 2).

Sex Differences Quantitative analyses revealed that the relative amount of the compound in peak 38 (squalene) was significantly higher in males and three compounds (peaks 2, 5, and 31) were higher in females (Table 3). Other compounds did not show differences between the sexes. GC peak 2 was only detected in one (Qiangqiang) of the eight males. Mass spectral data indicated that this peak from Qiangqiang only contained phenylacetic acid. However, in five of the eight female subjects, GC peak 2 was composed of 17.82 ± 27.37% ($N = 5$) phenylacetic acid and 82.18 ± 27.37% ($N = 5$) 5-methylhydantoin, which were estimated by each GC area percentage in peak 2 calculated after manually splitting GC peak 2. The latter was more abundant than the former ($t = 2.629$, $df = 4$, $P = 0.058$, paired t test). In other words, 5-methylhydantoin in GC peak 2 was not only female-specific in quality, but it was also more characteristic of chemical signals of females in quantity than phenylacetic acid. Further analysis indicated that phenylacetic acid showed no difference between the sexes (0.133 ± 0.236 vs. 0.022 ± 0.063 for females and males, respectively, both $N = 8$, $Z = 1.532$, $P = 0.125$). GC peak 4 (hydroquinone) was detected in two males (Didi and Longfei) and peak 5 (indole) in three females (Caocao, Zhuyun, and Zizhu). These compounds seem to be sex-specific in the AGS of the panda.

Intra-individual Similarity and Inter-individual Dissimilarity For males, cluster analysis showed that AGS composition in the samples remained relatively constant within individuals (i.e., Qiangqiang, Longsheng, Didi, and Longfei, cluster distance <1; Fig. 2). For females, two scent samples of Zizhu also showed a cluster distance less than one, but Caocao did not exhibit such a similarity in the dendrogram. Instead, the components of her AGS were closer to those of Ximei or Youyou (both females). This indicated that individual female AGS composition might fluctuate to a certain degree. However, further examination of each peak revealed that peaks 2 and 7 were present only in the extract from Caocao, whereas peaks 1 and 3 were present only in extracts from Ximei and Youyou. As a result, these compounds may be the keys for distinguishing Caocao from the other two whose individual attributes were not reflected as expected by the average linkage dendrogram. Likewise, the hierarchical cluster analysis failed to separate males from females despite the presence of sexual differences in several key compounds. In addition, peak 2 was present only in five females (Caocao, Meiqing, Zhuyun, Lesheng, and Zizhu) and peak 1 only in three other females (Yeye, Ximei, and Youyou).

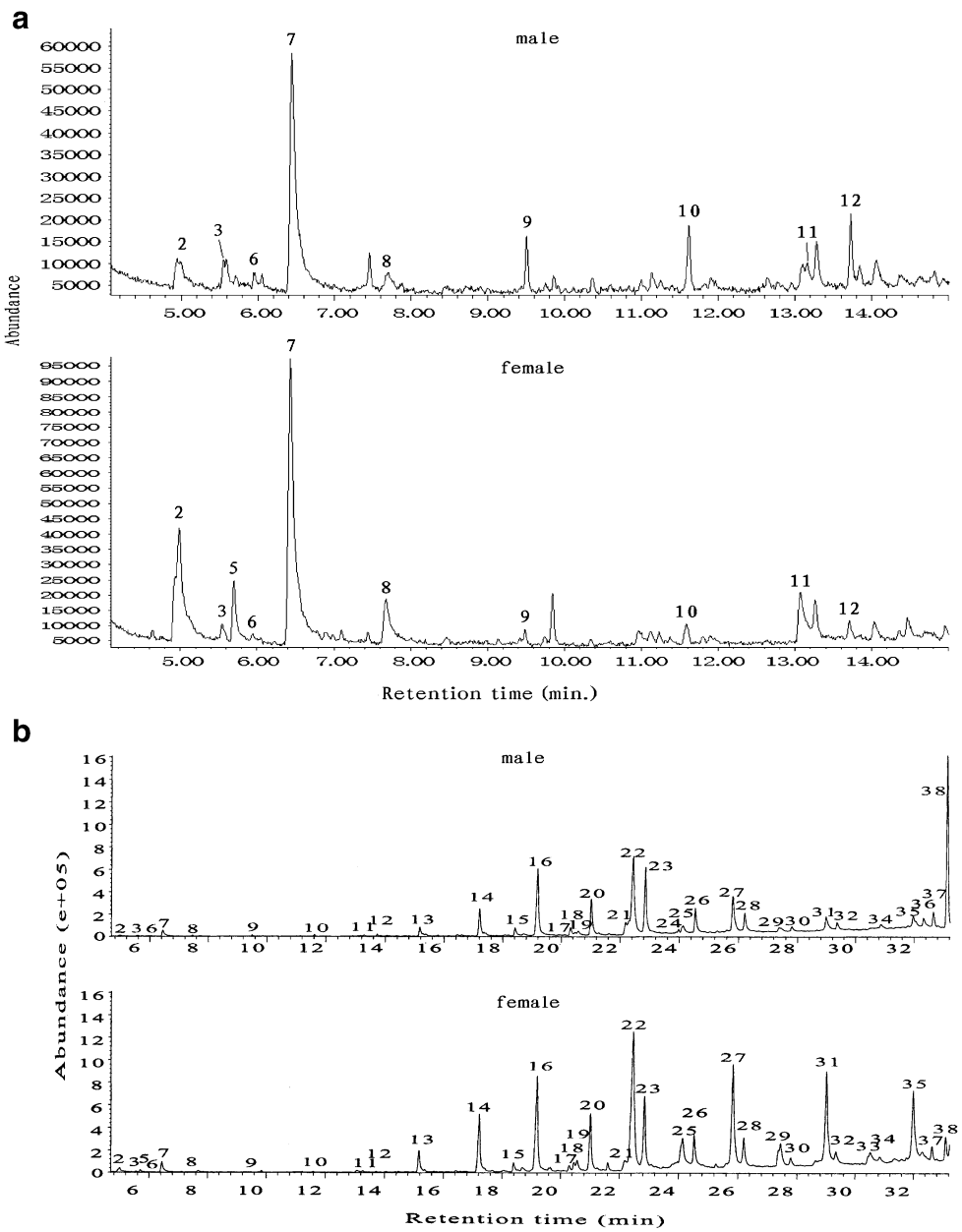

Fig. 1 Representative gas chromatogram of separation of the crude extract of the anogenital gland secretion of male (*top panels* of **a** and **b**, Qiangqiang) and female (*bottom panels* of **a** and **b**, Caocao) giant pandas, *A. melanoleuca*, on a 30-m HP5-MS capillary column. **a** Gas chromatogram (enlarged view) representing 4 to 15 min of retention time; **b** Gas chromatogram (normal view) representing 6–32 min of retention time. The numbers that label the GC peaks correspond to peak numbers in Table 2. Compounds 1 and 4 were undetectable in these two samples

Furthermore, the relative abundances of most compounds in inter-individual scent mark samples of either males or females exhibited much higher RSDs than those of samples drawn five times from the same individual (Ximei; Table 4). This further suggested that many AGS constituents may differ quantitatively among individuals.

Discussion

Our data show that many of the detectable 39 compounds (corresponding to 38 numbered GC peaks) from the AGS of giant pandas are straight-chain fatty acids. This, and the identification of squalene, in general, agrees with the previous findings of Yuan et al. (2004) and Liu et al. (2006), but shows a marked difference from the work of Hagey and MacDonald (2003) who found 111 volatiles smaller than indole. Moreover, we add several new compounds to the AGS of giant pandas that include decenal, decadienal, phenylacetic acid, 5-methylhydantoin, hydroquinone, phenylpropanoic acid, and erucic acid. The divergence in AGS compounds among studies might be explained by differences in the analytical systems, the concentration of samples analyzed, the season during which samples were collected, or the duration of sample storage. We analyzed our samples by injecting aliquots and concentrations that would not overload the column or cause overlap in retention times of the extract constituents. We

Table 3 Sexual differences in relative abundances of major compounds extracted from the anogenital gland secretion of giant pandas, *A. melanoleuca*

Peak No.	Relative Abundance		Statistical Significance	
	Males (N=8)	Females (N=8)	t or Z[a]	P
1[b]	0.479±0.686 (4)[c]	1.628±2.713 (3)	Z=0.116	0.908
2[b]	0.022±0.063 (1)	2.667±4.188 (5)	Z=2.233	0.026
3	0.164±0.201 (6)	0.382±0.479 (7)	Z=1.053	0.292
4[b]	1.112±2.444 (2)	0.000±0.000 (0)	Z=1.461	0.144
5[b]	0.000±0.000 (0)	0.026±0.039 (3)	Z=1.849	0.064
6[b]	0.164±0.245 (5)	0.336±0.492 (3)	Z=1.111	0.267
7[b]	0.157±0.443 (2)	0.316±0.636 (3)	Z=0.745	0.457
11	0.371±1.020 (2)	0.037±0.093 (4)	Z=0.138	0.890
13[b]	2.636±2.119 (7)	2.052±1.344 (7)	t=0.659	0.521
14	6.578±7.885 (8)	5.447±2.030 (8)	Z=1.365	0.172
16[b]	14.90±5.69 (8)	15.95±3.537 (8)	t=0.443	0.664
20[b]	8.911±6.993 (8)	5.932±2.377 (8)	Z=1.155	0.248
22[b]	20.76±7.938 (8)	26.33±7.956 (8)	t=1.402	0.183
23[b]	10.55±2.350 (8)	10.31±2.246 (8)	t=0.208	0.838
26	3.660±1.553 (8)	2.424±1.393 (7)	t=1.676	0.116
27	5.530±2.010 (8)	6.860±6.094 (8)	t=0.586	0.567
28	3.382±1.662 (8)	2.218±1.474 (8)	t=1.481	0.161
31[b]	1.586±1.102 (8)	6.237±6.115 (8)	Z=1.890	0.059
35	3.321±4.802 (8)	3.183±3.418 (7)	Z=0.000	1.000
38[b]	15.73±11.74 (8)	7.343±5.016 (8)	t=1.858	0.084

[a] Independent t test was used to analyze compounds 13, 16, 22, 23, 26–28, 38; Mann–Whitney U test was used to analyze the rest of the compounds. The level of significance for each test was set at α=0.1.
[b] Compounds identified using authentic standards
[c] Figures in the parentheses refer to the numbers of individuals for which the compound was detectable.

might have detected more compounds in our extracts by analyzing higher sample concentrations. Nonetheless, the 111 compounds reported by Hagey and MacDonald (2003) included compounds detected from both urine and vaginal secretions in addition to the AGS.

To further screen for pheromone candidates from the identified compounds, we conducted a quantitative analysis

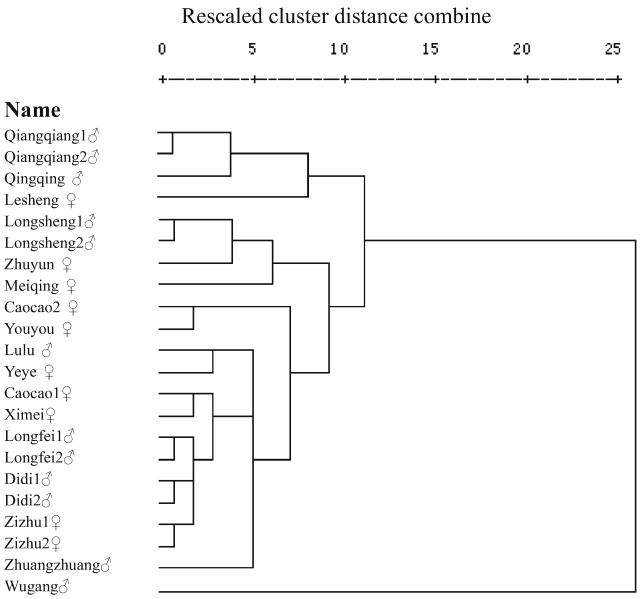

Fig. 2 Hierarchical linkage cluster (average linkage) dendrogram of the anogenital gland secretion of giant pandas, *A. melanoleuca*. The *numbers after the panda names* indicate the date [23 (=1) or 27 (=2) December 2006] on which AGS samples were collected. If no number is present, the sample was collected on 23 December 2006

to determine which compounds covary in relative abundance with sex or individual. For this analysis, all alkanes, which are pheromonally inactive, and high molecular weight fatty acids (present in trace amounts) were excluded (Singer et al. 1997; Novotny et al. 1999; Zhang et al. 2003, 2005, 2007a, b). We selected GC peaks for quantitative analysis by checking each GC profile of all samples; the GC profile in Fig. 1 is representative, but not inclusive of all data from all samples.

Some of the compounds that we detected (e.g., decenal and decadienal) might be products of oxidative degradation of fatty acids in the AGS, either while the secretion was associated with the animal or during our collection, storage, or extraction of the sample. In comparison, comparative analyses of fresh and 1-mo frozen extracts of flank gland samples from golden hamsters in our laboratory revealed no appreciable differences in chemical content (J.X. Zhang, unpublished data). Nonetheless, the relative roles of stable or degradative components in the semiochemical system of giant pandas bears further study. Compounds derived from decompositional processes may have ecological value in giant panda communication.

Our results suggest that the panda AGS contains a wealth of information that codes for sexes and individuality, which is consistent with previous behavioral tests (Swaisgood et al. 1999, 2000; White et al. 2002, 2003, 2004; Liu et al. 2005; Tian et al. 2007). The information concerning sex and individuality might be coded by the components in analog (quantitative differences of common compounds) and/or digital (unique compounds) forms proposed by Sun

Table 4 Variation in relative abundance of AGS constituents from male and female giant pandas, *A. melanoleuca*

GC Peak	RSD[a] of Inter-individuals		Intra-individuals (five replicates of Ximei's AGS sample, ♀)	
	Male (N=8)	Female (N=8)	RSD	Mean±SD
1	142.8	166.7	25.55	5.323±1.359
2	286.4	157.0	–	–
3	122.6	125.4	25.45	1.207±0.308
4	219.4	–	–	–
5	–	150.0	–	–
6	149.4	146.4	19.61	0.969±0.190
7	283.4	201.3	–	–
11	274.9	251.4	–	–
13	80.39	65.5	41.0	2.287±0.939
14	120.0	37.27	17.84	7.527±1.343
16	38.19	22.18	16.02	21.02±3.367
20	78.48	40.07	37.52	6.058±2.274
22	38.24	30.22	20.63	23.22±4.791
23	22.27	21.78	26.98	13.89±3.747
26	42.43	57.47	48.33	2.580±1.247
27	36.35	88.83	57.85	7.201±4.166
28	49.14	66.46	36.26	2.275±0.8237
31	69.48	98.04	68.46	2.973±2.033
35	144.5	107.4	97.14	1.268±1.234
38	74.63	68.31	46.36	2.204±1.020
Mean±SD	113.7±89.66	95.08±67.10	29.25±25.81	
t values[b]	3.662	3.789		
P values	0.002	0.001		

[a] RSD refers to relative standard deviation, which was calculated using the formula RSD = (SD/mean)×100, where mean and SD are the average of each compound peak area (in percentage) and their standard deviation, respectively

[b] An independent t test was used to compare RSDs from the same compounds between the male group and replicated data from Ximei and between the female group and replicated data from Ximei. The level of significance was set at α=0.05

and Müller-Schwarze (1998a, b) for the beaver and exemplified further by three *Mustela* species (Zhang et al. 2003, 2005). Other studies have documented that the odorant compounds that covary in quality or quantity with biological characters can be considered putative pheromones (Singer et al. 1997; Novotny et al. 1999). Based on qualitative and quantitative differences in the AGS extracts from the giant panda, we hypothesize that indole (female-specific), 5-methylhydydantoin, and erucic acid are potential female pheromones, whereas hydroquinone (male-specific) and squalene are potential male pheromones. This concurs with previous results in the elevation of the squalene level with sexual maturation in the panda (Liu et al. 2006). Squalene also has been shown to be active in tamarin, *Saguinus fuscicollis* (Epple et al. 1979) and male Canadian red-sided garter snakes, *Thamnophis sirtalis* (Mason et al. 1989). Indole and long-chain fatty acids pheromones are found in a variety of vertebrates including male ferret, *Mustela furo* (Clapperton et al. 1988), leopard geckos, *Eublapharis macularius* (Mason and Gutzke 1990), and golden hamsters (J.X. Zhang, unpublished data). Some straight-chain fatty acids and various isomers of decadienal are common components of insect pheromone blends (El-Sayed 2005). Such convergent uses of the same compounds in different species show that these compounds may possess some typical chemical properties of pheromones, one of which appears to be their volatility, which allows them to convey airborne cues over a distance.

Hierarchical cluster analysis and RSD of the relative abundances of 20 of the GC peaks in combination with individually unique compounds suggest that individual information may be borne in the giant panda by both the kind and degree of the scent constituents. Such coding patterns for individual identity have been found in three *Mustela* species (Zhang et al. 2003, 2005). In ferrets, for instance, Clapperton et al. (1988) identified inter-individual variation in the combination of five compounds in anal gland secretions. In female ferrets, the intra-individual similarity of urinary volatiles is not always elucidated by hierarchical cluster analysis; instead, dramatic fluctuations of a few compounds reveal an inter-individual dissimilarity (Zhang et al. 2005). Significant differences in RSD within and between individuals have also been used successfully to show the possibility of coding for information about individuality with the anal gland secretion in the Siberian weasel and steppe polecat and preputial gland secretion in the house mouse and Brandt's vole (Zhang et al. 2003, 2007a, b). The preputial glands in mice and voles seldom contain compounds that are unique to a specific individual or sex despite their sexual dimorphism in quantity, whereas the anal glands of carnivores always have noticeable individual-specific compounds (Zhang et al. 2003, 2007a, b). For

example, no two individuals share an identical compound composition in the urine of the lion, *Panthera leo* (Andersen and Vulpius 1999). In the giant panda, a fingerprint-like individual scent composition might be an alternative for the "genetic fingerprint," which provides a new powerful tool in the accurate census of wild giant pandas (Zhan et al. 2006). Preliminary comparisons of the data suggest that there is likely no correlation between the similarity of the AGS composition and genetic distance in the panda. For example, although Qingqing (♂), Longfei (♂), Longsheng (♂), Meiqing (♀), Zhuyun (♀), and Ximei (♀) all are sired by Dadi (♂), and Dadi (♂) and Didi (♂) are sired by Panpan (♂; Xie and Gipps 2005), their AGS compositions failed to reflect such genetic relationships. To conduct a thorough analysis of the extent of the role of genetic distance on AGS composition, one would need to include a chemical analysis of the AGS composition from all parents and exclude experimentally the effects of non-genetic factors, such as microbial community, housing condition, reproductive status, and food (Gosden and Ware 1976; Ferkin et al. 1997; Zhang et al. 2003).

In summary, according to the established methods used to screen for pheromones from numerous scent compounds detected by GC-MS, we propose several putative sex pheromones for the giant panda, especially male-produced squalene and female-produced erucic acid, which were present in all pandas in this study. Our results also show an individual "odor fingerprint" from the AGS of the giant panda. Verification of their pheromonal activity via bioassays and application of synthetic chemosignals to conserve this species will be subjects of our future studies.

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References

AGILENT TECHNOLOGIES 2002. Mass Spectral Libraries, NIST. Palo Alto, CA, USA.

ANDERSEN, K. F., and VULPIUS, T. 1999. Urinary volatile constituents of the lion, *Panthera leo*. *Chem. Senses* 24:179–189.

BROWN, R. E., and MACDONALD, D. W. 1985. Social Odours in Mammals. Oxford University Press, Oxford, UK.

CLAPPERTON, B. K., MINOT, E. O., and CRUMP, D. R. 1988. An olfactory recognition system in the ferret *Mustela furo* L. (Carnivora: Mustelidae). *Anim. Behav.* 36:541–553.

EL-SAYED, A. M. 2005. The Pherobase: Database of insect pheromones and semiochemicals. <http://www.pherobase.com/database/compound>.

EPPLE, G., GOLOB, N. F., and SMITH, A. B. I. 1979. Odor communication in the tamarin *Saguinus fuscicollis* (Callitrichidae): Behavioral and chemical studies, pp. 117–130, in F. J. Ritter (ed.). Chemical Ecology: Odour Communication in Animals. Elsevier, Amsterdam.

FERKIN, M. H., SOROKIN, E. S., JOHNSTON, R. E., and LEE, C. J. 1997. Attractiveness of scents varies with protein content of the diet in meadow voles. *Anim. Behav.* 53:133–141.

GASSETT, J. W., WIESLER, D. P., BAKER, A. G., OSBORN, D. A., MILLER, K. V., MARCHINTON, R. L., and NOVOTNY, M. 1996. Volatile compounds from interdigital gland of male white-tailed deer (*Odocoileus virginianus*). *J. Chem. Ecol.* 22:1689–1696.

GOSDEN, P. E., and WARE, G. C. 1976. The aerobic flora of the anal sac of the red fox (*Vulpes vulpes*). *J. Appl. Bacteriol*. 41:271–275.

HAGEY, L., and MACDONALD, E. 2003. Chemical cues identify gender and individuality in giant pandas (*Ailuropoda melanoleuca*). *J. Chem. Ecol.* 29:1479–1488.

LIU, D., FANG, J., SUN, R., ZHANG, G., WEI, R., and ZHANG, H. 1998. Behavioral comparison in individuals of different sexual ability in giant panda (*Ailuropoda melanoleuca*). *Acta Zool. Sin*. 44:27–34.

LIU, D., ZHANG, G., WEI, R., ZHANG, H., FANG, J., and SUN, R. 2002. The effects of sex and age on the behavior of captive giant pandas (*Ailuropoda melanoleuca*). *Acta Zool. Sin*. 48:585–590.

LIU, D., WANG, Z. P., TIAN, H., YU, C. Q., ZHANG, G. Q., WEI, R. P., and ZHANG, H. M. 2003. Behavior of giant pandas (*Ailuropoda melanoleuca*) in captive conditions: Gender differences and enclosure effects. *Zoo Biol*. 22:77–82.

LIU, D., ZHANG, G., WEI, R., ZHANG, H., FANG, J., and SUN, R. 2005. Behavioral responsiveness of captive giant pandas (Ailuropoda melanoleuca) to substrate odors from conspecifics of the opposite sex, pp. 101–109, in R. T. Mason, M. P. LeMaster, and D. Müller-Schwarze (eds.). Chemical Signals in Vertebrates 10Springer, New York.

LIU, D., YUAN, H., TIAN, H., WEI, R. P., ZHANG, G. Q., SUN, L., and SUN, R. Y. 2006. Do the anogenital gland secretions of giant panda code for their sexual ability? *Chin. Sci. Bull.* 51:1986–1995.

MASON, R. T., FALES, H. M., JONES, T. H., PANNELL, L. K., CHINN, J. W., and CREWS, D. 1989. Sex pheromones in snakes. *Science* 245:290–293.

MASON, R. T., and GUTZKE, W. H. N. 1990. Sex recognition in the leopard gecko, *Eublepharis macularius* (Sauria: Gekkonidae): Possible mediation by skin-derived semiochemicals. *J. Chem. Ecol*. 16:27–36.

NOVOTNY, M., MA, W., ZIDEK, L., and DAEV, E. 1999. Recent biochemical insights into puberty acceleration, estrus induction, and puberty delay in the house mouse, pp. 99–116, in R. E. Johnston, D. Müller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Kluwer, New York.

SCHALLER, G. B., HU, J., PAN, W., and ZHU, J. 1985. The Giant Pandas of Wolong. University of Chicago Press, Chicago, IL.

SINGER, A. G., BEAUCHAMP, G. K., and YAMAZAKI, K. 1997. Volatile signals of the major histocompatibility complex in male mouse urine. *Proc. Natl. Acad. Sci. U. S. A.* 94:2210–2214.

SPSS INC. 1999. SPSS for Windows. Release 10.0.1. Chicago, IL.

SUN, L., and MÜLLER-SCHWARZE, D. 1998a. Anal gland secretion codes for family membership in the beaver. *Behav. Ecol. Sociobiol*. 44:199–208.

SUN, L., and MÜLLER-SCHWARZE, D. 1998b. Anal gland secretion codes for relatedness in the beaver, *Castor canadensis*. *Ethology* 104:917–927.

SWAISGOOD, R. R., LINDBURG, D. G., and ZHOU, X. 1999. Giant pandas discriminate individual differences in conspecific scent. *Anim. Behav.* 57:1045–1053.

SWAISGOOD, R. R., LINDBURG, D. G., and OWEN, M. A. 2000. The effects of sex, reproductive condition and context on discrimina-

tion of conspecific odours by giant pandas. *Anim. Behav.* 60:227–237.

TIAN, H., WEI, R. P., ZHANG, G. Q., SUN, R. Y., and LIU, D. Z. 2007. Age differences in behavioral responses of male giant pandas to chemosensory stimulation. *Zoological Research* 28:134–140.

WHITE, A. M., SWAISGOOD, R. R., and ZHANG, H. 2002. The highs and lows of chemical communication in giant pandas (*Ailuropoda melanoleuca*): Effect of scent deposition height on signal discrimination. *Behav. Ecol. Sociobiol*. 51:519–529.

WHITE, A. M., SWAISGOOD, R. R., and ZHANG, H. 2003. Chemical communication in the giant panda (*Ailuropoda melanoleuca*): The role of age in the signaller and assessor. *J. Zool. Lond*. 259:171–178.

WHITE, A. M., SWAISGOOD, R. R., and ZHANG, H. 2004. Urinary chemosignals in giant pandas (*Ailuropoda melanoleuca*): Seasonal and developmental effects on signal discrimination. *J. Zool. Lond*. 264:231–238.

XIE, Z., and GIPPS, J. 2005. The International Studbook for Giant Panda (*Ailuropoda melanoleuca*). Chinese Association of Zoological Gardens, Beijing.

YUAN, H., LIU, D., SUN, L., WEI, R. P., ZHANG, G. Q., and SUN, R. Y. 2004. Anogenital gland secretions code for sex and age in the giant panda, *Ailuropoda melanoleuca*. *Can. J. Zool*. 82:1596–1604.

ZHAN, X. J., LI, M., ZHANG, Z. J., GOOSSENS, B., CHEN, Y. P., WANG, H. J., BRUFORD, M. W., and WEI, F. W. 2006. Molecular censusing doubles giant panda population estimate in a key nature reserve. *Curr. Biol*. 16:R451–R452.

ZHANG, J. X., NI, J., REN, X. J., SUN, L. X., ZHANG, Z. B., and WANG, Z. W. 2003. Possible coding for recognition of sexes, individuals and species in anal gland volatiles of *Mustela eversmanni* and *M. sibirica*. *Chem. Senses* 28:381–388.

ZHANG, J. X., SOINI, H. A., BRUCE, K. E., WIESLER, D., WOODLEY, S. K., BAUM, M. J., and NOVOTNY, M. V. 2005. Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30:727–737.

ZHANG, J. X., RAO, X. P., SUN, L., ZHAO, C., and QIN, X. 2007a. Putative chemical signals about sex, individuality and genetic background in the preputial gland and urine of the house mouse (*Mus musculus*). *Chem. Senses* 32:293–303.

ZHANG, J. X., RAO, X. P., ZHAO, C., LIU, X., and QIN, X. 2007b. Possible information about gender and individual recognition coded by insect pheromone analogs in the preputial glands in male Brandt's voles, *Lasiopodomys brandtii*. *Acta Zool. Sin*. 53:616–624.

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A Male-produced Aggregation Pheromone Blend Consisting of Alkanediols, Terpenoids, and an Aromatic Alcohol from the Cerambycid Beetle *Megacyllene caryae*

Emerson S. Lacey · Jardel A. Moreira · Jocelyn G. Millar · Lawrence M. Hanks

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Abstract Bioassays conducted with a Y-tube olfactometer provided evidence that both sexes of the cerambycid beetle *Megacyllene caryae* (Gahan) were attracted to odor produced by males. Odor collected from male *M. caryae* contained eight male-specific compounds: a 10:1 blend of (2*S*,3*R*)- and (2*R*,3*S*)-2,3-hexanediols (representing 3.2±1.3% of the total male-specific compounds), (*S*)-(−)-limonene (3.1±1.7%), 2-phenylethanol (8.0±2.4%), (−)-α-terpineol (10.0±2.8%), nerol (2.1±1.5%), neral (63.3±7.3%), and geranial (8.8±2.4%). Initial field bioassays determined that none of these compounds was attractive as a single component. Further field trials that used a subtractive bioassay strategy determined that both sexes were attracted to the complete blend of synthetic components, but the elimination of any one component resulted in a decline in trap captures. Blends that were missing (2*S*,3*R*)-2,3-hexanediol, (2*R*,3*S*)-2,3-hexanediol, or citral (a 1:1 mixture of neral and geranial) attracted no more beetles than did controls. A pheromone blend of this complexity, composed of alkanediols, terpenoids, and aromatic alcohols, is unprecedented for cerambycid species.

E. S. Lacey · L. M. Hanks (✉)
Department of Entomology,
University of Illinois at Urbana–Champaign,
320 Morrill Hall 505 South Goodwin Ave.,
Urbana, IL 61801, USA
e-mail: hanks@life.uiuc.edu

E. S. Lacey
e-mail: elacey@life.uiuc.edu

E. S. Lacey · J. A. Moreira · J. G. Millar
Department of Entomology, University of California,
Riverside, CA 92521, USA

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Introduction

Male-produced aggregation pheromones have been identified for nine species in four tribes of the cerambycid beetle subfamily Cerambycinae (Lacey et al. 2004, 2007b; Hanks et al. 2007). In another three species, male-produced pheromones attract only females (reviewed by Lacey et al. 2004). The pheromones of all these species are comprised of one to three compounds that share a similar structural motif, consisting of molecules that are 6, 8, or 10 carbons in length, with hydroxyl or carbonyl groups at C_2 and C_3 (reviewed by Lacey et al. 2004, 2007b). There are two exceptions to this trend: the male-produced aggregation pheromone of the cerambycine *Phymatodes lecontei* Linsley, (*R*)-2-methylbutan-1-ol (Hanks et al. 2007), and a component of the sex pheromone of *Hylotrupes bajulus* (L.), 1-butanol (Reddy et al. 2005). Volatile pheromones of species in the subfamily Cerambycinae apparently are produced by glands in the prothorax and secreted through pores lying within depressions in the cuticle (Ray et al. 2006; Hanks et al. 2007; Lacey et al. 2007b). Pheromone release by males of several species has been associated with a characteristic body posture, termed the "pushup stance" (Lacey et al. 2004, 2007a, b): Males fully extend their front legs, elevating the head and thorax, and remain motionless for extended periods.

The cerambycine species *Megacyllene caryae* (Gahan) is endemic to North America. The larvae develop in woody tissues of stressed, moribund, and dead trees of a variety of

hardwood species (Linsley 1964). Adults are active in early spring, and collection records from the area of our study, east-central Illinois, range from March 29 to June 7 (Illinois Natural History Survey, Champaign, IL). The adult beetles reportedly feed on flowers of trees in the genus *Crataegus* (Rosaceae; Dusham 1921), but dissections of field-collected adults of both sexes revealed that their guts contained pollen from trees in the genus *Quercus* (Fagaceae; unpublished data). In the laboratory, beetles can live for more than 30 d if provided sugar water (Lacey, personal observation). Adults are active from ~11:00 to 18:00 hours. Both sexes aggregate on larval hosts in numbers that may exceed 40 individuals. They mate soon after emergence, and females begin ovipositing immediately. Generation time is usually 1 yr.

M. caryae exhibits several characteristics that have been associated with production of volatile pheromones in other cerambycine species. For example, adults aggregate on larval hosts (Lacey, personal observation), males have sex-specific gland pores on their prothoraces (Ray et al. 2006), and males on larval hosts commonly assume the pushup stance (Lacey, personal observation). We report here the identification and testing of sex-specific volatile pheromone components produced by adult male *M. caryae*.

Methods and Materials

Source of Insects Adult *M. caryae* used in olfactometer bioassays and for pheromone collections were reared from logs of honey locust, *Gleditsia triacanthos* L. (Fabaceae), that were naturally colonized by beetles from April to May 2003 on the campus of University of Illinois at Urbana–Champaign (UIUC). Periodically, logs were moved to a 3×2×1-m rearing cage of window screen in a laboratory room (ambient conditions: fluorescent lighting, ~12:12 L/D, 20°C, 50% relative humidity) during January and March 2004. Most adults emerged within 14 d of moving logs into the laboratory. Freshly emerged adults were caged individually in 0.1-m^3 cylindrical cages of aluminum window screen with plastic Petri dishes at top and bottom, under ambient laboratory conditions. They were provided 10% sucrose solution dispensed from 8-ml vials plugged with cotton dental rolls (Patterson Dental Supply, South Edina, MN). Adults used in experiments appeared healthy and active.

Testing for Attractant Pheromones Bioassays for attractants produced by adult *M. caryae* were carried out with a horizontal glass Y-tube olfactometer (6 cm diameter, main tube 26 cm long, arm length 22 cm, 70° angle between arms). Bioassays were conducted outdoors in partial shade because beetles either were sedentary or appeared agitated when placed in the olfactometer under laboratory conditions. We conducted bioassays between 11:00 and 16:00 hours for 4 d in April 2004 (skies clear, air temperatures 17–24°C). A 2-l plastic chamber containing a cylinder of aluminum screen as a perch was attached to each arm of the Y-tube. One chamber contained six male beetles and the other six females. Ambient air was pulled through the olfactometer with a vacuum cleaner connected to a variable power supply (air speed ~1.0 m sec^{-1}). For each trial, a beetle was released at the base of the Y-tube and allowed 10 min to respond to an odor source by crossing a line 18 cm down one arm. Beetles that did not respond within 10 min were recorded as "no response." Chambers were alternated between arms of the Y-tube every three trials to control for positional bias. Chambers and the olfactometer were washed and rinsed with acetone each day. We bioassayed 20 individuals of each sex and compared numbers of beetles responding to treatments with a χ^2 goodness-of-fit test corrected for continuity (Sokal and Rohlf 1995).

Identification of Potential Pheromone Components Volatile compounds were collected from adult *M. caryae* by placing five females and males in separate glass vacuum traps (~0.3 l, custom manufactured by the glass shop, School of Chemistry, UIUC) that were lined with an aluminum screen to provide perches. A glass tube (6 cm×4 mm inner diameter [i.d.]) containing 100 mg of 80/100 mesh SuperQ® (Alltech Associates, Deerfield, IL) held between plugs of silanized glass wool was attached to one nipple of each chamber with an 8-cm-long section of Teflon® tubing, and charcoal-purified air was pulled through the apparatus with a water aspirator (0.7 l min^{-1}). Males and females were aerated simultaneously on a laboratory windowsill from 11:00 to 16:00 hours in April and May 2004. We selected this period because it was the only time during the day that males displayed the pushup stance. Collectors were eluted with three 0.5-ml aliquots of CH_2Cl_2 and the resulting extracts analyzed at the University of California Riverside on a Hewlett-Packard® (HP) 5973 mass selective detector interfaced to an HP 6890 gas chromatograph, fitted with a DB5-MS column (30 m×0.25 mm i.d., 0.25 μm film thickness; J&W Scientific, Folsom, CA), temperature-programmed from 40 (held for 1 min) to 250°C at 10°C min^{-1}. Injector temperature was 250°C, and injections were made in the splitless mode. Absolute configuration of the insect-produced compounds was determined by analysis of the extract on a Cyclodex-B gas chromatography (GC) column (30 m×0.25 mm i.d., 0.25 μm film thickness, J&W Scientific) with the GC programmed from 50 (held for 1 min) to 200°C at 5°C min^{-1}; injector and detector temperatures were 100 and 200°C, respectively. Identifications of peaks were confirmed by coinjections of extracts with authentic standards.

Synthesis of Pheromone Components The stereoisomers of 2-hydroxy-3-hexanone and 2,3-hexanediol (see "Results") were synthesized as described below and shown in Fig. 1. Racemic 2-hydroxy-3-hexanone and racemic (2*R**,3*S**)- and (2*R**,3*R**)-2,3-hexanediols were available from previous studies (Lacey et al. 2004, 2007b; Hanks et al. 2007). All other compounds were obtained from Sigma-Aldrich (St Louis, MO), including (*S*)-(−)-limonene (96%), 2-phenylethanol (99%), (−)-alpha-terpineol (90%), nerol (≥90%), and citral (95%; see "Results"). Because neral and geranial (see "Results") were not readily available as pure isomers, citral (a 1:1 mixture of neral and geranial; Sigma-Aldrich) was used in the bioassays.

Tetrahydrofuran was distilled from sodium/benzophenone ketyl under argon. ^{1}H- and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded with a Varian INOVA-400 (400 and 100.5 MHz, respectively) spectrometer as $CDCl_3$ solutions. Chemical shifts were expressed in parts per million relative to $CDCl_3$ (7.26 and 77.23 ppm for ^{1}H- and ^{13}C-NMR, respectively). Mass spectra were obtained with an HP 5890 GC interfaced to an HP 5970 mass selective detector, in electron impact mode (70 eV), with helium as the carrier gas. The GC was equipped with a DB5-MS column (25 m×0.20 mm i.d.×0.33 μm film; J&W Scientific). Solutions were dried over anhydrous Na_2SO_4 and concentrated under partial vacuum by rotary evaporation unless otherwise stated. Crude products were purified by flash or vacuum flash chromatography with silica gel (230–400 mesh, EM Science, Gibbstown, NJ). Reactions with air- or water-sensitive reagents were carried out in oven-dried glassware under argon. GC with a chiral stationary phase column was performed with an HP 5890 GC fitted with a Cyclodex B column (30 m×0.25 mm i.d.× 0.25 μm, J&W Scientific), programmed from 50 (held for 1 min) to 200°C at 5°C min^{-1}, with the injector at 100°C and helium as carrier gas (20 psi).

Ethyl (S)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate [(S)-2] 3,4-Dihydro-2H-pyran (53.4 g, 635 mmol) was added dropwise to a stirred solution of ethyl (*S*)-(−)-lactate (*S*)-1 (50.0 g, 423 mmol; Aldrich Chem., Milwaukee, WI)

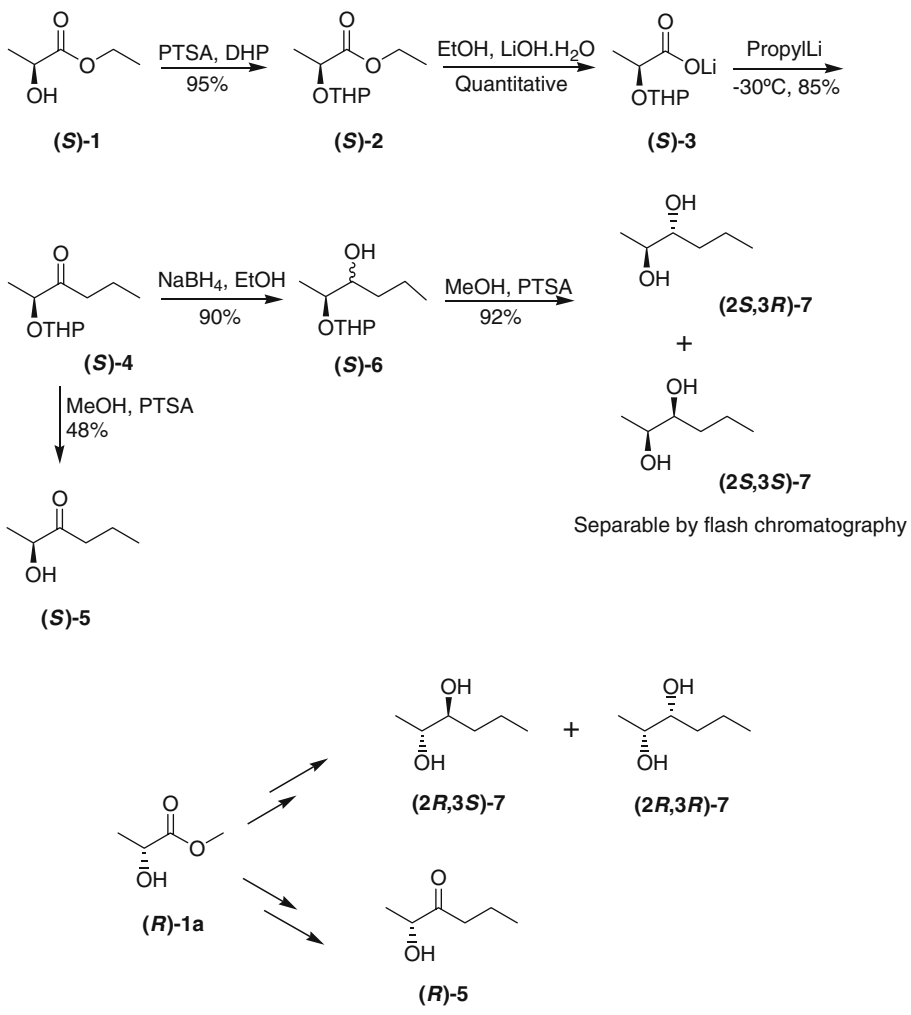

Fig. 1 Syntheses of all four 2,3-hexanediol stereoisomers and the two 2-hydroxy-3-hexanone enantiomers from ethyl (*S*)-lactate and methyl (*R*)-lactate

and 100 mg *p*-toluenesulfonic acid in 120 ml CH_2Cl_2 at 0°C under argon. The mixture was warmed to room temperature and stirred until all the starting material had been consumed (~5 hr). The mixture was then diluted with diethyl ether (60 ml) and washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. The crude product was Kugelrohr distilled (64–68°C, 0.25 mmHg), yielding 81.4 g of protected alcohol (*S*)-2 (95%) as a mixture of diastereoisomers (2:1—measured by GC). ^{1}H-NMR (major stereoisomer): δ 1.26 (t, *J*=7.2 Hz, 3H), 1.44 (d, *J*=7.0 Hz, 3H), 1.48–1.90 (m, 6H), 3.47–3.55 (m, 1H), 3.80–3.88 (m, 1H), 4.11–4.25 (m, 3H, two quadruplets overlapped with signals of other stereoisomer), 4.66–4.72 (m, 1H, dd overlapped with signals of other stereoisomer). ^{1}H-NMR (minor stereoisomer): δ 1.27 (t, *J*=7.2 Hz, 3H), 1.38 (d, *J*=6.8 Hz, 3H), 1.48–1.90 (m, 6H), 3.40–3.47 (m, 1H), 3.88–3.95 (m, 1H), 4.11–4.25 (m, 2H - quadruplet overlapped with signals of other stereoisomer), 4.40 (q, *J*= 7.0 Hz, 1H), 4.66–4.72 (m, 1H, dd overlapped with signals of other stereoisomer); mass spectrometry (MS; *m/z*: relative intensity): 144 (1), 130 (3), 129 (4), 101 (19), 85 (100), 73 (11), 67 (12), 57 (16), 55 (17), 45 (25), 43 (24).

Methyl (R)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate [(R)-2a] In the same manner as described above, methyl (*R*)-(+)-lactate (*R*)-1a (20.0 g, 192 mmol; Aldrich Chem., 96% enantiomeric excess [ee]) was converted to a mixture (2:1—measured by GC) of methyl (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate diastereoisomers (*R*)-2a (34.24 g) in 95% yield after Kugelrohr distillation (62–66°C,1.3 mmHg). ^{1}H-NMR (major stereoisomer): δ 1.44 (d, *J*=7.0 Hz, 3H), 1.46–1.90 (m, 6H), 3.40–3.54 (m, 1H), 3.72 (s, 3H), 3.76–3.93 (m, 1H), 4.41 (d, *J*=7.0 Hz, 1H), 4.65–4.71 (m, 1H) (some signals overlapped with signals of the other stereoisomer). ^{1}H-NMR (minor stereoisomer): δ 1.38 (d, *J*=6.8 Hz, 3H), 1.46–1.90 (m, 6H), 3.40–3.54 (m, 1H), 3.72 (s, 3H), 3.76–3.93 (m, 1H), 4.19 (d, *J*=6.8 Hz, 1H), 4.65 – 4.71 (m, 1H) (some signals overlapped with signals of the other stereoisomer); MS (relative intensity) *m/z*: 130 (2), 129 (6), 116 (2), 101 (28), 85 (100), 73 (7), 67 (15), 57 (20), 55 (26), 45 (25), 43 (30).

Lithium (S)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate [(S)-3] Ethyl (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate (*S*)-2 (80.00 g, 396 mmol) was added to a stirred suspension of $LiOH.H_2O$ (16.60 g, 396 mmol) in ethanol (200 ml) under argon at 0°C. The reaction was stirred for 30 min at 0°C, warmed to room temperature, and stirred for 3 hr. The mixture was concentrated under vacuum, and hexane (50 ml) was added to the concentrate, followed by concentration under vacuum to remove traces of ethanol and water as azeotropes. This procedure was repeated four times, and the residue was pumped under vacuum (0.1 mmHg) for 10 hr. ^{1}H-NMR showed that ethanol was still present in the salt, and so the crude lithium salt was suspended in benzene, the benzene distilled off at atmospheric pressure, and the residue again pumped under vacuum for 10 hr, affording lithium (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate (*S*)-3 as a light yellow solid in quantitative (99.7%) yield. ^{1}H-NMR (major stereoisomer): δ 1.39 (d, *J*=6.8 Hz, 3H), 1.42–1.92 (m, 6H), 3.40–3.55 (m, 1H), 3.82–3.92 (m, 1H), 4.18 (q, *J*=6.8 Hz, 1H), 4.70 (t, *J* 3.5 Hz, 1H). ^{1}H NMR (minor stereoisomer): δ 1.34 (d, *J*=6.8 Hz, 3H), 1.42–1.92 (m, 6H), 3.40–3.55 (m, 1H), 4.00–4.05 (m, 1H), 4.12 (q, *J*=7.0 Hz, 1H), 4.54 (dd, *J*=2.3 and 7.0 Hz, 1H). Some NMR signals from the two diastereomers overlapped.

Lithium (R)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate [(R)-3] Methyl (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate (*R*)-2a (34.20 g, 182 mmol) was converted to lithium (*R*)-2-(2-tetrahydropyranyloxy)-propanoate (*R*)-3 (32.70 g) as described above, in quantitative yield. ^{1}H-NMR and mass spectra matched those of the corresponding (*S*)-enantiomer mixture.

(S)-2-[(Tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone [(S)-4] Lithium wire (1% sodium content; 1.39 g, 200 mmol, previously rinsed with hexane) was cut into small pieces directly into a three-necked flask charged with anhydrous diethyl ether (85 ml) under argon. A few drops of a 1-bromopropane (12.3 g, 100 mmol) solution in 15 ml ether were added, and the mixture was stirred at ambient temperature until the reaction started. The mixture was then cooled to −20°C, and the remaining solution of bromopropane was added dropwise over 1.5 hr. When the addition was complete, the mixture was allowed to warm to room temperature and stirred for an additional hour before use.

The resulting propyllithium solution (~1.0 M in ethyl ether, 80 ml) was added dropwise to a suspension of lithium (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate (*S*)-3 (9.63 g, 53.5 mmol) in Et_2O (100 ml) under argon at −30°C. The reaction was allowed to warm to room temperature, stirred overnight, poured into crushed ice, and extracted with Et_2O (3×100 ml). The combined organic layers were washed with saturated aqueous NH_4Cl and brine, dried, and concentrated. The residue was purified by vacuum flash chromatography on silica gel (hexane: EtOAc, 9:1) affording (*S*)-2-[(tetrahydro-2H-pyran-2-yl) oxy]-3-hexanone (*S*)-4 (9.14 g, 85%). ^{1}H-NMR (major isomer): δ 0.90 (t, *J*=7.4 Hz, 3H), 1.34 (d, *J*=7.0 Hz, 3H), 1.45–1.95 (m, 8H), 2.40 (dt, *J*=7.2 and 17.3 Hz, 1H), 2.50 (dt, *J*=7.3 and 17.3 Hz, 1H), 3.40–3.53 (m, 1H), 2.77–3.90 (m, 1H), 4.27 (q, *J*=7.0 Hz, 1H), 4.54 (dd, *J*=3.1 and 4.5 Hz, 1H). Some NMR signals overlapped with the

signals from the minor stereoisomer. MS (major stereoisomer): 156 (1), 129 (3), 101 (1), 99 (1), 85 (100), 71 (9), 67 (13), 57 (14), 55 (9), 43 (30), 41 (22). ^{1}H-NMR (minor isomer): δ 0.91 (t, *J*=7.4 Hz, 3H), 1.25 (d, *J*=6.8 Hz, 3H), 1.45–1.95 (m, 8H), 2.56 (dt, *J*=7.2 and 17.8 Hz, 1H), 2.62 (dt, *J*=7.4 and 17.9 Hz, 1H), 3.40–3.53 (m, 1H), 2.77–3.90 (m, 1H), 4.07 (q, *J*=6.8 Hz, 1H), 4.60 (dd, *J*=2.8 and 5.3 Hz, 1H). MS (minor stereoisomer): 156 (1), 129 (3), 101 (1), 99 (1), 85 (100), 71 (7), 67 (12), 57 (15), 55 (10), 43 (32), 41 (22).

(R)-2-[(Tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone [(R)-4] Lithium (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-propanoate (*R*)-3 (10.52 g, 58.4 mmol) and *n*-propyllithium (0.73 M in ethyl ether, 120 ml) were reacted as described above, giving (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone (*R*)-4 (7.51g, 64%). NMR and mass spectra were in accord with those of the (*S*)-2-diastereomers.

(S)-2-Hydroxy-3-hexanone [(S)-5] Pyridinium-*p*-toluene sulfonate (100 mg) was added to a stirred solution of (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone (*S*)-4 (9.1 g, 45.5 mmol) in methanol (100 ml) at 0°C under argon. The reaction was allowed to warm to ambient temperature and stirred overnight. The methanol was removed by fractional distillation under reduced pressure. Water was added to the residue, and the mixture was extracted with Et_2O (4× 30 ml). The organic phase was washed with saturated $NaHCO_3$ and brine and dried, and the solvent was removed by distillation through a Vigreux column. The residue was purified by vacuum flash chromatography (silica gel, eluting with pentane/Et_2O 9:1), removing the solvent by distillation through a Vigreux column, followed by Kugelrohr distillation (34°C, 1.60 mmHg), giving 2.53 g (48%) of (*S*)-2-hydroxy-3-hexanone (*S*)-5. The moderate yield was primarily a result of losses during purification because of the volatility of the compound, rather than to any problem with the chemistry. ^{1}H-NMR: δ 0.92 (t, *J*=7.4Hz, 3H), 1.36 (d, *J*=7.2 Hz, 3H), 1.58–1.72 (m, 2H), 2.39 (dt, *J*=17.0 and 7.3 Hz, 1H), 2.48 (dt, *J*=17.0 and 7.2 Hz, 1H), 3.57 (d, *J*= 4.5 Hz, 1H), 4.22 (dq, *J*=4.5 and 7.2 Hz, 1H). ^{13}C-NMR: δ 13.96, 17.29, 20.03, 39.60, 72.80, 212.80. MS: 116 (M^+, 1), 87 (1), 83 (1), 74 (12), 73 (36), 72 (19), 71 (64), 55 (59), 45 (87), 43 (100), 41 (19).

(R)-2-Hydroxy-3-hexanone [(R)-5] (*R*)-2-Hydroxy-3-hexanone was obtained in 49.6% yield (2.10 g) from (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone (*R*)-4 after purification. NMR and mass spectra were identical to those of (*S*)-2-hydroxy-3-hexanone.

(S)-2-[(Tetrahydro-2H-pyran-2-yl)oxy]-3-hexanol [(S)-6] A solution of (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanone (*S*)-4 (10.00 g, 49.9 mmol) in ethanol (25 ml) was added dropwise to a stirred suspension of $NaBH_4$ (1.89 g, 50 mmol) in 25 ml ethanol at 0°C, and the mixture was stirred overnight. The mixture was concentrated, 100 ml brine added to the residue, and the product was extracted with ethyl acetate (5×50 ml). The combined organic phases were dried and concentrated. Purification by vacuum flash chromatography (hexane/EtOAc—9:1) afforded 9.08 g (90%) of product (*S*)-6. MS (*m/z*, rel. intensity): 158 (2), 157 (2), 129 (2), 101 (10), 85 (100), 67 (12), 57 (23), 56 (12), 55 (22), 45 (20), 43 (28), 41 (29). Because there were four stereoisomers in the product mixture, ^{1}H- and ^{13}C-NMR spectra were complex and not useful.

(R)-2-[(Tetrahydro-2H-pyran-2-yl)oxy]-3-hexanol [(R)-6] Compound (*R*)-6 was obtained in similar fashion with a 93% yield from (*R*)-2-[(tetrahydro-2H-pyran-2-yl) oxy]-3-hexanone (*R*)-4. Mass and NMR spectra were in accord with the (*S*)-6 mixture.

(2S,3S)-2,3-Hexanediol ((2S,3S)-7) and (2S,3R)-2,3-hexanediol [(2S,3R)-7] A solution of (*S*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanol (*S*)-6 (9.05 g, 45 mmol) and 100 mg *p*-toluenesulfonic acid in methanol (50 ml) was stirred overnight. After concentration, the residue was diluted with ethyl acetate (100 ml) and washed with saturated aqueous $NaHCO_3$ and brine, dried, and concentrated. Purification by vacuum flash chromatography (hexane/EtOAc—4:1) followed by Kugelrohr distillation (90–98°C, 4.5 mmHg) gave a mixture of (2*S*,3*S*)-2,3-hexanediol (2*S*,3*S*)-7 and (2*S*,3*R*)-2,3-hexanediol (2*S*,3*R*)-7 (48:52, respectively). The diastereoisomeric diols (6.18 g) were separated by flash chromatography in 2-g batches (hexane/acetone—5:1; column 32 cm long×5 cm diameter) followed by recrystallization at 4°C (50 ml hexane/g of diol) affording 2.55 g of pure (2*S*,3*S*)-2,3-hexanediol (diastereomeric excess [de]>98%) and 2.89 g of (2*S*,3*R*)-2,3-hexanediol (de>96%). (2*S*,3*R*)-7: ^{1}H-NMR: δ 0.96 (t, *J*=6.8 Hz, 3H), 1.15 (d, *J*=6.4 Hz, 3H), 1.30–1.46 (m, 3H), 1.46–1.60 (m, 1H), 1.99 (br s, 2H), 3.62–3.67 (m, 1H), 3.76–3.84 (m, 1H). ^{13}C NMR: δ 14.29, 16.84, 19.39, 34.13, 70.67, 74.86. MS: 103 (1), 85 (1), 75 (10), 73 (61), 72 (34), 57 (18), 55 (100), 45 (36), 43 (35), 41 (11). (2*S*,3*S*)-7: ^{1}H NMR: δ 0.94 (t, *J*=7.2 Hz, 3H), 1.19 (d, *J*=6.4 Hz, 3H),1.34–1.58 (m, 4H), 2.43 (br s, 2H), 3.31–3.37 (m, 1H), 3.59 quint, *J*=6.2 Hz, 1H). ^{13}C NMR: δ 14.26, 18.96, 19.70, 35.69, 71.13, 76.16. MS: *m/z*: 103 (1), 85 (1), 75 (16), 73 (59), 72 (35), 57 (21), 55 (100), 45 (38), 43 (37), 41 (12).

(2R,3S)-2,3-Hexanediol ((2R,3S)-7) and (2R,3R)-2,3hexanediol [(2R,3R)-7] In analogous fashion, a mixture of (2*R*,3*S*)-2,3-hexanediol (2*R*,3*S*)-7 and (2*R*,3*R*)-2,3-hexanediol (2*R*,3*R*)-7 (51:49, respectively) was obtained

in 92% yield from (*R*)-2-[(tetrahydro-2H-pyran-2-yl)oxy]-3-hexanol (*R*)-6. After purification, 2.51 g of (2*R*,3*S*)-2,3-hexanediol (de>97%) and 2.41 g (2*R*,3*R*)-2,3-hexanediol (de>98%) were obtained. NMR and mass spectra matched those of the (2*S*,3*R*)- and (2*S*,3*S*)-enantiomers, respectively.

Field Bioassays of Synthetic Pheromone Aeration extracts from male *M. caryae* contained eight male-specific compounds (see "Results"). Initially, we predicted that the hexanediols would have the greatest activity, based on pheromones that have been identified for closely related beetle species (see "Introduction"). Therefore, field bioassays of the individual components (including citral in place of neral and geranial) were first conducted before mixtures of compounds were tested. Bioassays were conducted at Allerton Park (Piatt, IL), a 600-ha mixed hardwood forest that harbors a population of *M. caryae*, between 22 and 31 May 2004 (skies clear, air temperatures ~19–28°C, average wind speed 8–20 kph). Sticky card traps ("mouse glue trap" cards, 12.6×22 cm, baited, Victor® Pest Control Products, Lititz, PA) were stapled to 1.5-m-tall wooden stakes, with the middle of the card ~1 m above the ground. Traps were positioned 10 m apart in a straight line approximately perpendicular to the prevailing wind direction. Traps were baited with a cotton dental wick that was loaded with 5 mg of the compound in 0.1 ml of methylene chloride and attached to the middle of the card. There were seven treatments that were randomly assigned to traps, as follows: racemic (2*R**,3*S**)-2,3-hexanediol, (*S*)-(−)-limonene, 2-phenylethanol, (−)-α-terpineol, nerol, citral, and a solvent control. Release rates of these lures were unknown, but similar lures loaded with 2,3-hexanediols remained attractive to *N. a. acuminatus* for at least 18 hr (Lacey et al. 2004). The study was replicated five times. For each replication, traps were set up at 11:00 hours, captured beetles were removed from traps the following morning, and the numbers and sex of beetles responding to treatments were recorded.

The 2004 bioassay was conducted late in the activity period for *M. caryae*, when populations were declining, and trap catches were low (see "Results"). Therefore, the bioassay was repeated on 8–20 May 2005 at the same site (skies clear, air temperatures ~16–27°C, wind speed 8–20 kph). By that time, we had discovered that adult beetles could extract themselves from sticky cards (Lacey et al. 2007b) and, therefore, adopted cross-vane flight-intercept panel traps (black, 1.2×0.30 m, Intercept™, model PT, APTIV, Portland, OR) that had proven efficient in capturing other species of cerambycid beetles (Lacey et al. 2007b). Traps were positioned as described above, and test chemicals were applied neat to cotton dental wicks (except [2*R**,3*S**]-2,3-hexanediols, which were dissolved in 0.025 ml of hexanes) in uncapped 1-dram vials that were hung in the open central slot of each trap (Lacey et al. 2007b). The study was replicated five times as described above. Too few beetles were captured in both the 2004 and 2005 studies to warrant statistical analysis (see "Results").

Field bioassays of blends of synthetic compounds were conducted in an abandoned plantation of mixed tree species ~1 km south of the campus of the UIUC in April to May 2006 (skies clear, air temperatures ~19–28°C, wind speed ~8–20 kph). A subtractive scheme was used to compare activity of the complete blend with seven treatments that each lacked a different component, as well as a solvent control. We used the same panel traps and lures as described above. The lures were baited with pheromone components, each diluted in 0.025 ml hexane, in ratios that approximated the mean relative abundances in extracts from aerations of males (see "Results") with two exceptions: (1) The relative proportions of hexanediols were increased because other cerambycine species had responded to relatively high quantities of similar compounds (see Lacey et al. 2004, 2007b) and (2) the total proportion of citral was decreased to compromise between the high proportion of geranial and lower proportion of neral that are produced by males (see "Results"). Lure quantities were: (2*S*,3*R*)-2,3-hexanediol (2.5 mg), (2*R*,3*S*)-2,3-hexanediol (0.5 mg), (*S*)-(−)-limonene (1.5 mg), 2-phenylethanol (5 mg), (−)-α-terpineol (5 mg), nerol (2 mg), citral (12 mg), and the control (0.175 ml hexane). Traps were positioned 8 m apart in transects approximately perpendicular to the direction of the prevailing wind. On each date, traps were baited at 10:00 hours, and beetles were collected at ~18:00 hours. Traps were cleaned with glass cleaner (Windex®, S. C. Johnson & Sons, Racine, WI) and lures replaced each day. The bioassay was replicated 14 times. Overall differences between treatments in numbers of beetles captured were tested by one-way analysis of variance (ANOVA), and differences between pairs of treatment means were subsequently tested with the least significant difference test (Analytical Software 2000). Three replicates that captured fewer than four beetles were excluded from the data analysis.

Results

Testing for Attractant Pheromones In olfactometer bioassays, 100% of female and 90% of male *M. caryae* responded by walking upwind. Of the 20 females that were tested, 19 were attracted to odor emitted by males and only one responded to odor of females. Males showed a similar response, with 16 responding to odor of males and only two responding to odor from females ($\chi^2_{1,\ 18}=21.8$, $P<0.001$).

Identification of Potential Pheromone Components GC-MS analysis of volatiles produced by male *M. caryae* revealed seven peaks that were absent in analogous samples of females. The first compound to elute (Fig. 2) was tentatively identified as 2,3-hexanediol, with a mass spectral base peak at *m/z* 55 (100) and characteristic mass fragments at *m/z* 75 (10), 73 (61), 72 (34), 45 (36), and 43 (35). The identification was confirmed by matching the mass spectrum and the retention time with those of an authentic standard of (2*R**,3*S**)-2,3-hexanediol; this diastereomer is completely resolved from the (2*R**,3*R**)-2,3-diastereomer on a DB5-MS column (Lacey et al. 2004). The enantiomeric composition of the insect-produced compound was determined to be a ~10:1 mix of (2*S*,3*R*)- and (2*R*,3*S*)-2,3-hexanediols by analysis on the Cyclodex-B column, with baseline resolution of the enantiomers. These diols represented 3.2±1.3% (*N*=7 aeration extracts) of the total male-specific compounds. The remaining compounds, in order of elution (Fig. 2), were (*S*)-(−)-limonene (3.1±1.7%), 2-phenylethanol (8.0±2.4%), (−)-α-terpineol (10.0±2.8%), nerol (2.1±1.5%), neral (63.3±7.3%), and geranial (8.8±2.4%). These compounds were tentatively identified by mass spectral fragmentation patterns, and the identifications were confirmed by retention time and mass spectral matches with authentic standards. The absolute configurations of those compounds that were chiral were determined by analyses and coinjections with appropriate standards on the Cyclodex-B GC column. Relative abundances of compounds varied between samples, with neral, 2-phenylethanol, (−)-α-terpineol, and geranial always being most abundant, whereas the hexanediols, (*S*)-limonene, and nerol were not detectable in some samples. We have found that beetles of other cerambycine species also produce pheromone sporadically under the conditions of aeration in closed chambers (unpublished data).

Syntheses of Pheromone Components Chiral 2,3-hexanediols were synthesized by modification and extension of the strategy developed by Hall et al. (2006) to produce chiral 2-hydroxy-3-decanones (Fig. 1). Thus, the alcohol function

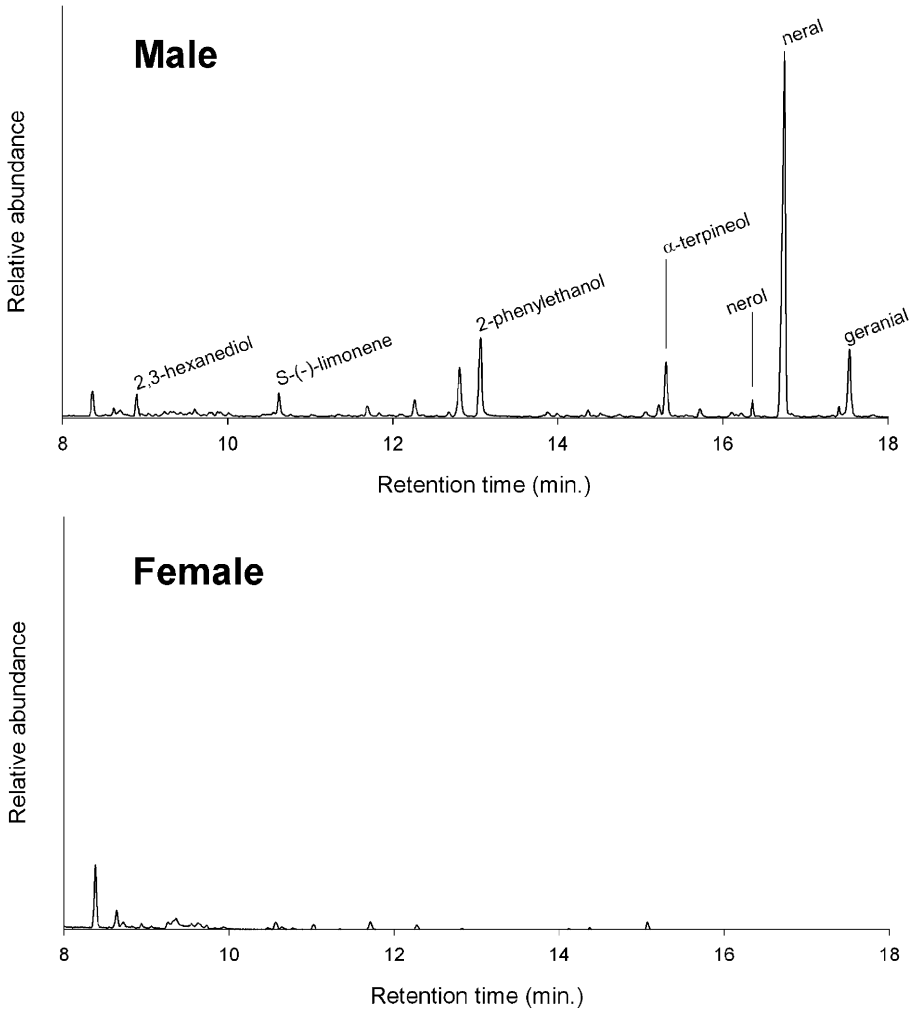

Fig. 2 Representative total ion chromatograms of extracts of headspace volatiles produced by male (*top*) and female (*bottom*) *Megacyllene caryae*

of ethyl (*S*)-(−)-lactate (*S*)-1 was protected as the tetrahydropyranyl (THP) ether (*S*)-2, followed by hydrolysis of the ester group to give the lithium salt of the carboxylic acid (*S*)-3 in quantitative yield. Alkylation of the salt with propyllithium in ether then yielded the THP-protected 2-hydroxy-3-hexanone (*S*)-4. (2*S*,3*R*)- and (2*S*,3*S*)-2,3-hexanediols were produced by reduction of THP-protected 2-hydroxy-3-hexanone (*S*)-4 with sodium borohydride in ethanol, giving the monoprotected diols mixture (*S*)-6. Acid-catalyzed removal of the THP group gave a diastereoisomeric mixture of (2*S*,3*R*)-2,3-hexanediol [(2*S*,3*R*)-7) and (2*S*,3*S*)-2,3-hexanediol ((2*S*,3*S*)-7]. The diastereomers were separated by flash chromatography on silica gel followed by recrystallization from hexanes, giving the two diols in high purity (>96% de). Analogous results were obtained for the syntheses of (2*R*,3*R*)-2,3-hexanediol [(2*R*,3*R*)-7) and (2*R*,3*S*)-2,3-hexanediol ((2*R*,3*S*)-7] starting from THP-protected hydroxyketone (*R*)-4.

Alternatively, removal of the THP group from compound 4 with acid catalysis in methanol gave (*R*)- and (*S*)-2-hydroxy-3-hexanones, (*R*)- and (*S*)-5, both of which are known pheromone components for cerambycid beetles (see Hanks et al. 2007). GC analysis on a Cyclodex-B column confirmed that no isomerization or racemization (see Lacey et al. 2007b) had occurred during the synthesis.

Field Bioassays of Synthetic Pheromone In field bioassays that tested the activity of individual compounds, sticky traps captured a total of two *M. caryae* in 2004, and panel traps captured a total of five in 2005. We concluded from this experiment that probably neither the 2,3-hexanediols nor any of the other compounds in the volatile blend produced by males were attractive to adult *M. caryae* as single components.

In bioassays that tested blends of components in 2006, traps captured 147 adult *M. caryae* (76 females and 71 males; sex ratio not significantly different from 1:1, $\chi^2_{1,\ 147}=0.17$, $P>0.05$). Treatments differed significantly in numbers of *M. caryae* that were captured (Fig. 3; sexes combined, overall ANOVA $F_{10,\ 147}=9.56$, $P<0.001$). The greatest numbers of beetles were captured in traps baited with the complete blend of pheromone components, whereas trap catches were reduced by ~50% in treatments that were missing (*S*)-(−)-limonene, 2-phenylethanol, (−)-α-terpineol, or nerol and reduced to levels not significantly different from controls in treatments missing (2*S*,3*R*)-2,3-hexanediol, (2*R*,3*S*)-2,3-hexanediol, or citral (Fig. 3).

Discussion

Attraction of both sexes of *M. caryae* to odor produced by live males in olfactometer bioassays provided the first evidence that males produce an aggregation pheromone. That this was an aggregation pheromone and not a sex pheromone was confirmed during field bioassays with synthetic pheromone, in which similar numbers of males and females were attracted to baited traps.

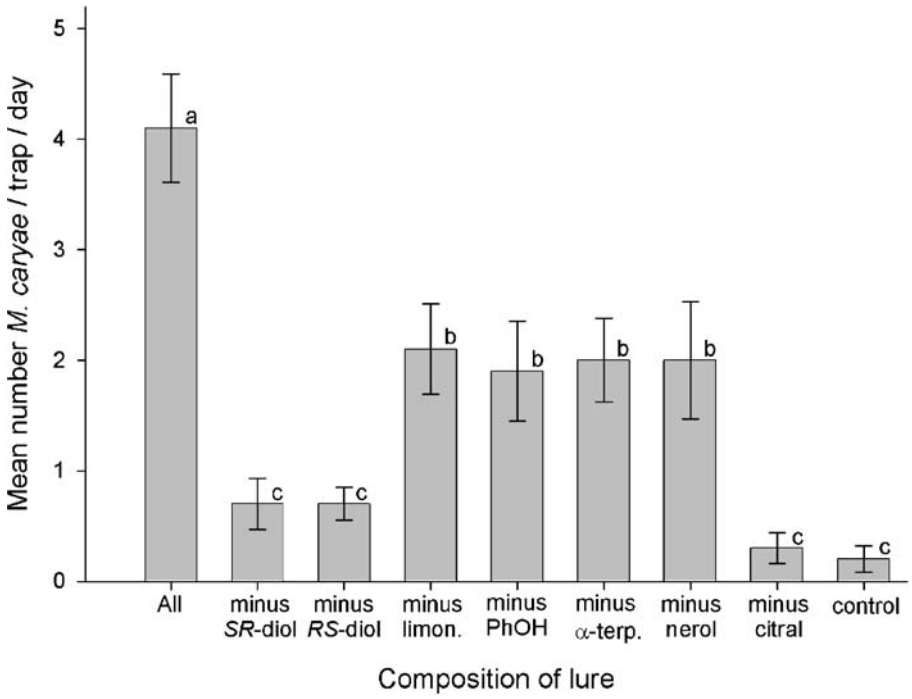

Fig. 3 Relationship between mean (±SEM) number of adult *Megacyllene caryae* caught in cross-vane panel traps (sexes combined) and composition of the lure. Treatments included the complete blend of synthetic components (All), and the complete blend minus (2*S*,3*R*)-2,3-hexanediol, (2*R*,3*S*)-2,3-hexanediol, (*S*)-(−)-limonene, 2-phenylethanol (*phoh*), (−)-α-terpineol, nerol, and citral (a 1:1 blend of neral and geranial), and a solvent control. *Means with different letters* are significantly different (LSD tests; $P<0.05$)

Although (2*S*,3*R*)- and (2*R*,3*S*)-2,3-hexanediol constituted only about 3 and 0.3%, respectively, of the total blend of volatile compounds produced by males, absence of either in synthetic blends resulted in a significant loss of activity. This finding indicated that adult *M. caryae* are sensitive to both components, unlike other clytine species that produce blends of enantiomers but respond strongly to the dominant component alone (see Hanks et al. 2007). Among the remaining components, one or both isomers of citral (neral or geranial) were equally as important to attraction as were the 2,3-hexanediols. Whereas the remaining components ([*S*]-[−]-limonene, 2-phenylethanol, [−]-α-terpineol, and nerol) increased attraction significantly, they were not necessary for attraction because beetles showed a partial response to blends lacking these compounds. The increased response of adult *M. caryae* to the complete blend of synthetic compounds confirmed that all of these compounds have a role in the natural pheromone blend.

The structure of the hexanediols produced by male *M. caryae* is consistent with the diol/hydroxyketone structural motif of pheromones reported for another 12 species in the subfamily Cerambycinae (see "Introduction"). To our knowledge, this motif is unique to cerambycine beetles. Whereas exceptions to the motif have been identified from two cerambycine species (see "Introduction"), the complex blend of compounds produced by male *M. caryae*, in addition to the components that are consistent with the structural motif, is unprecedented among cerambycines that have been studied to date.

All of the terpenoids and 2-phenylethanol produced by male *M. caryae* have been identified as pheromones of species in a broad range of insect and arachnid orders, serving a variety of behavioral functions (see Mayer and McLaughlin 1991). These compounds are also common in essential oils of plants (Budavari 1996), suggesting that beetles might acquire them from their host plants. However, it seems more likely that beetles produce the compounds *de novo* because adult male *M. caryae* from which volatile compounds were collected had been reared in the laboratory and so had not been exposed to host plants of adults.

Previous research suggests that the compounds produced by male *M. caryae* may have semiochemical functions in other species of cerambycids. For example, in electroantennograph studies of potential host plant volatiles, antennae of both sexes of *Xylotrechus pyrrhoderus* Bates and *Arhopalus tristis* (F.) responded to citral and α-terpineol, respectively (Iwabuchi et al. 1985; Suckling et al. 2001). Both α-terpineol and limonene were repellent to adult *Semanotus japonicus* Lacordaire in choice bioassays (Yatagai et al. 2002). Male *Xylotrechus quadripes* Chevrolat produce 2-phenylethanol, although it does not appear to be a pheromone component (Hall et al. 2006). A similar compound, 1-phenylethanol, is produced from metasternal glands of a congener of *M. caryae*, *M. robiniae*, and has been suggested to serve in defense against natural enemies (Wheeler et al. 1988). The fact that the terpenoids of *M. caryae* are produced by only one sex suggests that they are unlikely to have a defensive function.

The preparation of (2*S*,3*R*)- and (2*S*,3*S*)-2,3-hexanediols (and their enantiomers) in high diastereomeric and enantiomeric purity took advantage of the fact that during nonselective syntheses of mixtures of all four diol stereoisomers by the reduction of 2,3-hexanedione (Hanks et al. 2007), we observed that the diastereomeric 2,3-hexanediols were readily separable in gram quantities by flash chromatography. Thus, we reasoned that nonselective reduction of the ketone function of a chiral, THP-protected 2-hydroxy-3-hexanone precursor, in which the configuration at the carbon bearing the protected alcohol was fixed and known, should produce a mixture of the two diastereomers, each in high enantiomeric purity. Reduction of THP-protected (*S*)-2-hydroxy-3-hexanone (*S*)-4 gave an approximately 1:1 ratio of the two expected alcohol products. Removal of the THP-protecting group and separation of the resulting diol diastereomers by flash chromatography gave the (2*S*,3*R*)- and (2*S*,3*S*)-stereoisomers in quantities of several grams. Analogous reaction of the THP-protected (*R*)-2-hydroxy-3-hexanone (*R*)-4 gave the other two stereoisomers. Thus, this synthetic strategy provided ready access to all four 2,3-hexanediols in high stereoisomeric purity from readily available lactate ester synthons. In contrast, our previous syntheses of 2,3-hexanediol stereoisomers using the Sharpless asymmetric dihydroxylation protocols (Kolb et al. 1994) had produced diols with only 80–90% ee (Lacey et al. 2004). The synthetic strategy described above also has advantages in comparison to previously published syntheses of the 2,3-hexanediol stereoisomers, in which each stereoisomer was synthesized individually from a different chiral precursor (Schröder et al. 1994). It should also be noted that exactly the same strategy can be applied to the syntheses of the homologous 2,3-octanediols and 2,3-decanediols, compounds that also may be pheromone components of cerambycid beetles (e.g., Hall et al. 2006). This straightforward method of producing multigram quantities of all four stereoisomers of 2,3-alkanediols of any desired chain length should prove beneficial in the unraveling of pheromone blends for other cerambycid species.

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References

ANALYTICAL SOFTWARE. 2000. Statistix 7.1. Analytical Software, Tallahassee, FL.

BUDAVARI, S. (ed.). 1996. The Merck Index. Merck, Whitehouse Station, NJ.

DUSHAM, E. H. 1921. The painted hickory borer. *Bull. Cornell Univ. Ag. Ext. Stn.* 407:170–203.

HALL, D. R., CORK, A., PHYTHIAN, S. J., CHITTAMURU, S., JAYARAMA, B. K., VENKATESHA, M. G., SHEEDHARAN, K., VINOD KUMAR, P. K., SEETHARAMA, H. G., and NAIDU, R. 2006. Identification of components of male-produced pheromone of coffee white stem-borer *Xylotrechus quadripes*. *J. Chem. Ecol.* 32:195–219.

HANKS, L. M., MILLAR, J. G., MOREIRA, J. A., BARBOUR, J. D., LACEY, E. S., MC ELFRESH, J. S., REUTER, F. R., and RAY, A. M. 2007. Using generic pheromone lures to expedite identification of aggregation pheromones for the cerambycid beetles *Xylotrechus nauticus*, *Phymatodes lecontii*, and *Neoclytus modestus modestus*. *J. Chem. Ecol.* 33:889–907.

IWABUCHI, K., TAKAHASHI, J., NAKAGAWA, Y., and SAKAI, T. 1985. Electroantennogram responses of grape borer *Xylotrechus pyrrhoderus* Bates (Coleoptera: Cerambycidae) to its male sex pheromone components. *J. Chem. Ecol.* 11:819–828.

KOLB, H. C., VANNIEUWENHZE, M. S., and SHARPLESS, K. B. 1994. Catalytic asymmetric dihydroxylation. *Chem. Rev.* 94:2483–2547.

LACEY, E. S., GINZEL, M. D., MILLAR, J. G., and HANKS, L. M. 2004. Male-produced aggregation pheromone of the cerambycid beetle *Neoclytus acuminatus acuminatus*. *J. Chem. Ecol.* 30:1493–1507.

LACEY, E. S., RAY, A. M., and HANKS, L. M. 2007a. Calling behavior of the cerambycid beetle *Neoclytus acuminatus acuminatus*. *J. Insect Behav.* 20:117–128.

LACEY, E. S., MOREIRA, J. A., MILLAR, J. G., RAY, A. M., and HANKS, L. M. 2007b. Male-produced aggregation pheromone of the longhorned beetle *Neoclytus mucronatus mucronatus*. *Entomol. Exp. Appl.* 122:171–179.

LINSLEY, E. G. 1964. The Cerambycidae of North America, Part V: Taxonomy and classification of the subfamily Cerambycinae, Tribes Callichromini through Ancylocerini. *Univ. Calif. Publ. Entomol.* 22:1–197.

MAYER, M. S., and MCLAUGHLIN, J. R. (eds.). 1991. Handbook of Insect Pheromones and Sex Attractants. CRC, Boca Raton, FL.

RAY, A. M., LACEY, E. S., and HANKS, L. M. 2006. Predicted taxonomic patterns in pheromone production by longhorned beetles. *Naturwissenschaften* 93:543–550.

REDDY, G. V. P., FETTKÖTHER, R., NOLDT, U., and DETTNER, K. 2005. Capture of female *Hylotrupes bajulus* as influenced by trap type and pheromone blend. *J. Chem. Ecol.* 31:2169–2177.

SCHRÖDER, F., FETTKÖTHER, R., NOLDT, U., DETTNER, K., KÖNIG, W. A., and FRANCKE, W. 1994. Synthesis of (3*R*)-3-hydroxy-2-hexanone, (2*R*,3*R*)-2,3-hexanediol and (2*S*,3*R*)-2,3-hexanediol, the male sex pheromone of *Hylotrupes bajulus* and *Pyrrhidium sanguineum* (Cerambycidae). *Liebigs Annalen der Chemie* 1994:1211–1218.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry, 3rd edn. Freeman, New York, NY.

SUCKLING, D. M., GIBB, A. R., DALY, J. M., CHEN, X., and BROCKERHOFF, E. G. 2001. Behavioral and electrophysiological responses of *Arhopalus tristis* to burnt pine and other stimuli. *J. Chem. Ecol.* 27:1091–1104.

WHEELER, J. W., ABRAHAM, M., HIGHET, R. J., and DUFFIELD, R. M. 1988. Metasternal gland secretion of the locust tree borer, *Megacyllene robiniae* (Coleoptera: Cerambycidae). *Comp. Biochem. Physiol.* 91B:771–775.

YATAGAI, M., MAKIHARA, H., and OBA, K. 2002. Volatile components of Japanese cedar cultivars as repellents related to resistance to *Cryptomeria* bark borer. *J. Wood Sci.* 48:51–55.

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A Simple, Convenient, and Efficient Preparative GC System that Uses a Short Megabore Capillary Column as a Trap

Satoshi Nojima · Charles S. Apperson · Coby Schal

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Abstract A simple, convenient, and highly efficient preparative GC system has been developed that uses short sections of megabore capillary columns as sample collection (sorbent) traps. The performance of this system with various types of capillary column traps and under various collection conditions was systematically investigated with model compounds, including C4 to C20 normal alkanes, esters, and alcohols. The thickness and polarity of the sorptive stationary phase and the temperature of the collection trap affected trap performance. Each group of compounds was efficiently trapped above a critical Kovat's index, and the type of trap (deactivated, methyl polysiloxane, polyethylene glycol), film thickness, and whether or not the trap was cooled significantly shifted this threshold index. Above this critical index, recovery efficiencies of traps with methyl polysiloxane films were 80–100% for a wide range of injected sample mass. For example, a DB-1 collection trap with a film thickness of 1.5 μm methyl polysiloxane operated at ambient temperature trapped >84% of the mass of injected compounds of all three chemical classes with Kovat's index >1,100 (determined on a nonpolar column) with injected sample mass ranging from 10 to 1,000 ng of each compound. This preparative GC system is technically and economically feasible for most researchers. Furthermore, it is suitable for the preparation of NMR samples of volatile and semivolatile compounds, especially with sample sizes ranging from several nanograms to several micrograms.

Keywords Preparative GC · Megabore capillary column · Open tubular trap · Semiochemicals · Fractionation · Isolation · Purification · NMR · Sample preparation

S. Nojima (✉) · C. S. Apperson · C. Schal
Department of Entomology and W. M. Keck Center for Behavioral Biology, North Carolina State University, Raleigh, NC 27695, USA
e-mail: toshi_nojima@ncsu.edu

Introduction

The identification of semiochemicals is an essential step for understanding chemically mediated behavioral and ecological interactions. Numerous substances have been identified, and the databases of these compounds have grown exponentially in recent years. Nonetheless, identification of semiochemicals, some of which occur in trace amounts, remains an arduous but indispensable prelude for the ensuing basic and applied research.

The isolation and purification of active compounds are key steps in a bioassay-guided identification process, and preparative GC is a powerful purification technique for volatile and semivolatile compounds (Heath and Dueben 1998). However, there are several technical and practical constraints associated with preparative GC: (1) the necessity to condense volatile compounds by cooling the collection trap with a refrigerant, commonly dry ice, dry ice/acetone, or liquid nitrogen (Heath and Dueben 1998), a rather inconvenient process that makes rapid exchanges of traps cumbersome and time-consuming; (2) low, unsatisfactory recovery efficiency of volatile compounds; and (3) expense and difficulty of setting up a commercial instrument or of fabrication of a custom-built system.

Capillary collection traps, or open tubular traps (OTT), have been used to enrich and concentrate volatile organic compounds from the headspace of various samples for GC/

GC-MS analyses of environmental and food samples (Baltussen et al. 2002; Pillonel et al. 2002; Kloskowski et al. 2007). This technique was first developed by Grob and Habich (1985), and later, Blomberg and Roeraade (1987) applied it to volatile collection from preparative GC with the advantage of high recovery efficiencies of volatiles without cryogenic trapping. They suggested that this preparative GC technique could be an attractive approach for many applications. However, in the last two decades, there has been limited practical application of their innovation (Shimoda et al. 1993, 1996).

The parameters involved in sample collection for both instrumental analyses and preparative GC by using OTT appear to be the same. Although many parameters that influence the trapping efficiency of OTT for sample enrichment have been studied (Grob and Habich 1985; Burger and Munro 1986, 1987; Blomberg and Roeraade 1987; Cao and Hewitt 1992; Zhirong et al. 1999; Pettersson et al. 2004), most previous methodological studies were done with custom-made super thick film OTT along with custom-made sample collection and desorption devices and custom-modified instruments mainly for gaseous and solvent-like highly volatile compounds such as environmental pollutants. For example, Blomberg and Roeraade (1987) used custom-made 200 cm, 80 μm silicon film collection traps for preparative GC for compounds ranging from solvent-like to C12 hydrocarbon. In practical applications, however, it is convenient and cost-effective to use commercially available materials with standardized parameters that should yield more consistent results. Moreover, there has not been extensive and systematic evaluation of OTT performance for preparative GC collection especially for volatile and semivolatile semiochemicals.

Nojima et al. (2004) previously reported on a manual preparative GC system, which is relatively simple and inexpensive and achieves high recovery efficiency by using cryogenic trapping with a short section of a deactivated megabore capillary tube. This system has facilitated the efficient purification of semiochemicals for NMR analysis even at the submicrogram scale and has been used for structure elucidation of a few micrograms of a thermally and chemically unstable female sex pheromone of the German cockroach (Nojima et al. 2005). Nevertheless, this preparative system requires some familiarity with instrumentation, a refrigerant for cryogenic trapping, and a major drawback is that it is not suitable for sequential fractionation of multiple compounds within a single GC run.

In this study, we report a further improvement of this preparative GC approach that consists of a simple modification of a regular GC system coupled to an OTT sample collection by utilizing only commercially available materials and systematic evaluation of the system by using a wide range of model compounds.

Methods and Materials

Chemicals Straight chain methyl esters (C4–C16), hydrocarbons (C7–C20), and alcohols (C4–C16) were obtained from Supelco (Bellefonte, PA, USA) or Aldrich (Milwaukee, WI, USA).

Preparative GC

Modification of the GC An HP5890 gas chromatograph was converted to a preparative GC. Schematic diagrams of the preparative GC configuration and outlet port assembly are shown in Fig. 1. A split–splitless (S/SL) injection port assembly equipped with heat sink, heater cartridge, and sensor was used for the preparative GC outlet port and installed adjacent to the FID port. The heater and sensor were connected to the control ports assigned to detector B so that the temperature of the preparative GC outlet port could be controlled and monitored as "detector B."

The S/SL injection port was modified to an outlet port for the preparative GC as follows (Fig. 1a–c). The septum well on the weldment nut, which is welded to 1/16″ tubing

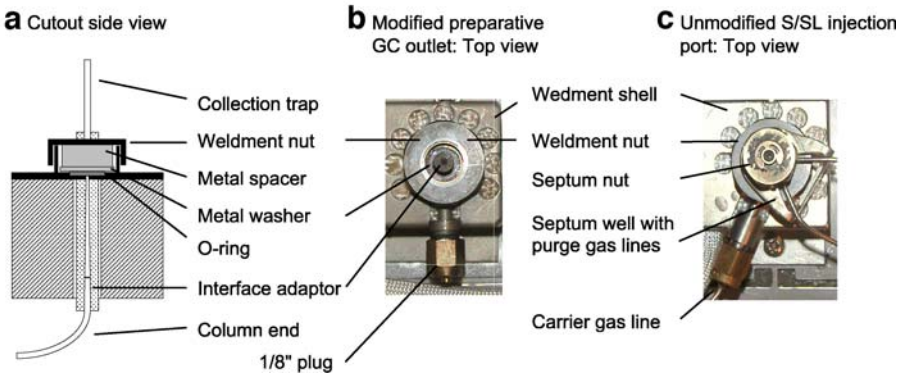

Fig. 1 **a–c** Schematic diagrams of the preparative GC configuration and outlet port assembly

for the purge gas and retained on the weldment nut by a retainer ring, was removed from the injection port. The metal spacer, used for supporting the weldment nut against the septum well, was kept and utilized for fixing an interface adaptor between the column end and collection traps. The carrier gas line welded to the shell weldment was sealed with a 1/8″ stainless steal plug (Swagelok®) (Fig. 1b).

Installation of a megabore capillary column The distal column end was cut with a column cutter, and finger lipids and other contaminants were removed by wiping with methanol-wetted tissue paper. The column end was inserted into the preparative GC outlet port so that its end protruded several centimeters above the port. A direct injection glass liner (1 mm Uniliner® for 0.32/0.53 mm ID, columns, 1.0 mm ID, 6.3 mm OD×78.5 mm; Restek, Bellefonte, PA, USA) was used as an interface adaptor between the column end and collection trap (Fig. 1a and b). The column was pushed into the longer tapered seat of the liner to make a securely tight seal. Then, the glass liner–column assembly was retracted into the outlet port. An O-ring for inlet glass liners for Agilent GCs (6.3–6.5 mm ID), a flat stainless steel washer (6.7 mm ID, 17.2 mm OD), and the metal spacer that was taken from the septum well were put on the liner in this order (Fig. 1a). Then, the weldment nut was put on the glass liner, finger-tightened, and then tightened with a wrench while holding the glass liner with clean forceps. It was important to avoid rotating the glass liner while tightening the weldment nut to prevent twisting and stressing the connection between the glass liner and the column. Any tension at this connection may result in an unexpected detachment of the column under frequent temperature/pressure fluctuations and vibrations from the oven fan. The end of the column was not fixed to the modified S/SL outlet port with a column nut and ferrule to provide some free rotation for the column.

Collection traps Sections (40 cm) of various types of megabore (0.53 mm ID) capillary columns were used as collection traps: (a) collection traps without stationary phase whose inner surface was deactivated (Agilent Technologies, Santa Clara, CA, USA); (b) nonpolar collection traps on which a methyl polysiloxane film was bonded at a film thickness of 0.5, 1.5, or 5.0 μm (DB-1, Agilent Technologies); and (c) a polar collection trap on which polyethylene glycol film was bonded at a film thicknesses of 1.5 μm (Stabilwax®, Restek, Bellefonte, PA, USA). The collection traps were rinsed twice with 100 μl of methylene chloride and dried at room temperature overnight. Both ends of the collection traps were cut and cleaned with methanol on tissue paper. For reuse, the traps were reconditioned by rinsing with solvent, and the trap was carefully examined for a square and even end for a secure connection to the interface adaptor.

Preparative GC conditions A nonpolar EC-5 megabore capillary column (1.0 μm film thickness, 0.53 mm ID× 30 m, Alltech Associates, Deerfield, IL, USA) was used as a separation column, and a 2-m deactivated column (0.53 mm ID, no stationary phase; Alltech Associates) was connected to the front of the separation column with a press fit connector (Alltech Associates). Helium was used as the carrier gas at a head pressure of 27.6 kPa and a flow rate of 5.5 ml min^{-1}. The oven temperature was set at 45°C for 2 min, increased at 15°C min^{-1} to 250°C, and held for 5 min. The injector and collection port temperatures were held at 270°C and 250°C, respectively. The septum purge flow rate was set at 3 ml min^{-1} with a total flow rate of 22 ml min^{-1} in the split injection mode (split ratio of 1:5), whereas the total flow rate was set at 50 ml min^{-1} in the splitless injection mode with the purge valve off for 1 min. Retention times of model compounds were established before preparative GC work by FID under the same analytical conditions. The Kovat's retention indices (Heath and Dueben 1998) of model compounds were estimated by retention times of straight chain hydrocarbons.

Sample Collection and Compound Recovery from Traps

Sample collection For collection with cryogenic trapping, a detachable and well-insulated sheath equipped with a reservoir for a refrigerant was used to cool the collection traps (Fig. 2a; see detail in Nojima et al. 2004). The reservoir cup was filled with dry ice, and a collection trap was inserted into the sheath for equilibration. To collect compounds, the trap–sheath assembly was connected to the interface adaptor in the outlet port by gently pushing the trap end into the tapered seat of the adaptor just before a sample collection window. A gentle connection gave a secure seal that resulted in high recovery efficiencies and facilitated easy, smooth, and quick exchanges of collection traps for multiple collections. A tighter connection may cause breakage of the tip of the collection trap during trap exchanges, resulting in poor recovery when column fragments were in the glass liner. We found that a syringe cleaning wire (0.17 mm OD; Hamilton, Reno, NV, USA) worked well to remove column fragments from the adaptor. During the collection, the lower end of the cooling sheath was kept in contact with the interface adaptor so that a gradual cooling zone was generated along the collection trap for better recovery efficiencies (Brownlee and Silverstein 1968). At the end of a sample collection window, the collection trap was withdrawn together with the sheath; the trap was pulled out from the sheath and set up on a lab

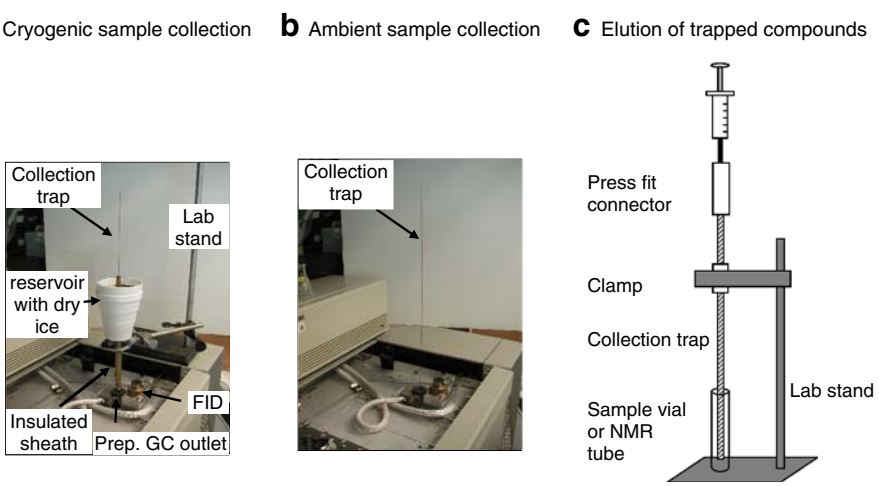

Fig. 2 Sample collections under cryogenic (**a**) and ambient (**b**) conditions, and sample elution from the trap (**c**)

stand for the extraction step. For a long collection, a lab stand and a clamp were used to hold the sheath (Fig. 2a).

For sample collection at ambient temperature, a clean collection trap was connected to the interface adaptor as described above. The connection between the trap and the adaptor was secure enough to hold the 40-cm collection trap without any other support (Fig. 2b).

Elution of compounds from the traps The collection trap was set up vertically on a lab stand as shown in Fig. 2c. A press fit connector was attached to the upper end of the trap, and the other end was put into a sample vial. Then, a GC syringe was used to introduce solvent containing an internal standard into the press fit connector. In a preliminary experiment, compounds trapped on various types of collection traps were fully extracted with 40 μl of hexane, methylene chloride, or ether; 10 μl methylene chloride was found to be sufficient to extract trapped compounds for qualitative analyses.

Analytical GC conditions Sample analysis was conducted on an HP5890 GC equipped with a nonpolar EC-5 capillary column (0.25 μm film thickness, 0.25 mm ID×30 m; Alltech Associates). Helium was used as the carrier gas at a head pressure of 115 kPa (flow rate, 1.5 ml min^{-1}). Oven temperature was set at 50°C for 2 min, increased at 15°C min^{-1} to 250°C, and held for 5 min. The injector and detector temperatures were set at 270°C. Septum purge flow rate was set at 3 ml min^{-1} with a total flow rate of 14 ml min^{-1} in the split injection mode (split ratio of 1:10), whereas a total flow rate was set at 50 ml min^{-1} in the splitless injection mode with the purge valve off for 1 min.

Experiment 1: qualitative comparison of various types of collection traps under ambient and cryogenic conditions The trapping capabilities of deactivated capillary tubes (0 μm film), DB-1 capillary tubes with a film thickness of 1.5 μm, and Stabilwax® capillaries with a film thickness of 1.5 μm were compared. A mixture of model compounds that included straight chain methyl esters (C4–C16), hydrocarbons (C7–C20), and alcohols (C4–C16) in methylene chloride at a concentration of 300 ng μl^{-1} each was used for this experiment. One microliter of the mixture was injected into the preparative GC in split mode (split ratio of 1:5) so that the C4 ester, methyl butanoate, separated from the solvent peak that obscured it in the splitless injection mode. The collection window was 2 to 18.5 min, so that all compounds from a single injection were collected in the same collection trap (Fig. 3). The collections were made both under cryogenic and ambient conditions. Trapped compounds were eluted with 10 μl methylene chloride, and 2 μl of each extract were analyzed in split mode at a split ratio 1:5. Two replicates were made for each trapping condition.

Experiment 2: qualitative comparison of various film thicknesses of DB-1 collection traps under ambient and cryogenic conditions. The same collections were made using DB-1 capillary traps with film thicknesses of 0.5, 1.5, and 5.0 μm. Trapped compounds were recovered and analyzed as described above.

Experiment 3: quantitative comparison of the trapping efficiencies of various film thicknesses of DB-1 collection traps at ambient temperature. Mixtures of model compounds at concentrations of 10, 100, and 1,000 ng μl^{-1} of each compound in hexane were used for this experiment. For deactivated collection traps (0 μm film), a mixture of C13–C16 methyl esters, C16–C19 hydrocarbons, and C13–C16 alcohols was used, whereas for DB-1 collection traps (which exhibited better trapping capabilities in preliminary studies), a mixture of C8–C11 methyl esters, C11–C14 hydrocarbons, and C8–C11 alcohols was used. One microliter of each mixture was injected into the preparative GC in

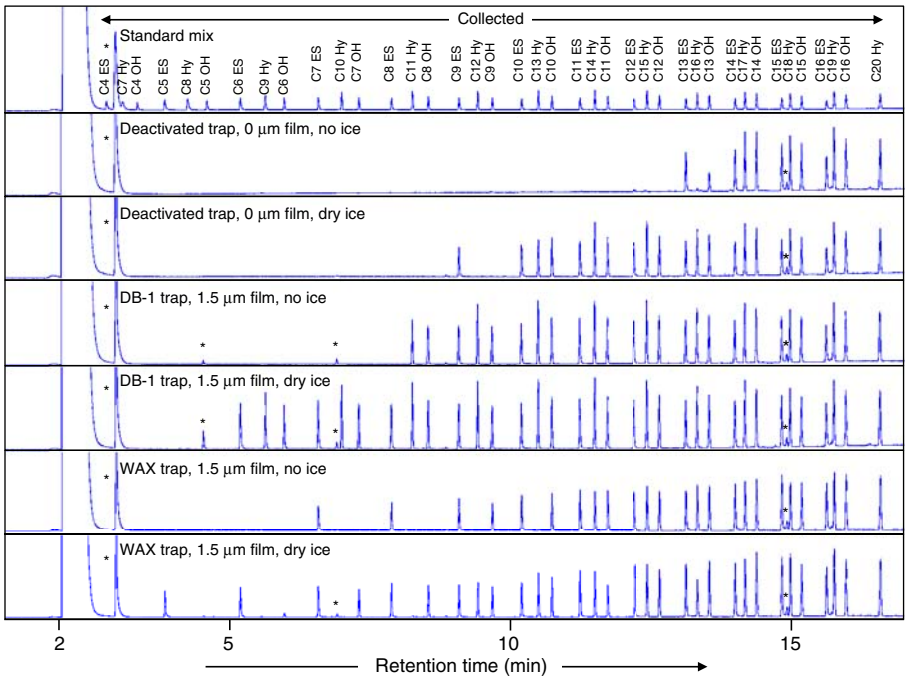

Fig. 3 A comparison of trapping of model compounds by three types of collection traps under ambient and cryogenic conditions. One microliter of a mixture of straight chain methyl esters (C4–C16), hydrocarbons (C7–C20), and alcohols (C4–C16) at a concentration of 300 ng μl^{-1} per compound was injected into the preparative GC in split mode, and all compounds were collected in a single collection trap between 2 and 18.5 min of each run. Collections were made with both dry ice and at ambient temperature. Trapped compounds were eluted with methylene chloride, and the extracts were analyzed by GC-FID. *Asterisks* indicate impurities that likely originated from the instrument, the solvent, and not from the traps (see text)

splitless mode, and all compounds in the mixture were collected in the same collection trap during each run. The collection windows were 13–18 min for deactivated collection traps and 8–13.5 min for DB-1 collection traps. All collections were made at ambient temperature. Trapped compounds were eluted with 20 µl hexane, then 10 µl hexane containing 500 ng pentacosane internal standard, and then an additional 20 µl hexane. Extracts were combined, and 1 µl of each sample was subjected to quantitative (FID) GC analysis in splitless mode. Five replicates were made for each trap condition.

Experiment 4: qualitative analysis of distribution of trapped compounds in the collection traps. We used the same mixtures of compounds at a concentration of 1,000 ng μl^{-1} each compound, and the same conditions described above for deactivated and DB-1 collection traps. After the collections, each 40 cm collection trap was cut into 10 cm sections, and each was eluted with 20 µl hexane, then 10 µl hexane containing 500 ng pentacosane internal standard, and then an additional 20 µl hexane. One microliter of each extract was analyzed by GC-FID in splitless mode. Two replicates were made for each collection trap.

Experiment 5: a practical collection trial. A mixture of C4–C12 methyl esters, C7–C15 hydrocarbons, and C4–C12 alcohols in methylene chloride, at a concentration of 300 ng μl^{-1} each compound, was used for this experiment. One microliter of this mixture was injected into the preparative GC in splitless mode, and compounds were collected in groups of similar retention index (a C_n methyl ester, $C_{(n+3)}$ hydrocarbon and C_n alcohol; Fig. 7), except for the last three groups that were collected together in the same DB-1 trap with 1.5 µm film thickness. Each collection window for a group was about 1 min except for the last three groups. Trapped compounds were eluted with 20 µl hexane, and 1 µl of each extract was injected into a GC-FID in split mode at a split ratio 1:16.

Results and Discussion

Experiment 1: Qualitative Comparison of Various Types of Collection Traps under Ambient and Cryogenic Conditions

Trapping of model compounds in megabore collection traps varied with trap type and whether or not we used cryogenic conditions (Fig. 3). Cooling the collection traps with dry ice greatly increased the trapping capabilities of various types of collection traps. At ambient temperature, a deactivated megabore column (0 µm film) failed to trap all three compound types at or below a retention index of about 1600 (on an EC-5 column), but with cryogenic trapping the same trap was effective down to 1300. Addition of a nonpolar stationary phase (DB-1, 1.5 µm film thickness) extended the range of compounds trapped down to 1100 without cooling and to 900 with dry ice. Although a polar megabore collection trap (Stabilwax®, 1.5 µm film thickness) was less effective at trapping hydrocarbons than the DB-1 trap, the WAX trap effectively trapped the C7 ester (retention index 1000) at ambient temperature and down to a C5 ester (retention index 800) with dry ice (Fig. 3). Thus,

the presence of a stationary phase greatly improved the trapping capabilities of collection traps, and cryogenic conditions (with or without stationary phase) extended the range of compounds trapped to a lower retention index. As in gas chromatography, it appears that both the affinity of compounds to the film and their volatility play important roles in the effectiveness of WAX collection traps. Although the WAX trap was more effective for esters, DB-1 traps are more suitable for practical use because this nonpolar stationary phase effectively traps highly volatile compounds and is less selective of the three chemical classes we tested. Moreover, bonded DB-1 columns tolerate both high temperatures and repeated solvent extractions.

It is important to note that even without cooling the collection trap, the addition of a stationary phase to the megabore traps vastly improved their trapping effectiveness of early eluting compounds, which practically covers a wide range of volatile–semivolatile semiochemicals. The stationary phase alleviates the need to use a refrigerant, which has been a rather inconvenient process that makes rapid exchanges of traps cumbersome and time-consuming.

Contamination is often a serious issue in preparative GC, especially when collecting minute amounts of sample. Several minor contaminants (asterisks in Fig. 3; see also experiment 2; Fig. 4) likely originated from the instrument or extraction solvents and not from traps because the contaminants became negligible after repeated sample processing (see Figs. 6 and 7).

Experiment 2: Qualitative Comparison of Various Film Thicknesses of DB-1 Collection Traps under Ambient and Cryogenic Conditions

We compared the trapping effectiveness of various film thicknesses of DB-1 collection traps. Under ambient trap conditions, a collection trap with a film thickness of 5.0 μm consistently trapped compounds of retention index 1000 and above, whereas 1.5 and 0.5 μm films trapped above 1100 and 1200, respectively (Fig. 4). All three film thicknesses trapped smaller hydrocarbons and alcohols, but not esters. The addition of cryogenic conditions extended the range of compounds trapped by 2C units to retention indices of 800 for 5.0 μm, 900 for 1.5 μm, and 1000 for 0.5 μm films. Thus, traps with thicker films were more effective than traps with thinner films. However, the addition of more stationary phase to traps already containing the same phase yielded only moderate benefits compared to the initial addition of a sorptive phase to traps without film or the choice of polar or nonpolar phase. Nevertheless, with dry ice cooling, the effectiveness of DB-1 collection traps with a film thickness of 5.0 μm extended to solvent-like compounds, C5 ester, C8 hydrocarbon, and C5 alcohol. Based on these results, we expect that thicker films on WAX collection traps will also extend the trapping effectiveness of polar compounds that elute earlier than C4 alcohol.

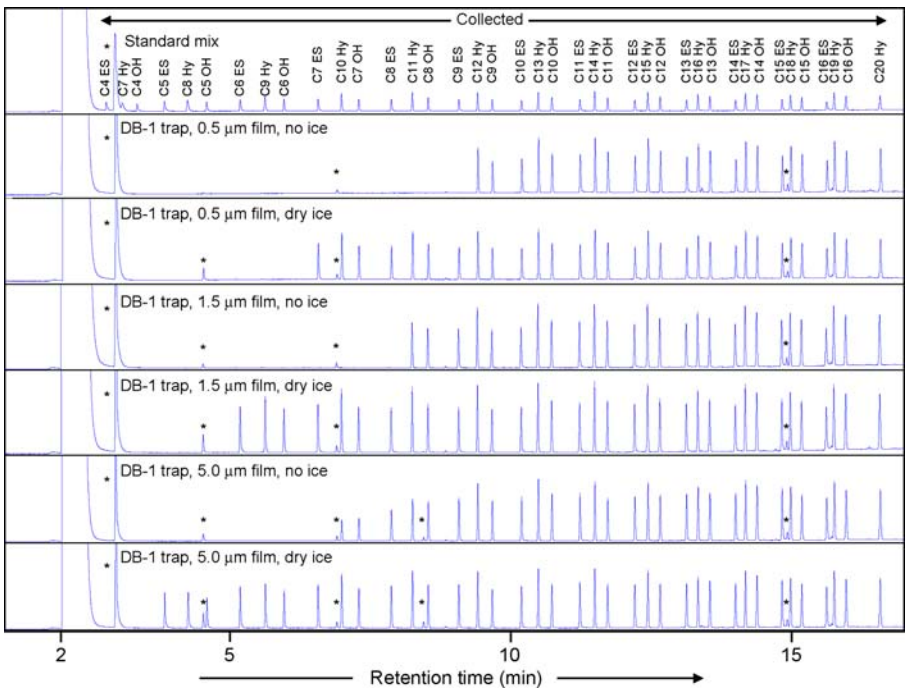

Fig. 4 A comparison of trapping of model compounds on DB-1 traps with various film thicknesses. The same mixture as in Fig. 3 was used for this experiment. The mixture was injected into the preparative GC in split mode, and all compounds were collected in a single collection trap between 2 and 18.5 min of each run. Collections were made with both dry ice and at ambient temperature. Trapped compounds were eluted with methylene chloride, and the extracts were analyzed by GC-FID. *Asterisks* indicate impurities that likely originated from the instrument, the solvent, and not from the traps

Experiment 3: Quantitative Comparison of the Trapping Efficiencies of Various Film Thicknesses of DB-1 Collection Traps at Ambient Temperature

The recovery efficacy of deactivated traps (0 μm film) varied with chemical class and sample loading (Fig. 5a). With injections of 1000 ng per compound, these traps efficiently collected >92% of all three chemical classes tested at and above the retention index of 1600. However, early eluting compounds exhibited a clear dose–response with poor trapping at low sample loadings. For alcohols, this pattern extended for all compounds in the mixture, from C13 to C16 ($P<0.05$, ANOVA). There are two main explanations for this observation. First, irreversible adsorption of alcohols to the inner surface of the deactivated trap might occur, and it would be more significant at lower sample loading sizes. Second, at high sample loading, the early eluting trapped compounds may form a stationary phase on the deactivated column, thus retarding the loss of other compounds.

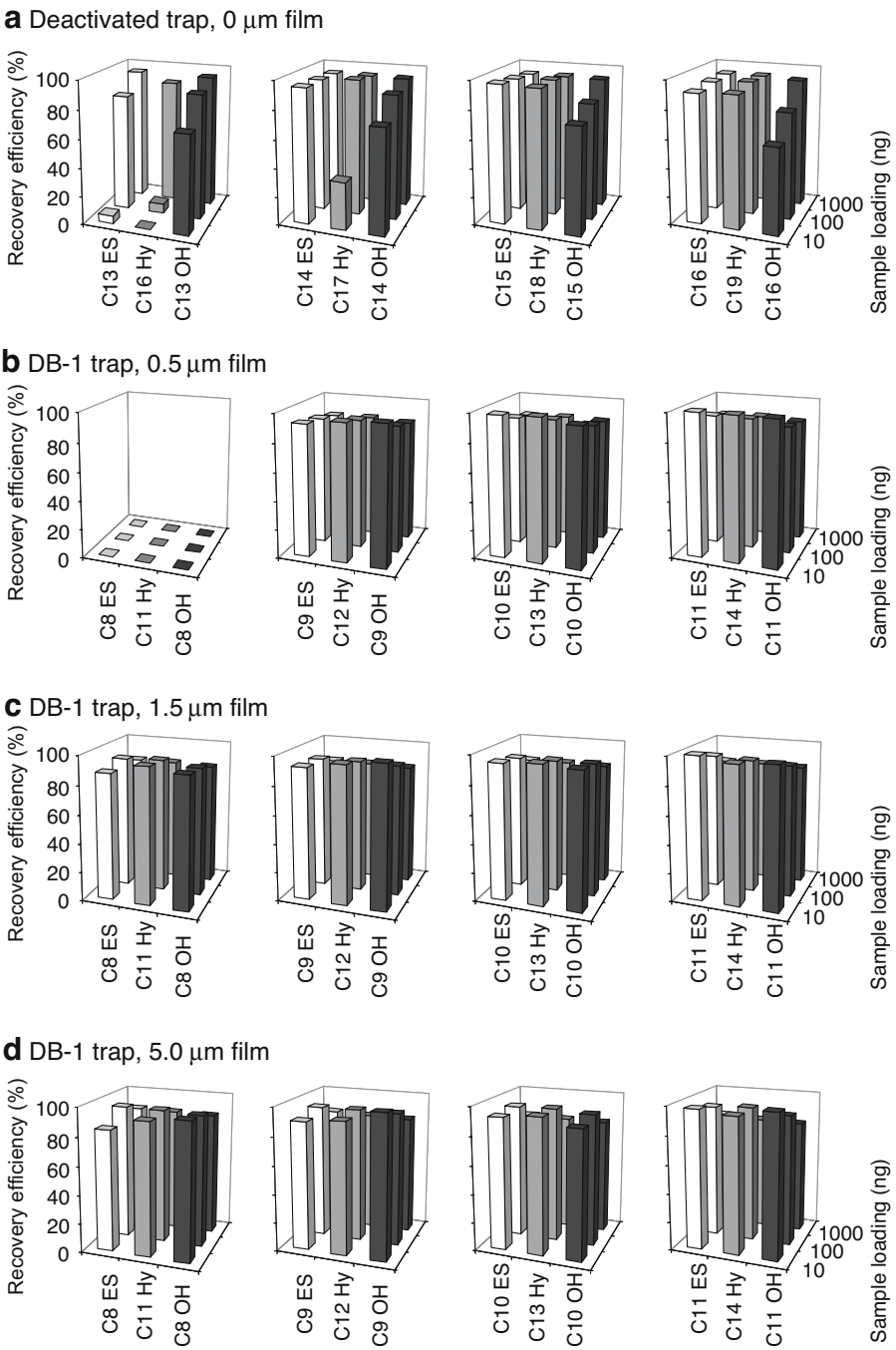

Fig. 5 a–d Trapping and recovery efficiencies of DB-1 traps with various film thicknesses and at different sample loadings conducted at room temperature. A mixture of straight chain methyl esters (C13–C16), hydrocarbons (C16–C19), and alcohols (C13–C16) was used with deactivated traps (0 μm film), and another mixture of methyl esters (C8–C11), hydrocarbons (C11–C14), and alcohols (C8–C11) was used with DB-1 traps. Preparative collections were made at sample loadings of 10, 100, and 1,000 ng of each compound. All compounds were collected in a single collection trap between 13.5 and 18.0 min for deactivated traps and between 8.0 and 13.5 min for DB-1 traps. Trapped compounds were eluted with hexane, and the extracts were analyzed by GC-FID

The recovery efficacies of DB-1 traps with a 0.5-μm film showed a distinct threshold retention index at about 1,200, below which almost none of the compounds were trapped (Fig. 5b). Adding more stationary phase (1.5 or 5.0 μm) greatly improved trapping efficiency to >80% for all tested compounds down to a retention index of 1100, and at all sample loadings (Fig. 5c,d). It is interesting to note that the DB-1 traps with a 1.5-μm film showed much better trap capability for C8 ester in this experiment in which the collection window was 8–13.5 min than in the qualitative experiment (#1) in which the collection window was 2–18.5 min (Fig. 4). It is probably because of the shorter collection window of this experiment than the earlier experiments (see the results and discussion of experiment 5). The threshold retention indices for the traps with 1.5 or 5.0 μm films could not be quantified but should be lower than 1000–1100 based on the results of qualitative experiments (Fig. 4). There was an overall inverse relationship between recovery efficiency of all DB-1 traps and the amount of sample injected ($P<0.05$, ANOVA). This is opposite to what was expected based on the classical Brownlee and Silverstein (1968) method in which recovery efficiencies decreased with decreasing amounts of injected sample. Nevertheless, the efficiency of trapping was 90–100% for almost all small samples, and even with injections of 1,000 ng per compound the trapping efficiency was >80%, generally considered satisfactory.

The connection between the column end and the collection trap is critical for achieving satisfactory recovery of eluting compounds in this preparative GC technique. An uneven cut on the column and trap ends can introduce reactive surfaces and a loose connection, both of which will diminish trapping efficiency. In our quantitative experiments, the recovery efficiencies were overall satisfactory through a wide range of sample loading sizes and different chemical classes, indicating that the interface adaptor system that used a direct injection glass liner between the column end and the traps was secure.

In preparative GC, it is thought that a gradual temperature gradient along the collection trap is necessary to achieve high recovery (Brownlee and Silverstein 1968) because a mist could be formed as the eluting compounds in the gas phase are rapidly cooled as they exit the hot GC, and the mist could then be propelled out through the trap by the relatively high linear velocity of capillary GC. In this experiment, the collection traps were kept at ambient temperature with no insulation before and during collections. This configuration probably resulted in a short gradual cooling zone along the trap in which compounds were quickly cooled from 250°C at the preparative GC outlet to about 25°C within the trap. However, the recovery efficacies were overall satisfactory. In principle, the trap collection at ambient temperature may be similar to the Grob splitless injection procedure whereby compounds are vaporized in the injection port and then condensed (solvent or thermally refocused) onto the head of the column to achieve narrow bands and sharp signals (Grob and Grob 1969). Nevertheless, as shown in experiments 1 and 2, a gradual cooling zone along the trap by a well-insulated sheath in combination with cryotrapping with dry ice significantly improved the efficiency of trapping.

Experiment 4: Qualitative Analysis of Distribution of Trapped Compounds in the Collection Traps

Under ambient collection conditions, in the deactivated collection trap (0 μm film), all injected compounds were condensed in the first 10 cm section of the trap (Fig. 6a). With the addition of a nonpolar stationary phase, not only the range of trapped compounds extended to a retention index of 1100 (again, better than in experiment 1 because a shorter collection window was used), but the condensed compounds were distributed farther up the trap, depending on the film thickness (Fig. 6b–d); more volatile compounds were trapped in sections farther from the GC outlet. Thus, threshold compounds that were trapped in the lowest 10 cm on a 5.0-μm stationary phase (e.g., retention index of approximately 1200) were trapped in the 10- to 20-cm zone on a 1.5-μm phase, and in the 10- to 30-cm zone on 0.5 μm DB-1 film thickness (Fig. 6b–d). Likewise, more volatile compounds, for example, with a retention index of 1100, were trapped in the 10- to 20-cm section on a 5.0-μm stationary phase, in the 20- to 40-cm zone on a 1.5-μm phase, and were not trapped at all on a DB-1 megabore trap with only 0.5 μm film thickness. This finding undoubtedly relates to greater interaction with the thicker stationary film, as also evidenced by later retention times on thicker films under otherwise identical GC analytical conditions. Therefore, for compounds with a retention index >1200, even a 10-cm section of megabore trap would yield satisfactory results in preparative GC. We have used 10 cm DB-1 traps with 1.5 μm film thickness to study the behavior of model compounds (data not shown). In practice, however, a longer trap is easier to handle and is more amenable to cryogenic trapping of solvent-like compounds. It is worth mentioning that when a mixture at a concentration of 1,000 ng per compound was injected, a narrow 10 mm band approximately 5 mm from the GC outlet became opaque as compounds condensed at the base of the traps.

Experiment 5: A Practical Collection Trial

Practical preparative GC generally targets single peaks for bioassay or analytical procedures that require pure compound (microchemical, spectrometric). The collection window is, therefore, much narrower than the 5–16.5 min used

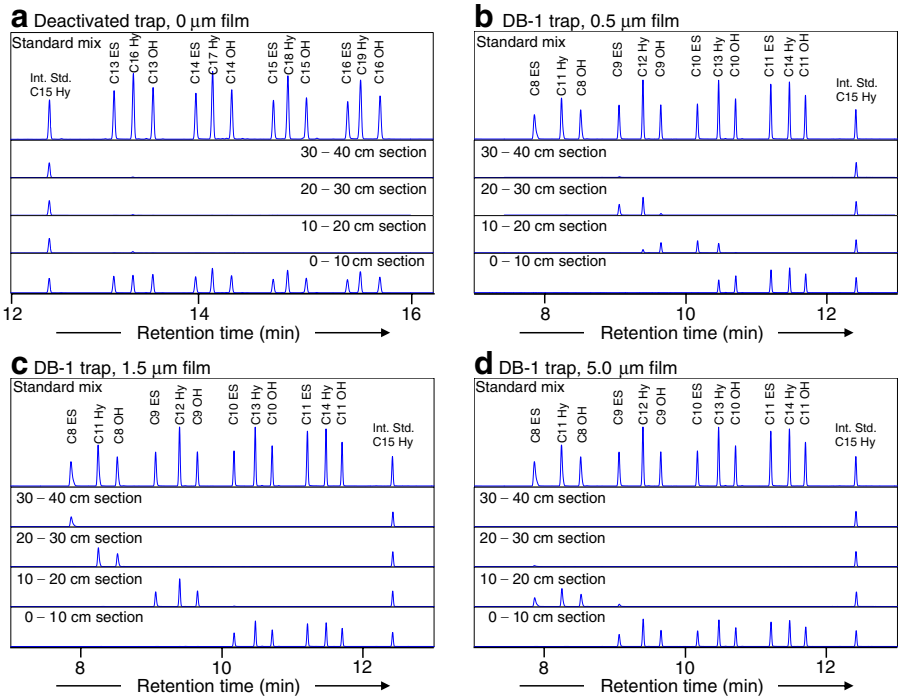

Fig. 6 a–d Qualitative analysis of the distribution of condensed compounds along the collection traps. A mixture of straight chain methyl esters (C13–C16), hydrocarbons (C16–C19), and alcohols (C13–C16) was used with deactivated traps (0 µm film), and another mixture of methyl esters (C8–C11), hydrocarbons (C11–C14), and alcohols (C8–C11) was used with DB-1 traps at a concentration of 1,000 ng μl^{-1} of each. One microliter of the mixture was injected into the preparative GC in splitless mode, and all compounds were collected in the same collection trap at each run at ambient temperature. After the collection, the 40-cm collection traps were cut into 10 cm sections and each section was extracted with hexane and subjected to GC-FID analysis

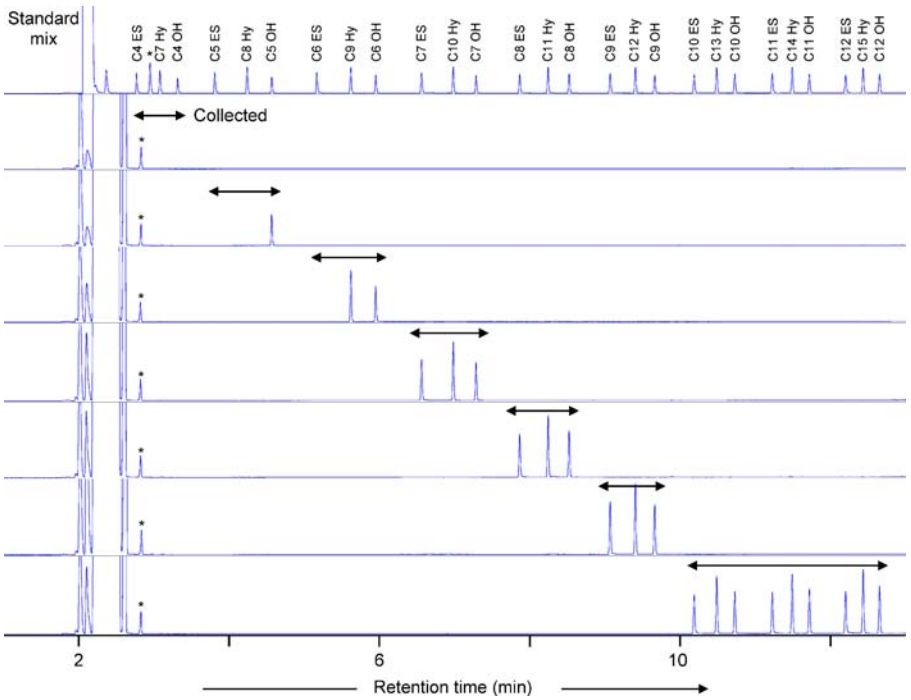

Fig. 7 A practical fractionation trial using DB-1 traps with 1.5 µm film at ambient temperature. A mixture of methyl esters (C4–C12), hydrocarbons (C7–C15), and alcohols (C4–C12) at a concentration of 300 ng μl^{-1} of each compound was used for this experiment. One microliter of the mixture was injected into the preparative GC in splitless mode, and compounds were collected in groups based on similar retention index, such that each group consisted of a methyl ester (C_n), hydrocarbon (C_{n+3}), and alcohol (C_n). The last three groups (retention index 1300 to 1500) were trapped together in the same trap. Each collection window for the groups was about 1 min except for the last three groups. Trapped compounds were eluted with hexane, and the extracts were analyzed by GC-FID. *Asterisks* indicate impurities that likely originated from the solvent and not from the traps

in the previous experiments. To verify the performance of our system with a more practical collection window, we conducted sequential fractionations of single injections by using 40 cm DB-1 megabore collection traps with 1.5 μm film. Compounds were fractionated in groups, each comprising a methyl ester (C_n), hydrocarbon (C_{n+3}), and alcohol (C_n) (Fig. 7). All collection windows for each fraction were about 1 min, except the last fraction, which was approximately 3 min. The results showed that each group was perfectly fractionated with ease and without any cross contamination (Fig. 7). The shorter collection times revealed even greater efficacy of this preparative GC approach. All three compounds around retention index 1000 were now entirely trapped, whereas this threshold was at retention index 1100 with long collection windows (see Figs. 3 and 4). Also, more volatile compounds, such as C5 alcohol, which could not be trapped even under cryogenic conditions (Figs. 3, 4), were now trapped at ambient conditions with a short collection window. It thus appears that highly volatile compounds condense in the stationary phase of the trap, but if the trap remains coupled to the GC outlet, a combination of the high velocity of the carrier gas and gradual heating of the trap might chromatograph the trapped compounds and discharge them out of the trap. In practice, on a long megabore column, the peak width near the base of early eluting compounds (i.e., collection window) is only one to several seconds, and we expect that our preparative GC system will perform even more efficiently under these conditions.

General Discussion and Conclusions

There are several technical and practical constraints associated with preparative GC, as pointed out earlier. Major advantages of our system are: (1) a wide range of volatile compounds can be effectively trapped without cooling the collection traps; (2) the collection traps can be rapidly and easily exchanged for trapping multiple but discrete GC peaks with 80–100% recovery; (3) the modification of a regular GC system into this preparative system can be achieved easily and inexpensively by using commercially available materials; and (4) commercially available capillary columns with a wide range of stationary phases, widths, and film thicknesses facilitate optimization of traps to the target compounds and to the analytical column to minimize pressure drop as compounds enter the trap. Indeed, this system could be used in a hybrid mode with longer traps with thicker film and cryotrapping for early eluting compounds and short traps with thinner film operated at ambient conditions for less volatile compounds.

A minor disadvantage of this system is that compounds are collected based on their retention times, which are determined by FID in a prior injection. Although this disadvantage is rather negligible because switching between the FID and preparative GC outlets is easy and takes only minutes, a variable or fixed splitter could be introduced at the end of the column to alleviate this problem.

In conjunction with a large-volume injection system coupled to a megabore capillary column, this preparative GC system can be used practically like a preparative HPLC system for samples up to microgram amounts. Furthermore, our preparative GC system is suitable for a simple two-step preparation of NMR samples of volatile and semivolatile compounds: a compound of interest can be optimally trapped on a short section of the megabore column and eluted directly into an NMR tube with minimal NMR solvent, resulting in high recovery of a clean sample with minimal background noise.

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References

BALTUSSEN, E., CRAMERS, C. A., and SANDRA, P. J. F. 2002. Sorptive sample preparation—a review. *Anal. Bioanal. Chem* 373:3–22.

BLOMBERG, S., and ROERAADE, J. 1987. Preparative capillary gas-chromatography. II. fraction collection on traps coated with a very thick-film of immobilized stationary phase. *J. Chromatogr* 394:443–453.

BROWNLEE, R. G., and SILVERSTEIN, R. M. 1968. A micro-preparative gas chromatograph and a modified carbon skeleton determinator. *Anal. Chem* 40:2077–2079.

BURGER, B. V., and MUNRO, Z. 1986. Headspace gas-analysis: quantitative trapping and thermal desorption of volatiles using fused-silica open tubular capillary traps. *J. Chromatogr* 370:449–464.

BURGER, B. V., and MUNRO, Z. 1987. Headspace gas analysis: liquid desorption of headspace volatiles trapped on activated carbon open tubular traps. *J. Chromatogr* 402:95–103.

CAO, X. L., and HEWITT, C. N. 1992. Trapping efficiencies of capillary cold traps for C2–C10 hydrocarbons. *J. Chromatogr* 627:219–226.

GROB, K., and GROB, G. 1969. Splitless injection on capillary columns. Part I. The basic technique steroid analysis as an example. *J. Chromatogr. Sci* 7:584–586.

GROB, K., and HABICH, A. 1985. Headspace gas-analysis: The role and the design of concentration traps specifically suitable for capillary gas chromatography. *J. Chromatogr* 321:45–58.

HEATH, R. R., and DUEBEN, B. D. 1998. Analytical and preparative gas chromatography, pp. 85–126, in J. G. Millar, and K. F. Haynes (eds.). Methods in Chemical Ecology: Chemical Methods, vol. 1. Kluwer, New York.

KLOSKOWSKI, A., CHRZANOWSKI, W., PILARCZYK, M., and NAMIESNIK, J. 2007. Modern techniques of sample preparation for determination of organic analytes by gas chromatography. *Crit. Rev. Anal. Chem* 37:15–38.

NOJIMA, S., KIEMLE, D. J., WEBSTER, F. X., and ROELOFS, W. L. 2004. Submicro scale NMR sample preparation for volatile chemicals. *J. Chem. Ecol* 30:2153–2161.

NOJIMA, S., SCHAL, C., WEBSTER, F. X., SANTANGELO, R. G., and ROELOFS, W. L. 2005. Identification of the sex pheromone of the German cockroach, *Blattella germanica*. *Science* 307:1104–1106.

PETTERSSON, J., ALDAEUS, F., KLOSKOWSKI, A., and ROERAADE, J. 2004. Ultra thick film open tubular traps with an increased inner diameter. *J. Chromatogr. A* 1047:93–99.

PILLONEL, L., BOSSETT, J. O., and TABACCHI, R. 2002. Rapid preconcentration and enrichment techniques for the analysis of food volatile. A review. *Lebensm.-Wiss. Technol* 35:1–14.

SHIMODA, M., SHIRATSUCHI, H., MINEGISHI, Y., and OSAJIMA, Y. 1993. Flavor deterioration of nonfermented coarse-cut sausage during storage—flavor as a factor of quality for nonfermented sausage. 2. *J. Agric. Food Chem* 41:946–950.

SHIMODA, M., SHIRATSUCHI, H., NAKADA, Y., WU, Y., and OSAJIMA, Y. 1996. Identification and sensory characterization of volatile flavor compounds in sesame seed oil. *J. Agric. Food Chem* 44:3909–3912.

ZHIRONG, Z., WENQUI, L., and LINXIAN, S. 1999. A very thick film open-tubular trap for headspace capillary gas chromatography. *Chromatographia* 49:321–326.

J Chem Ecol (2008) 34:429–437
DOI 10.1007/s10886-008-9428-0

Olfactory Responses of Neotropical Short-Tailed Singing Mice, *Scotinomys teguina*, to Odors of the Mid-Ventral Sebaceous Gland: Discrimination of Conspecifics, Gender, and Female Reproductive Condition

Marcela Fernández-Vargas · Zuleyma Tang-Martínez · Steven M. Phelps

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Abstract We examined the olfactory communication of the Neotropical short-tailed singing mouse, *Scotinomys teguina*, by investigating whether *S. teguina* responded to odors produced by the mid-ventral sebaceous gland of conspecifics. Females spent significantly more time investigating male odor than an odorless stimulus or a female odor. Males spent significantly more time investigating female odor than an odorless stimulus, but not that of a male odor. This latter result does not seem to be explained by differences in age or sexual experience of test subjects, but may be influenced by reproductive condition of the female odor donor. Male *S. teguina* spent significantly more time (1) investigating and (2) in total proximity to odors of estrous than non-estrous females. Males spent more time (1) investigating and (2) in total proximity to odors of males than non-estrous females. Finally, given the choice between odor of males vs proestrous females, males showed no preference. Thus, the mid-ventral gland in *S. teguina* seems to convey information about conspecifics, sex, and female reproductive condition. Male odor, compared with proestrous female odor, is equally interesting to males, suggesting that the gland also plays an important role in male–male communication. Sexual dimorphism in the size of the gland and in the amount of secretion produced by the gland may be related to either male–male competition or female choice.

Keywords Chemical communication · Mid-ventral sebaceous gland · Neotropical rodent · Odor preferences · *Scotinomys teguina* · Sex differences

M. Fernández-Vargas (✉) · Z. Tang-Martínez
Department of Biology, University of Missouri-St. Louis,
One University Blvd.,
St. Louis, MO 63121, USA
e-mail: mf976@umsl.edu

S. M. Phelps
Department of Zoology, University of Florida,
Gainesville, FL 32611, USA

Introduction

In animal communication, information is obtained through several sensory channels, and the olfactory channel is a commonly used channel by the majority of mammalian species (Johnston 1983; Duvall et al. 1986). In many species of rodents, the sense of smell is an important source of information about their conspecifics and their environments (Blaustein 1981; Johnston 1983, 2003; Kleiman and Eisenberg 1983; Halpin 1980, 1986; Brown and MacDonald 1985). In fact, odor cues play an important role in sexual selection. Small mammals such as rodents that lack sexual dimorphism in size may be sexually dimorphic in terms of odor, thus evolving alternative traits for sexual selection (Kleiman 1977; Blaustein 1981).

The literature on rodents is replete with evidence of sex-specific effects of olfactory signals in species such as house mice, voles, and hamsters. Reproductive behavior and physiology in both sexes could be affected easily by the odor cues from conspecifics (Koyama 2004). Many rodents can distinguish odors from conspecifics, heterospecifics (Stoddart 1986; Hurst et al. 1994), and the opposite sex (Hurst 1990c; Sawrey and Dewsbury 1994; Ferkin and Johnston 1995; Solomon et al. 1999), in addition to recognizing sexual receptivity (Bakker et al. 1996; Xiao et al. 2004), age (Solomon and Rumbaugh 1997; Drickamer

and Brown 1998; Ferkin 1999), social status (White et al. 1986; Hurst 1990a,b,c), kin (Mateo 2003), and sexual experience (Taylor et al. 1983). Rodents have also been shown to detect parasite infection by odors, an ability that can affect mate choice (Kavaliers and Colwell 1995; Kavaliers et al. 2004; Zala et al. 2004).

Mammalian skin glands frequently are the sources of odoriferous secretions involved in scent-communication (Müller-Schwarze 1983). In rodents, mid-ventral sebaceous glands are commonly found in association with the hair follicles on the surface of the skin (Quay and Tomich 1963; Müller-Schwarze 1983; Brown 1985; Flood 1985), and their development and activity depend on androgenic steroids from testes, ovaries, or the adrenal cortex (Brown 1985; Flood 1985). The product (sebum) secreted by the sebaceous gland varies in composition, but usually is oily and rich in lipids with antimicrobial properties (Flood 1985). Secretion rates of sebaceous glands are usually slow, and the sebum reaches the skin surface from body movement, rubbing of the skin, grooming, and contractions of the arrector muscles of the hair (Flood 1985).

Beyond contributing to an individual's odor and maintaining quality of the pelage, the role of the secretion produced by sebaceous glands is not completely clear in many species (Flood 1985), and it is likely to have multiple functions (Müller-Schwarze 1983). Secretions from the mid-ventral sebaceous gland may be involved in reproduction by signaling sexual identity and reproductive condition. Chemical constituents of urine, vaginal products, and scent gland secretions can all change with the estrous cycle as a means of advertisement of female readiness to mate (Johnston 1983). Studies that test male responsiveness to female odors in different reproductive conditions have shown that males of many species are more attracted to odors of females in behavioral estrus than to those in non-estrus (Johnston 1983; Bakker et al. 1996; Portillo and Paredes 2004).

Animal communication in Neotropical rodents is an area that is almost completely unexplored. *Scotinomys teguina* (Family Cricetidae) is a rodent from the highland mountains of southeast Mexico and Central America that shows an interesting system of vocal communication (Hooper and Carleton 1976). As expected in rodent species, communication by *S. teguina* seems to also involve chemical components. However, chemical communication in *S. teguina* has never been examined systematically. *S. teguina* has a mid-ventral sebaceous gland beneath a small patch of dusky-white fur, from which it is suspected that the noticeable musky odor (characteristic of the genus) is produced (Hooper and Carleton 1976). The mid-ventral sebaceous gland is sexually dimorphic. It is larger and more visible in males, and is probably associated with sexual maturity (Hooper and Carleton 1976). Although a promiscuous mating system has been demonstrated only in *Scotinomys xerampelinus*, the closest related species (Blondel 2006), the social and mating system of *S. teguina* is unknown. However, pair bonding and male parental care have been suggested because females are tolerant of male's presence at the nest during a few days after the birth of pups (Hooper and Carleton 1976).

In this study, we focused on olfactory signals produced by the sebaceous gland of *S. teguina*. The objective was to determine whether *S. teguina* responds to the olfactory cues produced by the mid-ventral sebaceous gland of conspecifics. We hypothesized that this rodent communicates chemically by using the odor cues produced by its mid-ventral gland, and that those cues may provide information about conspecifics, sex, and female reproductive state. In experiment 1, we gave both males and females a choice between an opposite sex odor and an odorless stimulus. In experiment 2, males and females had a choice between male and female odors, but we did not control for the reproductive condition of target females. In experiment 3, we explored the effects of female reproductive condition by examining whether *S. teguina* males discriminate (a) between mid-ventral sebaceous gland odor from estrous and non-estrous females, (b) between the odors of a male and a female in non-estrus, or (c) between the odors of a male and a female in vaginal proestrus (behavioral estrus).

Methods and Materials

Animals and Rearing Conditions The animals used in this study came from a laboratory colony of *S. teguina* at the Department of Zoology, University of Florida at Gainesville. The laboratory colony was started with 22 wild animals caught in 2003 from Monteverde, Costa Rica. In 2005, the colony was composed of 19 males and 22 females and in 2006 of 15 males and 19 females. Eight males and 16 females used in 2005 were also present in the 2006 colony. Ages of the females ranged between 2 and 23 mo (in 2005) and 11 and 35 mo (in 2006). The ages of males ranged between 4 and 21 mo (in 2005) and 11 and 32 mo (in 2006). The mice were kept in a climate-controlled environment with a constant temperature of 21±2°C and on a 12:12-hr light:dark cycle, with lights turning on at 8:00 EST. All animals were housed in glass terrariums (26×51×35 cm), with sterile bedding (Harlan Teklad lab grade "sani chips," Madison, WI, USA) lining the bottom, sterile moss for humidity, a running wheel for exercise, and a "log" for shelter (half of an 18 cm long PVC tube cut transversally). All animals were housed individually in their cages with the exception of breeding pairs (in 2005 and 2006) and same litter sibling pairs (only five pairs in 2005). Food (a mixture of dry cat chow, nuts, and seeds) and water

were provided *ad libitum*. Furthermore, because they are insectivorous, each mouse received mealworms three times a wk.

Test Subjects We used 17 males and 18 females as test subjects for experiments 1 and 2, and 15 males for experiment 3. All test subjects were sexually mature [sexual maturity in females is between 28 and 39 d; in males puberty is reached at approximately 6–8 wk (Hooper and Carleton 1976)]. Nine (experiments 1 and 2) and six (experiment 3) test subjects were sexually experienced, and the rest were nonsexually experienced. Sexually experienced individuals were those that had previously been breeding animals and had shared a cage with an individual of the opposite sex. Nonsexually experienced individuals were virgin animals that had never been in a cage with another opposite sex individual. Each test subject was used only once in each experimental session, and there was a 2- to 4-d interval between experiments. The order of experimental sessions was random for each test subject.

Odor Collection In all experiments, we collected the odor stimulus immediately before testing. We collected the odor stimulus by gently rubbing a cotton swab (Q tip) against the mid-ventral sebaceous gland for 20 sec (10 sec for each cotton tip). We then placed the swab inside a Petri dish. The Petri dish was constructed of two separate plastic dishes fitted together with a screw in the middle. The plastic dishes had 3 mm perforations scattered over the upper surface, allowing odors to be detected from outside the dish. In experiment 1, the odorless stimulus consisted of a cotton swab with distilled water inside the Petri dish. We prevented odor contamination by changing surgical gloves and rinsing hands with 70% ethanol after manipulating each mouse. All equipment was washed between trials with hot water and soap, and then rinsed with 70% ethanol and allowed to dry to prevent contamination or residual odors.

As a consequence of a relatively small number of animals in the colony, our choices for test subjects and odor donor animals were constrained. Animals used as test subjects were also used as odor donors in different trials. However, we used the odor of each animal only once in each experiment, and there was an interval of 2 d before any odor donor was used as a test subject. The odor of a test subject was never used as an odor stimulus during its own test. We selected the odor donors randomly, but made sure that the odor donor was not closely related and was unfamiliar to the test subject. Thus, pairs of animals sharing a cage (e.g., breeding pairs or same litter siblings), familiar with each other, were never paired as test subject-odor donor in a given trial. In this study, we did not test the response to odors from familiar and unfamiliar conspecifics. In addition, subjects and donors varied in age and sexual experience because of the small number of available animals.

Assessment of Female Reproductive Condition Fernández-V (2006) described the estrous cycle in *S. teguina* and developed the protocol for the vaginal cytological assay used in these experiments. In brief, *S. teguina* shows an estrous cycle similar to the one described for laboratory rats and house mice. The cycle lasts about 4 to 5 d and consists of proestrus (PRO), estrus (ES), metestrus (ME), and diestrus (DI) stages. Before the behavioral experiments and over a 20-d period, we determined the estrous cycle and its phases for each female by daily vaginal cytological assays. Although we assayed each female on the day of the test, the estrous stage was not only determined by the smear obtained on the test day, but also was confirmed by information from earlier cycles. Lastly, it is important to emphasize that in accordance with Nelson (2000), we classified proestrus as the vaginal phase that coincides with behavioral estrus. Thus, odor donors revealing a vaginal smear in proestrus were considered to be in behavioral estrus and physically prepared to mate.

We compared odors from non-estrous and proestrous females. We considered a female to be in a non-estrous condition when she presented in the vaginal smear a phase in transition of ES–ME, metestrus (ME), or transition ME–DI. We determined that a female was in proestrus when the vaginal smear indicated a transition DI–PRO phase or vaginal proestrus (behavioral estrus). During this phase, the vaginal smear contained large numbers of nucleated epithelial cells outnumbering and completely dominating the number of cornified epithelial cells.

Behavioral Assays At the beginning of each experimental trial, we moved the subject animal to the experimental room (away from the colony room) and allowed it to acclimate for 20 min. During experimentation, room lights were off, and a red light was turned on. Experiments 1, 2, and 3 shared the design of the preference test. In all tests, the experimental arena was a rectangular glass terrarium (26×51×35 cm) with the bottom covered with 1.5 cm of clean bedding.

Each trial started with a 4-min acclimation period of the test subject inside a vertical PVC tube set in the middle of the arena (terrarium) half-way between two different odor stimuli placed at each end of the terrarium. For each trial, we randomly selected the left or right position for each odor stimulus in the arena. After the acclimation period, we removed the tube and recorded the amount of time the test subject spent sniffing and investigating the odor stimulus (Petri dish) during a 5-min test. We defined "sniffing and investigating" whenever the test subject had its nose directly over the odor stimulus (Petri dish) and was drawing

in air (nostrils wiggling). Sniffing and investigating was our measure of discrimination in experiments 1, 2, and 3. In addition, only in experiment 3, we also recorded the time each male spent in proximity to, but not sniffing, the odor stimulus. We defined proximity as the time spent sitting near (within 2 cm of the Petri dish) or on top of the Petri dish containing an odor stimulus. We used these data to calculate a second measure of discrimination: "total proximity" (calculated as time spent sniffing and investigating the Petri dish plus time spent sitting in proximity). Because of inconclusive results obtained in session 2.2, we recorded time in proximity only in experiment 3 to obtain additional behavioral data that could be potentially useful for the interpretation of male odor responses.

The rationale behind this experimental design was that if one odor is detected as different from the other odor presented during the test, then the test subject is likely to spend more time investigating one odor compared to the other. A significant difference in time spent investigating demonstrates odor discrimination and possible preference. A nonsignificant difference demonstrates that there is no preference, but cannot be taken as evidence that the subject cannot detect a difference between the odors. It is possible that an animal that spent an equal amount of time with each stimulus was able to detect the difference between stimuli, but simply was not motivated to spend more time with one or the other (Rothblat and Kromer 1991; Englemann et al. 1995; Tang-Martinez 2001).

Experiment 1: Discrimination of Heterosexual Conspecific Odor This experiment attempted to establish whether *S. teguina* can distinguish between an odorless stimulus and an odor stimulus collected from the mid-ventral sebaceous gland of an opposite sex conspecific. We divided this experiment into two sessions. Session 1.1 consisted of a female as the test subject (N=11), distilled water as an odorless source, and odor of a male collected from the mid-ventral sebaceous gland as the conspecific odor stimulus. Session 1.2 consisted of a male as the test subject (N=10), distilled water as an odorless source, and odor of a female collected from the mid-ventral sebaceous gland as the conspecific odor stimulus. We predicted that if males or females distinguish an odor from an opposite sex individual, they should spend more time sniffing and investigating the odor sample than the water sample.

Experiment 2: Discrimination between Male and Female Odor The aim of this experiment was to determine whether females and males distinguish mid-ventral sebaceous gland odors collected from the same vs opposite sex conspecifics. We divided experiment 2 into two sessions. In session 2.1, a female was the test subject (N=9), whereas in session 2.2 a male was the test subject (N=11). In both sessions, we presented the test subject with two odor stimuli from the mid-ventral sebaceous gland, one collected from a male and the other from a female. In this experiment, we selected female odor donors at random, and we did not control for the stage of the estrous cycle of the females. A difference in time spent sniffing and investigating one odor stimulus vs the other will demonstrate the ability of the experimental subject to discriminate male vs female odors and possibly also show a preference for a particular sex odor.

Experiment 3.1: Male Discrimination of Female Reproductive Condition The goal of this experiment was to determine whether males discriminate between mid-ventral gland odors of a non-estrous female vs a proestrous female. The experiment consisted of exposing a male test subject to the two female odors collected from the mid-ventral sebaceous glandular area. Our prediction was that the male would prefer the odor of a proestrous female to that of a non-estrous one.

Experiment 3.2: Male Discrimination of Male Odor vs Non-estrous Female Odor The aim of experiment 3.2 was to determine whether males spent more time with, and in proximity to, male vs female mid-ventral gland odors, when the female is in a non-estrous stage of the cycle. We presented the male subject with two odor stimuli, one taken from a male and the other taken from a female in non-estrus. Given the results obtained in experiment 2.2., we predicted that the male will prefer male over female odor, if the female odor donor was in a non-estrous phase.

Experiment 3.3: Male Discrimination of Male Odor vs Vaginal Proestrus Female Odor The objective of this experiment was to determine whether males investigated and spent more time with, and in proximity to, an odor stimulus collected from a male or from a female in vaginal proestrus (behavioral estrus). Thus, the design was exactly the same as that of Experiment 3.2, except that the female odor stimulus was collected from a female in proestrus. Our prediction was that males would prefer this female odor compared to the male odor.

Data Analyses Paired-sample tests were used to examine significant differences in time spent sniffing and investigating and time in total proximity to the two stimuli. In all comparisons, the critical level of α was set at 0.05 (significant) or 0.001 (highly significant). In experiment 1, a one-tailed test was employed because the obvious prediction is that the test subject will be more attracted to the odor of a conspecific than to an odorless stimulus. In experiment 2, we used a two-tailed test because it was difficult to make unequivocal, clear-cut predictions. In experiment 3.1, we used a one-tailed test because the *a*

priori prediction is that the test subject will be more attracted to the odor of a proestrous female over a non-estrous female. In experiments 3.2 and 3.3, we used two-tailed tests because unequivocal predictions were not possible. Time spent sniffing and investigating and time in total proximity to each odor stimulus are presented as mean seconds (sec)±standard error (SE). In all cases, we assessed normality of the data by using the Shapiro and Wilk method (JMP, SAS Institute Inc. 1989–1997). To determine differences in the response to each odor stimulus, we used a paired *t*-test when data were normally distributed and Wilcoxon matched-pairs signed-ranks test (*W*) when data were not normally distributed (JMP, SAS Institute Inc. 1989–1997 and InStat, GraphPad Software Inc. 1998). We used three-factor analysis of variance (ANOVA) to determine whether potentially confounding variables such as sexual experience or age had an effect on the results obtained in experiments 2.2 and 3.3. The factors analyzed were odor stimuli (male and female odor) × sexual experience (sexual and nonsexual) × age (young <18 mo and old ≥18 mo; JMP, SAS Institute Inc. 1989–1997).

Results

Experiment 1 Discrimination of Heterosexual Conspecific Odor In experiment 1, both females and males spent significantly more time investigating the conspecific odor when compared with the odorless stimulus. Specifically, female *S. teguina* spent more time investigating the odor stimulus obtained from the sebaceous gland of a male than the odorless source (distilled water; t=4.23, df=10, P< 0.001; Fig. 1). Males also spent more time investigating the odor stimulus from a female than the odorless source (t= 2.17, df=9, P=0.028; Fig. 1).

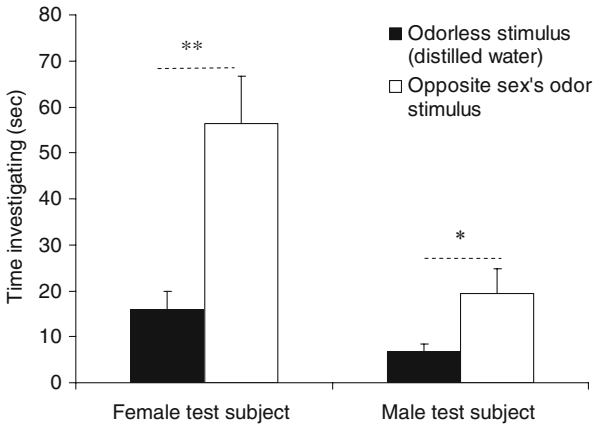

Fig. 1 Time (sec; mean±SE) spent by *S. teguina* test subjects sniffing and investigating an odorless stimulus (distilled water) vs an odor stimulus collected from an opposite sex individual. Female test subjects in session 1.1 (N=11) and male test subjects in session 1.2 (N=10). Significant differences between odor pairs, *P<0.05, **P< 0.001

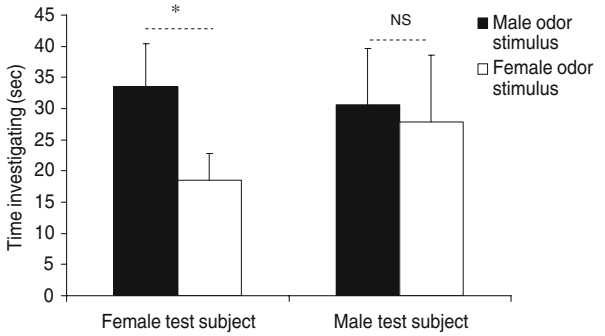

Fig. 2 Time (sec; mean±SE) spent by *S. teguina* test subjects sniffing and investigating the odor stimulus of a female vs the odor stimulus of a male. Female test subjects in session 2.1 (N=9) and male test subjects in session 2.2 (N=11). Significant differences between odor pairs, *P< 0.05. Nonsignificant difference (NS) between odor pairs, P>0.05

Experiment 2 Discrimination between Male and Female Odor The results for sexual discrimination by odors in experiment 2 demonstrated a different pattern in females when compared with males. Females sniffed or investigated male odors more than the same sex odors (t=3.26, df=8, P= 0.011; Fig. 2). However, males spent relatively the same amount of time investigating the gland odor of females and males (W=−7, P=0.765; Fig. 2). This unexpected result was not related to the sexual experience or age of the male subjects. A three-factor ANOVA performed on log-transformed data demonstrated that there were no significant effects of odor stimuli, sexual experience, or age on the time *S. teguina* spent sniffing and investigating the odor sources ($F_{1,16}$<2.43, P>0.139). Interactions between factor pairs were also not significant. The outcome in experiment 2.2 may have resulted from the fact that the reproductive state of female odor donors was uncontrolled; this led us to design experiment 3.

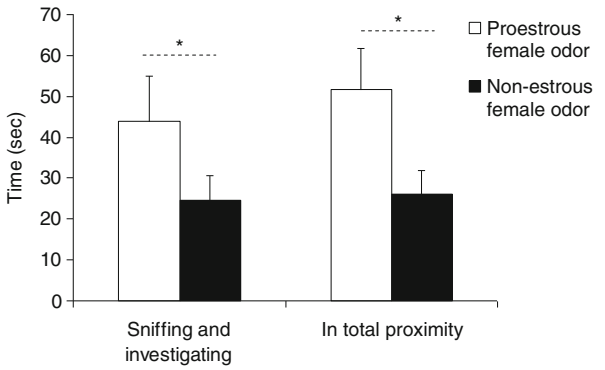

Fig. 3 Time (sec; mean±SE) spent by *S. teguina* male test subjects (N=10) sniffing and investigating, and in total proximity to the odor of a proestrous female vs the odor of a non-estrous female. Significant differences between odor pairs, *P<0.05

Experiment 3.1 Male discrimination of Female Reproductive Condition In experiment 3.1, *S. teguina* males spent more time sniffing and investigating the odor stimulus from a female in proestrus (behavioral estrus) than from a female in non-estrus (W=39.0, P=0.024; Fig. 3). We found the same odor preference pattern for time in total proximity (time spent investigating plus time in proximity to the Petri dish). Specifically, males spent more time in total proximity to the proestrous female odor stimulus compared to the non-estrous female odor stimulus (W=45.0, P=0.01; Fig. 3).

Experiment 3.2 Male Discrimination of Male odor vs Non-estrous Female Odor In experiment 3.2, the time spent by the male sniffing and investigating the male odor was different from the time spent sniffing and investigating the non-estrous female odor (W=−45.0, P=0.004) (Fig. 4). When one considers the time the male subject spent in total proximity, males spent more time in total proximity to the male odor compared to the non-estrous female odor (t=2.88, df=9, P=0.018; Fig. 4).

Experiment 3.3 Male Discrimination of Male Odor vs Vaginal Proestrous Female Odor Contrary to our prediction, results obtained in experiment 3.3 showed no male preference for any odor stimulus. Neither male odor nor proestrous female odor stimulated more sniffing or investigation (t=0.55, df=11, P=0.592; Fig. 5). The difference in the time the male subjects spent in total proximity to the two stimuli was also not significant (t=1.28, df=11, P=0.227; Fig. 5). Finally, there were no significant effects of odor stimuli, sexual experience, or age on the time male *S. teguina* spent sniffing and investigating the odor sources (three-factor ANOVA; $F_{1,18}$<2.73, P>0.116). Interactions between factor pairs were not significant either.

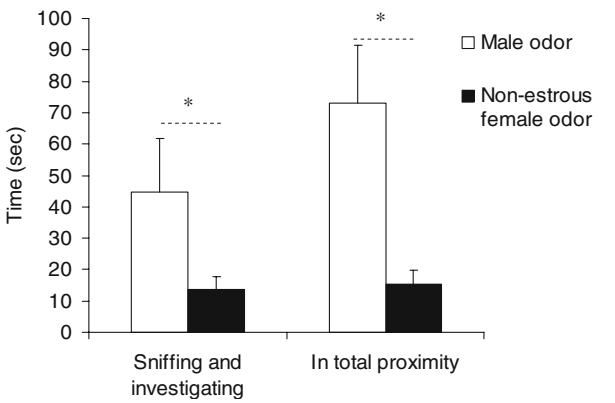

Fig. 4 Time (sec; mean±SE) spent by *S. teguina* male test subjects (N=10) sniffing and investigating, and in total proximity to the odor of a male vs the odor of a non-estrous female. Significant differences between odor pairs, *P<0.05

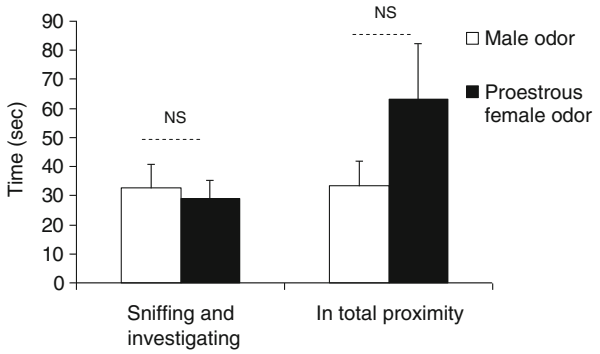

Fig. 5 Time (sec; mean±SE) spent by *S. teguina* male test subjects (N=12) sniffing and investigating, and in total proximity to the odor of a male vs. the odor of a proestrous female. Nonsignificant difference (NS) between odor pairs, P>0.05

Discussion

We found that there is chemical communication in *S. teguina* mediated by the odor produced by the mid-ventral sebaceous gland. Both male and female *S. teguina* distinguished between opposite sex conspecific odor and distilled water. In rodents, heterosexual conspecific identification mediated by olfactory cues is important during social encounters. During dyadic interactions, *S. teguina*, as is the case in many species of rodents (e.g., Gavish et al. 1983; Johnston 1985), engage in mutual nasal, oral, and anal sniffing, followed by genital and mid-ventral gland investigation (Hooper and Carleton 1976; personal observation). These typical behaviors may provide information about individual differences (Halpin 1980, 1986; Johnston and Jernigan 1994; Mateo 2006), which may be essential in identifying mates and enhancing mating success (Newman and Halpin 1988; Tang-Martínez et al. 1993). Individual identification through olfactory cues in *S. teguina* remains to be demonstrated in future studies.

Generally, sexual discrimination through odor cues is indicated by preference for the odor from those individuals that are of the opposite sex (Johnston 1983; Brown 1985; Ferkin and Johnston 1995). Female *S. teguina* showed a significant preference for male sebaceous gland odors compared to female odors. Therefore, *S. teguina* females can discriminate gender through mid-ventral gland odors. Female preference for male odor has also been demonstrated in other species of rodents (e.g., in Sawrey and Dewsbury 1994; Ferkin and Johnston 1995; Petrulis et al. 1999; Solomon et al. 1999; Zenuto et al. 2004). In montane voles (*Microtus montanus*), the female attraction to male bedding is related to the species' reproductive physiology because exposure to scent from territorial males promotes ovulation in the female (Sawrey and Dewsbury 1994). However, in *S. teguina*, male secretions from the mid-ventral sebaceous gland do not induce ovulation in females likely because *S. teguina* is a spontaneous ovulator

(Fernández-V 2006). Possibly, *S. teguina* male odor is a sexual attractant to females. Such a function would explain the strong preference for male sebaceous gland odors demonstrated by female *S. teguina.*

Male *S. teguina* were able to distinguish a female's reproductive state from chemical cues produced by the female's mid-ventral sebaceous gland. Specifically, males spent significantly more time sniffing/investigating and in total proximity to odors of estrous females than non-estrous females (Fig. 3). Therefore, the mid-ventral sebaceous gland seems to provide information to males about female sexual receptivity. Based on studies of other species (Johnston 1983; Bakker et al. 1996; Portillo and Paredes 2004), these results are not surprising because for males, the ability to distinguish proestrous from non-estrous females and their preference for proestrous odors are likely to be highly adaptive and may enhance a male's reproductive success. Likewise, advertisement of estrous condition by females is likely to attract males, thereby also increasing female reproductive success.

The lack of preference in male *S. teguina* for female odors, when compared with male odors (experiment 2.2) suggested that males, in contrast to females, may not be able to discriminate gender through mid-ventral gland odors. However in experiment 3.2, *S. teguina* males preferred to spend significantly more time sniffing and investigating and in total proximity to the odor samples collected from male glands compared to those collected from non-estrous female glands (Fig. 4). Thus, it appears that if a female is nonreproductive, males might be more interested in investigating another male's odor. These results suggest that males can distinguish gender identity through ventral gland odors, but they are more attracted to male than non-estrous female odors. Finally, given the choice between male odor and proestrous female odor, males again as in experiment 2.2, showed no preference for female vs male stimuli (Fig. 5).

It is intriguing that when males were asked to choose between male odors and female odors (either non-estrous or proestrous), the male subjects either showed no preference or preferred the male odors. These results raise the question of why male odor is so interesting to other males. There are two hypotheses that could explain these results.

First, it is possible that *S. teguina* males were unable to distinguish male odors from proestrous female odors because of hormonal abnormalities in the males. It has been shown that manipulation of circulating levels of testosterone and differences in sexual motivation, affect odor preferences in males (Bakker et al. 1996; Portillo and Paredes 2004; Xiao et al. 2004). In our study, we did not test the hormonal levels of each male subject, nor, for that matter, of male odor donors. However, these animals had never experienced any treatment related to hormonal studies such as hormone injection or castration. Unless rearing *S. teguina* in captivity has an effect on hormonal levels and behavior in males, as has been reported in a few other rodent species (Gottreich et al. 2000; Künzl et al. 2003), it is likely that the males in our study were hormonally and motivationally normal. Consequently, we do not consider that this is a plausible explanation for the pattern observed.

The second and most likely explanation of the observed pattern is that *S. teguina* males did not discriminate behaviorally between odors from males and from proestrous females because *the chemical signal produced by the gland in males is important in male–male communication*. The results from experiment 3.1 demonstrated that the female mid-ventral gland provided chemical cues that allowed males to discriminate female reproductive condition. However, if those chemical signals are also important for male–male communication (possibly in agonistic contexts), it might be expected that males would show more interest in these male odor cues than in those of non-estrous females. Consequently the odors of females in proestrus and the odors of males may be equally important for males.

In Nearctic cricetines (e.g., *Neotoma, Peromyscus, Rattus*), mid-ventral glands commonly are more developed in males than in females (Quay and Tomich 1963), and male odor is believed to contain more compounds than female odor (Blaustein 1981; Brown 1985). In female *S. teguina*, the gland is smaller, less visible, and lacks the heavy staining of the pelage observed in males (Hooper and Carleton 1976). Possibly, the secretion is also less copious and the odor less strong or chemically complex than in males. Sexual differences in the secretion and size of the mid-ventral gland may be related to sexual selection, through either female choice or male–male competition. This could explain the sexual dimorphism of the mid-ventral sebaceous gland and the different sexual responses to odors in *S. teguina*.

During male–male competition, dominant male rodents might produce different cues, or different amounts of the same cue, when compared with subordinate male rodents, and females could choose on the basis of the signal emitted (Johnston 1983; Brown 1985; White et al. 1986). Dominant males of many species have a larger scent gland than subordinates, and their odor may potentially indicate their dominance (Brown 1985). Although the social system in *S. teguina* is not well known, aggression and dominance interactions in male adults after male–male encounters have been reported for both *S. teguina* and *S. xerampelinus* (Hooper and Carleton 1976). We also observed agonistic interactions between *S. teguina* males during experiments with dyadic interactions (unpublished data).

Finally, males in territorial species such as montane voles, *Microtus montanus*, and tuco-tucos, *Ctenomys talarum*,

also have failed to show a preference for female odor when compared with male odor (Sawrey and Dewsbury 1994; Zenuto et al. 2004). Presumably, in these species, males are interested in male odor for attainment and maintenance of a breeding territory and in female odor for reproduction (Sawrey and Dewsbury 1994; Zenuto et al. 2004). Likewise in *S. teguina*, male and proestrous female odors seem to be equally interesting. However, it is not known whether *S. teguina* actively defends a territory. In contrast to both territorial montane voles and tuco-tucos (Jannett 1981; Zenuto et al. 2004), *S. teguina* does not deposit scent on substrates by using their mid-ventral gland. During experiments designed to stimulate scent marking in *S. teguina*, we did not observe scent deposition on the substrate with their mid-ventral gland (Fernández-V 2006). In contrast, the only circumstances in which we have observed *S. teguina* individuals use the mid-ventral sebaceous gland have been during self-grooming sessions (spreading the secretion over their own fur) or during allo-grooming bouts (spreading the secretion over a conspecific's fur; unpublished data). These behaviors may suggest that in *S. teguina*, the gland is used to mark individuals (e.g., females) rather than a territory. Such a lack of territoriality would be in agreement with the absence of male intrasexual exclusive space use found in *S. xerampelinus* (Blondel 2006).

In summary, this study demonstrates that chemical communication is important in *S. teguina* and that the mid-ventral sebaceous gland mediates discrimination of heterosexual conspecifics, sex, and female reproductive condition. However, the social organization and mating system in *S. teguina* need to be studied further in order to fully understand how the communication system evolved and how it currently works.

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References

BAKKER, J., OPHEMERT, J. V., and SLOB, A. K. 1996. Sexual differentiation of odor and partner preference in the rat. *Physiol. Behav.* 60:489–494.

BLAUSTEIN, A. R. 1981. Sexual selection and mammalian olfaction. *Am Nat* 117:1006–1010.

BLONDEL, D. V. 2006. Social organization of a species of Singing Mouse, Scotinomys xerampelinus. MSc Thesis. University of Florida.

BROWN, R. E. 1985. The rodents II: suborder Myomorpha, Social Odours in Mammals. pp. 345–457, in R. E. Brown, and D. W. MacDonald (eds.). Clarendon Press, Oxford, UK.

BROWN, R. E., and MACDONALD, D. W. 1985. Social Odours in Mammals. Clarendon Press, Oxford, UK.

DRICKAMER, L. C., and BROWN, P. L. 1998. Age-related changes in odor preferences by house mice living in seminatural enclosures. *J. Chem. Ecol* 24:1745–1755.

DUVALL, D., MÜLLER-SCHWARZE, D., and SILVERSTEIN, R. M. 1986. Chemical Signals in Vertebrates. Plenum Press, New York, NY.

ENGLEMANN, M., WOTJAK, C. T., and LANDGRAF, R. 1995. Social discrimination procedure: an alternative method to investigate juvenile recognition abilities in rats. *Physiol. Behav* 58:315–321.

FERKIN, M. H. 1999. Attractiveness of opposite-sex odor and responses to it vary with age and sex in meadow voles (*Microtus pennsylvanicus*). *J. Chem. Ecol* 25:757–769.

FERKIN, M. H., and JOHNSTON, R. E. 1995. Meadow voles, *Microtus pennsylvanicus*, use multiple sources of scent for sex recognition. *Anim. Behav* 49:37–44.

FERNÁNDEZ-V. 2006. Olfactory responses of Neotropical singing mice (*Scotinomys teguina*) to the odors of the mid-ventral sebaceous gland. MSc. Thesis. University of Missouri-St. Louis.

FLOOD, P. 1985. Sources of significant smells: the skin and other organs, Social Odours in Mammals. pp. 19–36, in R. E. Brown, and D. W. MacDonald (eds.). Clarendon Press, Oxford, UK.

GAVISH, L., CARTER, C. S., and GETZ, L. L. 1983. Male-female interactions in prairie voles. *Anim. Behav* 31:511–517.

GOTTREICH, A., ZURI, I., BAREL, S., HAMMER, I., and TERKEL, J. 2000. Urinary levels in the male blind mole rat (*Spalax ehrenbergi*) affect female preference. *Physiol. Behav* 69:309–315.

HALPIN, Z. T. 1980. Individual odors and individual recognition: Review and commentary. *Biol. Behav* 5:233–243.

HALPIN, Z. T. 1986. Individual odors among mammals: Origins and functions. *Adv. Study Behav* 16:39–70.

HOOPER, E. T., and CARLETON, M. 1976. Reproduction, growth and development in two contiguously allopatric rodent species, genus *Scotinomys*. *Misc. Public. Mus. Zool., Univ. Michigan* 151:1–52.

HURST, J. L. 1990a. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). I. Communication between males. *Anim. Behav.* 40:209–222.

HURST, J. L. 1990b. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). II. Communication between females. *Anim. Behav.* 40:223–232.

HURST, J. L. 1990c. Urine marking in populations of wild house mice (*Mus domesticus* Rutty). III. Communication between the sexes. *Anim. Behav.* 40:233–243.

HURST, J. L., HAYDEN, L., KINGSTON, M., LUCK, R., and SORENSEN, K. 1994. Response of the aboriginal house mouse, *Mus spretus* Lataste, to tunnels odoured by conspecifics. *Anim. Behav.* 48:1219–1229.

InStat, GraphPad Software Inc. 1998. Version 3.0. San Diego, CA, USA. http://www.graphpad.com.

JANNETT, F. J. Jr. 1981. Scent mediation of intraspecific, interspecific, and intergeneric agonistic behavior among sympatric species of voles (Microtinae). *Behav. Ecol. Sociobiol* 8:293–296.

JMP, SAS Institute Inc. 1989–1997. Version 3.2.1. Cary, NC, USA. http://www.jmp.com.

JOHNSTON, R. E. 1983. Chemical signals and reproductive behavior, Pheromones and Reproduction in Mammals. pp. 3–33, in J. G. Vandenberg (ed.). Academic Press, New York, NY.

JOHNSTON, R. E. 1985. Communication, The Hamster. Reproduction and Behavior. pp. 121–154, in H. I. Siegel (ed.). The Plenum Press, New York, NY.

JOHNSTON, R. E. 2003. Chemical communication in rodents: From pheromones to individual recognition. *J. Mammal.* 84:1141–1162.

JOHNSTON, R. E., and JERNIGAN, P. 1994. Golden hamsters recognize individuals, not just individual scents. *Anim. Behav* 48:129–136.

KAVALIERS, M., and COLWELL, D. D. 1995. Odors of parasitized males induced aversive responses in female mice. *Anim. Behav* 50:1161–1169.

KAVALIERS, M., CHOLERIS, E., AGMO, A., and PFAFF, D. W. 2004. Olfactory-mediated parasite recognition and avoidance: linking genes to behavior. *Horm. Behav.* 46:272–283.

KLEIMAN, D. G. 1977. Monogamy in mammals. *Quart. Rev. Biol* 52:36–69.

KLEIMAN, D. G. and EISENBERG, J. F. 1983. Part III Mechanisms of communication, pp. 147–149, in J. F. Eisenberg and D. G. Kleiman (eds.). Advances in the Study of Mammalian Behavior. *Am. Soc. Mammal. Spec. Public.* 7:1–751.

KOYAMA, S. 2004. Primer effects by conspecific odors in house mice: a new perspective in the study of primer effects on reproductive activities. *Horm. Behav* 46:303–310.

KÜNZL, C., KAISER, S., MEIER, E., and SACHSER, N. 2003. Is a wild mammal kept and reared in captivity still a wild animal? *Horm. Behav* 43:187–196.

MATEO, J. M. 2003. Kin recognition ground squirrels and other rodents. *J. Mammal.* 84:1163–1181.

MATEO, J. M. 2006. The nature and representation of individual recognition cues in Belding's ground squirrels. *Anim. Behav.* 71:141–154.

MÜLLER-SCHWARZE, D. 1983. Scent glands in mammals and their functions, pp. 150–185, in J. F. Eisenberg and D. G. Kleiman (eds.). Advances in the Study of Mammalian Behavior. *Am. Soc. Mammal. Spec. Public*. 7:1–751.

NELSON, R. J. 2000. An Introduction to Behavioral Endocrinology. Sinauer Associates, Sunderland, MA.

NEWMAN, K. S., and HALPIN, Z. T. 1988. Individual odors and mate recognition in the prairie voles, *Microtus ochrogaster. Anim. Behav* 36:1779–1787.

PETRULIS, A., PENG, M., and JOHNSTON, R. E. 1999. Effects of vomeronasal organ removal on individual odor discrimination, sex-odor preference, and scent marking by female hamsters. *Physiol. Behav* 66:73–83.

PORTILLO, W., and PAREDES, R. G. 2004. Sexual incentive motivation, olfactory preference and activation of the vomeronasal projection pathway by sexually relevant cues in non-copulating and naïve males. *Horm. Behav* 46:330–340.

QUAY, W. B., and TOMICH, P. Q. 1963. A specialized midventral sebaceous glandular area in *Rattus exulans*. *J. Mammal* 44:537–542.

ROTHBLAT, L. A., and KROMER, L. F. 1991. Object recognition memory in the rat: the role of the hippocampus. *Behav. Brain Res* 42:25–32.

SAWREY, D. K., and DEWSBURY, D. A. 1994. Conspecific odor preferences in montane voles (*Microtus montanus*): Effects of sexual experience. *Physiol. Behav* 56:339–344.

SOLOMON, N. G., and RUMBAUGH, T. 1997. Odor preferences of weanling and mature male and female pine voles. *J. Chem. Ecol* 23:2133–2142.

SOLOMON, N. G., FERKIN, M. H., and O'BOYLE, R. 1999. Do pine voles (*Microtus pinetorum*) use numerous types of olfactory cues to discriminate gender? *J. Chem. Ecol* 25:2147–2159.

STODDART, D. M. 1986. The comparative responses of mice and voles to conspecific and heterospecific odors in the field - A lesson in social behavior, Chemical Signals in Vertebrates. pp. 533–540, in D. Duvall, D. Müller-Schwarze, and R. M. Silverstein (eds.). Plenum Press, New York, NY.

TANG-MARTINEZ, Z. 2001. The mechanisms of kin discrimination and the evolution of kin recognition in vertebrates: a critical re-evaluation. *Behav. Process* 53:21–40.

TANG-MARTÍNEZ, Z., MUELLER, L. L., and TAYLOR, G. T. 1993. Individual odors and mating success in the golden hamster, *Mesocricetus auratus*. *Anim. Behav* 45:1141–1151.

TAYLOR, G. T., EGAN, D., and HALLER, J. 1983. Sexual experience, androgens and female choice of a mate in laboratory rats. *J. Endocrinol* 96:43–52.

WHITE, P. J., FISHER, R. B., and MEUNIER, G. F. 1986. Female discrimination of male dominance by urine odor cues in hamsters. *Physiol. Behav* 37:273–277.

XIAO, K., KONDO, Y., and SAKUMA, Y. 2004. Sex-specific effects of gonadal steroids on conspecific odor preference in rat. *Horm. Behav* 46:356–361.

ZALA, S. M., POTTS, W. K., and PENN, D. J. 2004. Scent-marking displays provide honest signals of health and infection. *Behav. Ecol* 15:338–344.

ZENUTO, R. R., FANJUL, M. S., and BUSCH, C. 2004. Use of chemical communication by the subterranean rodent *Ctenomys talarum* (Tuco-tuco) during the breeding season. *J. Chem. Ecol* 30:2111–2126.

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A Sterol-Like Odorant in the Urine of Mozambique Tilapia Males Likely Signals Social Dominance to Females

Eduardo N. Barata · Jared M. Fine · Peter C. Hubbard · Olinda G. Almeida · Pedro Frade · Peter W. Sorensen · Adelino V. M. Canário

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Abstract Many species of freshwater fish with relatively simple mating strategies release hormonally derived sex pheromones in urine. However, it is not known whether species with more complex reproductive strategies use specialized urinary chemical signals. We addressed this by using the Mozambique tilapia (*Oreochromis mossambicus* Peters 1852), a lek-breeding species in which males establish dominance hierarchies and visiting females mate preferentially with territorial/dominant males. We measured urination frequency of territorial males in social isolation and in the presence of females that were either ready to spawn or had finished spawning. In groups of fish, we monitored the volume of urine stored in subordinate and dominant males to determine if urine volume and olfactory potency (by recording electro-olfactograms, EOG, in females) are related to the male's social rank. Dominant, territorial males stored more urine than subordinates and released it in short pulses, the frequency of which increased in the presence of females ready to spawn but not in the presence of post-spawn females. Urine from subordinate and dominant males was fractionated by liquid chromatography and fractions tested for olfactory potency by using the EOG, with the most potent fraction analyzed by mass spectrometry (MS). The olfactory system of females was sensitive to a urinary compound that was more abundant in the urine of dominant males than in that of subordinates. MS analysis suggested the compound is a sulfated amino-sterol-like compound with a formula of $C_{29}H_{40}N_2O_{10}S$. Therefore, we suggest that dominant/territorial tilapia males dramatically increase urination frequency in the presence of females ready to spawn and that the urinary odorant acts as a pheromonal signal of dominance, thereby influencing female spawning.

Keywords Behavior · Electro-olfactogram · Fish · HPLC · LC–MS · Mass spectrometry · Reproduction · Olfaction · Sex pheromones · Social dominance

E. N. Barata (✉) · P. C. Hubbard · O. G. Almeida · P. Frade · A. V. M. Canário
CCMAR–CIMAR Laboratório Associado,
Universidade do Algarve,
Campus de Gambelas,
8005-139 Faro, Portugal
e-mail: ebarata@ualg.pt

E. N. Barata
Departamento de Biologia, Universidade de Évora,
Apartado 94,
7002-554 Évora, Portugal

J. M. Fine · P. W. Sorensen
Department of Fisheries, Wildlife and Conservation Biology,
University of Minnesota,
St. Paul, MN 55108, USA

Present address:
J. M. Fine
Alzheimers Research Center, Regions Hospital,
St. Paul, MN 55101, USA

Introduction

Although sex pheromones are known to mediate reproduction in fish, detailed information on chemical identities, physiological origin, control of release, and function is available only from a small number of species with relatively simple reproductive strategies (Stacey and Sorensen 2006). Among these, the goldfish (*Carassius auratus*) is the best studied. It has a scramble-competition polygynous mating system, representative of many other cyprinids; females

release steroids and prostaglandins via the gills and/or the urine, which act as hormonal pheromones that affect the reproductive physiology and behavior of males. Sexually receptive females control the release of urinary prostaglandin pheromones while exhibiting simple behaviors. This is thought to represent a specialization of pheromone release related to marking spawning habitat, so as to lure males (Appelt and Sorensen 2007). Cichlids, in contrast, employ a range of relatively complex mating systems (Barlow 1991). Typical among the mouth-brooding cichlids is polygynandry, wherein males defend a spawning territory that is visited by females and where males display conspicuous courtship behaviors (Barlow 1991; Nelissen 1991). The possibility of chemical communication in these reproductive interactions, however, has been little studied in this group.

The Mozambique tilapia (*Oreochromis mossambicus* Peters 1852) is a maternal mouth-brooding cichlid that spawns repeatedly throughout the year, with the females having a regular ovulatory cycle of 15–20 days (Coward and Bromage 2000). Males aggregate in breeding arenas or leks, where territorial males adopt a characteristic black coloration and defend small territories centered on the nests (pits) that they dig in the sand (Bruton and Boltt 1975). Females visit the leks when ready to spawn but then collect and brood the fertilized eggs and subsequent fry away from the leks (Fryer and Iles 1972). In captivity, male tilapia form a stable linear hierarchy (Oliveira and Almada 1996, 1998b), with the largest alpha males receiving the majority of spawning females (Oliveira and Almada 1996). Previous studies have found that the olfactory system of females is highly sensitive to substances released to the water by territorial males and to male body fluids, with urine being of special importance (Frade et al. 2002). This suggests the possibility of male urinary pheromones being involved in tilapia reproduction and, if males control their release, for chemical communication in this species.

In the present study, we tested whether male tilapia send chemical signals to females and whether this ability depends on male social status. Three experiments were conducted. First, we assessed urination frequency of males, with and without pre-ovulatory (ready to spawn) and post-ovulatory (post-spawn) females, to determine whether they may use urinary pheromone(s) to communicate. Second, we formed groups of fish and monitored the volume of urine stored in subordinate and dominant males to determine if urine volume and urine olfactory potency on females are related to male social rank. Finally, to characterize the urinary odorants, samples of urine collected from subordinate and dominant males were fractionated by liquid chromatography, the resulting fractions were tested for olfactory potency on females, and the most potent fraction was analyzed by mass spectrometry.

Methods and Materials

Experimental Animals

Before the experiments, groups of fish (one male and three or four females) were housed for several weeks in re-circulating aquaria. The aquaria (93×60×55 cm) contained 200 l dechlorinated tap water at 27°C and sand substrate. The photoperiod was 12 L:12 D, and the fish were fed twice a day with commercial cichlid food (Nutrafin basix®; Rolf C. Hagen, Inc., Montreal, Canada). Spawning occurred in all fish groups, but the eggs were removed to stimulate the initiation of a new ovulatory cycle. Each female was observed over at least two ovulatory cycles before use in behavioral experiments to ensure the predictability of ovulation. "Pre-ovulatory" females were used in experiments on the day before or on the day predicted for their next ovulation; their ovulation cycle ranged from 9 to 16 days (mean ± SD; 15.0±3.1 days; N=7). "Post-ovulatory" females were used 1–3 days after their last ovulation.

Visualization of Urine and Quantifying Patterns of Urination Behavior

To determine whether males use urine to communicate during reproductive behavior, the urination frequency of a male was assessed in social isolation and while a female was present. In each replicate, a male was taken from its family tank and isolated in a glass tank (79×35×45 cm, 50 l, 27°C, aerated), with a sand substratum, for 24 h before the experiment. The male was lightly anesthetized by immersion in iced water for 2–3 min and injected in the dorsal musculature with 100 µl patent blue violet (100 mg ml^{-1} in 0.9% NaCl; Sigma-Aldrich), a dye that is released mainly through the urine and allows monitoring of the frequency of urine release (Appelt and Sorensen 1999, 2007; Barata et al. 2007). The fish was then placed back into its tank, and after the first urine pulse, urination was measured and fish behavior was recorded on video for 45 min (social isolation). Then, a pre- or post-ovulatory female (taken from a different family tank) was introduced to the tank and urine release and behavior recorded for another 45 min. Frequency and duration of each urine pulse was recorded by the observer with a hand-held computer (Psion Organizer LZ64) programmed for timing the start and end of each urine pulse. The procedure was replicated with seven males (mean ± SD, standard length = 156.3±18.6 mm, and body weight = 119.5±18.6 g) and seven pre-ovulatory females (length = 147.0±20.9 mm; weight = 100.1±41.8 g), and with another group of seven males (length = 155.0±26.2 mm; weight = 120.7±48.2 g) and seven post-ovulatory females (length = 146.1±18.5 mm; weight = 96.6±45.9 g). After each replicate, the males were returned to their

original family tanks, but the females were deeply anesthetized (500 mg l^{-1} 3-aminobenzoic acid ethyl ester, MS222, Sigma-Aldrich), and then a sharp blow to the head was applied to assure death before assessing their ovarian condition. The ovaries of each female were weighed and the gonadosomatic index (GSI = gonads mass/body mass × 100) calculated. A random sample of ten eggs was also weighed, and their diameters measured under a binocular microscope.

Male behavior was classified as not displaying (hovering or active swimming when the female was present), courting (including nest digging), or aggressive. Female behavior was classified as not displaying, receptive (following the male during its courtship displays), or submissive. These behaviors are described in detail by Baerends and Baerends van Roon (1950) and by Oliveira (1995). Total duration of all behaviors was quantified by using The Observer Video-Pro 4.0 software (Noldus Information Technology, Wageningen, The Netherlands).

Quantifying Urine Stored in Subordinate and Dominant Males

To determine if urine volume stored in the bladder is related to male social behavior and to obtain urine for testing olfactory potency and mass spectrometry analysis, males in social groups were monitored daily. Two groups (A and B) of 12 males and ten females (previously kept in family tanks as described above) were sequentially housed for 15 days in a plastic tank (128×110×50 cm) containing dechlorinated tap water (approximately 600 l) at 25–27°C; the photoperiod was 12 h, and feeding was once a day. The males were tagged with colored plastic labels (T-Bar extra small anchor—FF-94, Floy Tag Inc., Seattle, WA, USA) attached to the muscle near the dorsal fin and their behavior observed each morning, midday, and afternoon over 10 days (30 observations per male). Systematic behavioral observations started on the four day after formation of each fish group. The frequency of submissive (escape from an aggressive opponent, submission displays during agonistic interactions, or absence of dark coloration without social interaction) and dominant behaviors (aggressive displays, nest-digging, courtship displays towards females, or dark coloration without social interaction) was recorded over 5 min for each male. Although the tank had no sand, some males exhibited nest-digging behavior by nipping the bottom, as they normally do with a sand substratum. At the end of each afternoon's observations, each male was slightly anesthetized (50 mg l^{-1} MS222), and urine was taken by gently squeezing the area immediately above and anterior to the genital papilla. Squeezing of this abdominal area caused erection of the genital papilla, and a jet of urine was collected. Urine volume was measured by weighing, and the urine was stored at −20°C until use (see below). The fish were allowed to recover and placed back in the tank.

The standard length and weight of each fish were measured at the start of each replicate (mean ± SD; group A: male length = 137.8±5.6 mm, male weight = 81.6± 11.3 g, female length = 103.7±9.6 mm, female weight = 39.9±9.1 g; group B: male length = 144.4±6.3 mm, male weight = 86.4±10.0 g, female length = 111.2±10.4 mm, female weight = 46.6±10.2 g) and at the end to calculate their daily growth. The GSI of males of different social rank was compared between six dominant males (three from each group) and six subordinates (two from fish group A and four from group B) following the procedure described above for females. Samples of urine obtained from these males were assessed for olfactory potency on females, fractionated by liquid chromatography, and the resulting fractions assessed for olfactory potency and analyzed by mass spectrometry (see below). Samples of urine collected daily from all males in the two replicates were also assessed for olfactory potency to determine whether there is a relationship between males' social rank and the olfactory potency of their urine (see below).

Liquid Chromatography Coupled with Mass Spectrometry To determine which odorants are found in the urine of dominant and subordinate males, solid-phase extraction (SPE) of urine samples was carried out followed by fractionation and analysis by liquid chromatography–mass spectrometry (LC–MS). Urine samples from the same male were pooled for analysis. Urine (2 ml) was passed through a C18 cartridge (Sep-Pak®, Waters, Milford, MA, USA) and eluted with methanol (2 ml). Half of the eluent was kept at −20°C until used for assessment of olfactory potency on females by recording the electro-olfactogram (EOG; see below), while the other half was fractionated by LC–MS. Distilled water (2 ml) was subjected to the same procedure to generate a control eluent.

For LC–MS, the unconjugated bile acid chenodeoxycholic acid (CDCA; Steraloids Inc, Newport, RI, USA) was added (1 μg) to each of the sep-pack eluates (internal standard) to standardize retention times and injection volumes between samples of male urine. Eluates were dried under a stream of nitrogen, reconstituted in 110 μl methanol/water (60/40, *v*/*v*) and transferred to autosampler glass vials for LC–MS. The LC column (Nova-Pak reversed-phase C18, 4 μm, Waters Chromatography Division, Milford, MA, USA) was coupled to a mass spectrometer with electrospray ionization (ESI; LCQ Classic, Thermo Electron Corporation, Waltham, MA, USA). The mobile phase was isocratic at 15% methanol for 4 min, increased linearly from 15% to 100% methanol from 4 to 91 min, and allowed to run at 100% methanol for 5 min before ramping back to 15% methanol for the next sample.

The flow was 1 ml min^{-1}. After passing through the column, the sample was routed to a splitter—10% to the mass spectrometer and 90% collected into 30 3-min fractions for 90 min by using an autosampler (FC203, Gilson Inc., Madison, WI, USA). The 12 samples were run in succession, alternating between subordinate and dominant males. The fractions from each male were kept at −20°C until assessment of olfactory potency on females (see below).

Mass spectra were acquired with an ion trap mass spectrometer equipped with an ESI source and analyzed with Xcalibur software (Thermo Electron Corporation). The ion trap was operated in the negative ion mode with a spray voltage of 5 kV. A stream of 99% pure nitrogen at 60 psi was used as the sheath gas. Data were collected continuously in the range *m*/*z* 200–950. The relative peak areas (normalized to the area of the internal standard, CDCA) in the LC fractions that had significant olfactory potency were determined with Xcalibur software. Positive-ion MS was performed on olfactory-active fractions to determine whether any other compound could be present that was not detectable in the negative-ion mode. MS–MS at various energies was performed in the negative-ion mode in an attempt to ascertain more structural information about compounds in active fractions.

To deduce the chemical formula of compounds in active fraction(s), negative-ion high-resolution electrospray ionization was performed with a QSTAR1 MS (Applied Biosystems, Foster City, CA, USA) equipped with a hybrid quadrupole/time-of-flight detector. The most likely formulae were deduced by making several conservative assumptions about structure: (1) the presence of between 0 and 40 carbon atoms (high numbers were used so as to be conservative), (2) the presence of between 0 and 90 hydrogen atoms, (3) an even number of (or no) nitrogen atoms with a maximum of 12 nitrogens, (4) at least one sulfur and four oxygen atoms (consistent with the presence of at least one sulfate group as observed with MS/MS) with a maximum of three sulfur and 12 oxygen atoms, and (5) a mass accuracy within 15 ppm (a conservative measure, as a properly calibrated instrument should measure within 10 ppm). We identified all potential molecular formulae that fit this description and then systematically eliminated remaining formulae when the measured isotopic distribution pattern (i.e., the entire spectra, including the parent ion and all of its isotopes) did not match a structure's theoretical isotopic distribution pattern (for details on this procedure, see Sorensen et al. 2005 and Hoye et al. 2007).

Olfactory Sensitivity of Females to Male Urine Whole urine, urine extracts obtained by SPE, and LC fractions were tested for olfactory potency on females. The EOG was recorded as previously described (Frade et al. 2002). Briefly, male tilapia were anesthetized in water containing 100 mg l^{-1} MS222 and immobilized with gallamine triethiodide (3 mg kg^{-1} in 0.9% saline). The fish were placed in a padded Perspex® V-clamp, and their gills irrigated with aerated water containing MS222 (50 mg l^{-1}). The olfactory rosette was exposed by removing the ring of cartilage surrounding the nostril and continually irrigated with dechlorinated, charcoal-filtered tap water (6 ml min^{-1}). Stimulus-containing water was introduced into this flow via a three-way solenoid valve. At least 1 min was allowed to elapse between successive stimuli. The DC voltage was recorded by two glass micropipettes filled with 0.9% NaCl in 4% agar, one being placed close to the olfactory epithelium, and the other placed lightly on the skin of the head. The signal was amplified ($\times 10^3$) and recorded on a PC running Axoscope software (version 1.1, Axon Instruments, Inc., Foster City, CA, USA). The peak amplitude of the EOG to a given stimulus was blank-subtracted and normalized to the response to 10^{-5} M L-serine (standard).

Stimuli were made up on the day of recording by using the same dechlorinated, charcoal-filtered tap water used to irrigate the fish's nostril. Urine collected each day from males in the social groups was tested at a dilution of $1{:}10^4$ in water (*v*/*v*) on the same female, with six females (mean ± SD; length = 114.0±4.7 mm; weight = 47.6±6.1 g) used to assess the olfactory potency of all urine samples. Untreated urine from six subordinate and six dominant males and the corresponding urine SPE eluents were tested on each of the three females at a dilution of $1{:}10^4$ (*v*/*v*). Using another group of females, the SPE urine eluent and corresponding LC fractions of one male of each social rank were tested on the same female; the stimuli from each male were tested on three females. Therefore, 18 females (mean ± SD; length = 123.1±18.1 mm; weight = 62.0±33.4 g) were used to assess the olfactory potency of these male-derived stimuli at a dilution of $1{:}10^4$ (*v*/*v*; an equivalent dilution of the untreated sample urine). As a control, methanol was tested at 0.015% to 0.1% in water (the range of methanol concentration in urine extracts and LC fractions).

Statistical Analysis Frequency and duration of urine pulses from males and behavioral data of both sexes were compared between social contexts or between female reproductive conditions (social isolation, pre- or post-ovulatory female) by either the Student's *t* tests (paired samples) or one-way analysis of variance (ANOVA). The non-parametric Kendall's correlation was used to evaluate (within each of the two social groups of fish) the relationship between the mean urine volume per day and male size or mean dominance index per day (calculated as the ratio of the summed frequency of all dominant behaviors and the summed frequency of all dominant and submissive behaviors). The daily growth rate (length and

weight) within each fish group was tested for difference from zero with the Student's *t* test; the relationship between growth rate and the mean dominance index per day was evaluated by the Kendall's correlation. Univariate regression was used to evaluate the relationship between the mean EOG amplitudes evoked by daily urine samples of each male and fish group (fixed factor), mean urine volume per day, dominance index per day, and two-way interactions between the continuous covariates and the fixed factor. The Student's *t* test for independent samples was used to compare the relative area of LC peaks in fractions that had significant olfactory potency between subordinate and dominant males. Normalized EOG amplitudes (mean from three females for each stimulus) evoked by crude urine, SPE eluents, and LC fractions from subordinate and dominant males were compared by one-way repeated measures ANOVA with male type as between-group variable and stimulus type as within-subject variable. Statistical analyses were carried out with SPSS® 14.0 for Windows software (SPSS Inc., 1989–2005), and in all cases, statistical significance was set at $P<0.05$. All data are shown as mean ± SEM.

Ethical Statement Our research has followed the European Convention for the Protection of Vertebrate Animals Used for Experimental and Other Scientific Purposes (European Treaty Series No. 123), the ASAB/ABS Guidelines for the Use of Animals in Research (published in Animal Behavior, 2003, 65:249–255), and the Guidelines for the Use of Fishes in Research by the American Fisheries Society (http://www.fisheries.org/afs/publicpolicy/guidelines2004.pdf).

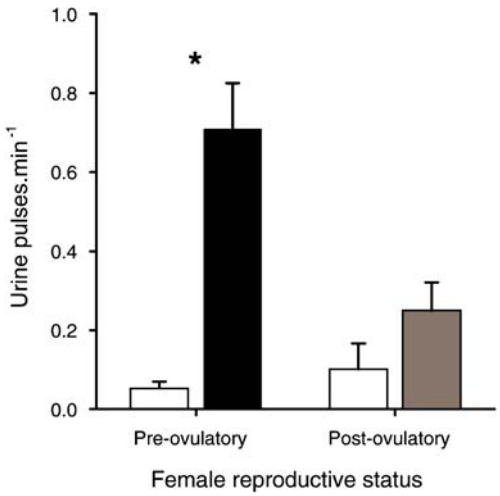

Fig. 1 Urination of male tilapia in isolation and in the presence of a female. Urination frequency (mean ± SEM) with a pre-ovulatory female (*black bar*, $N=7$) was significantly higher than in isolation (*open bar*, $N=7$; *asterisk* Student's *t* test for paired samples, $t_6=5.46$, $P<0.005$). With a post-ovulatory female (*gray bar*, $N=7$), the frequency of urination was not different from that when in social isolation (*open bar*, $N=7$; $t_6=1.77$, $P=0.127$)

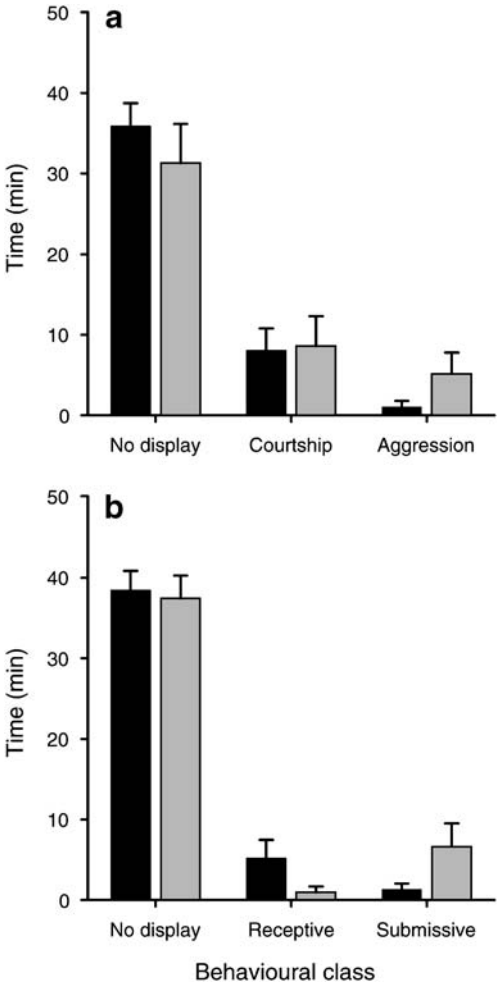

Fig. 2 Behavior of male and female tilapia. **a** Males in the presence of pre-ovulatory females (*black bars*, $N=7$) or post-ovulatory females (*gray bars*, $N=7$) did not differ (Student's *t* tests for independent samples, $df=12$, $P>0.05$) in the time (mean ± SEM) spent in different behavioral classes. **b** Pre-ovulatory females (*black bars*, $N=7$) and post-ovulatory females (*gray bars*, $N=7$) did not differ in the time spent in different behavioral classes in the presence of a male (Student's *t* tests for independent samples, $df=12$, $P>0.05$)

Results

Male Urination Frequency and Behavior In isolation, male tilapia urinated at low frequency (about once every 10 min) and expelled urine in short-duration pulses (about 1 s). When together with a pre-ovulatory female, males significantly increased their urination frequency; this did not happen with a post-ovulatory female (Fig. 1). No obvious changes in the release of feces or intestinal fluids were seen. The duration of urinary pulses was not different among the three social contexts (isolation, 1.43±0.27 s, $N=44$; with pre-ovulatory female, 1.26±0.14 s, $N=225$; with post-ovulatory female, 1.31±0.12 s, $N=78$; one-way ANOVA, $F_{2,344}=0.16$, $P=0.856$).

Male behavior was not different in the presence of pre- or post-ovulatory females. Males were never indifferent to

the presence of a female and spent most of the time swimming in her vicinity (no display), sometimes displaying obvious courtship behaviors and, more rarely, aggressive behaviors (Fig. 2a). Pre- and post-ovulatory females did not differ in their behavior either. The females spent most of the time swimming in the vicinity of the male and sometimes exhibiting receptive or submissive behaviors, depending on the male's behavior (Fig. 2b). The pattern of urine release varied among males; higher urination frequency was not necessarily associated with specific behaviors of either the male or the female. For example, a male might urinate at high frequency during clear courtship displays or while he was swimming in the vicinity of a pre-ovulatory female without displaying obvious courtship (Fig. 3, example 1). Also, a male would sometimes urinate with high frequency in the presence of a pre-ovulatory female without displaying obvious courtship (Fig. 3, example 2). Finally, in the presence of a post-ovulatory female, the male would sometimes exhibit clear courtship behaviors without urinating at high frequency (Fig. 3, example 3).

Analysis of females' ovarian states confirmed that they were in different reproductive condition. The GSI of pre-ovulatory females (4.4±1.7%) was higher than that of post-ovulatory females (1.4±0.4%; Mann–Whitney U, z=3.1, N_1=N_2=, P<0.005). The average egg weight was also higher in pre-ovulatory females (2.48±1.40 mg) than in post-ovulatory females (0.17±0.06 mg; z=3.1, P<0.005). This was due to larger egg sizes between 1.6 to 2.5 mm (54.3±24.4% of the eggs) in pre-ovulatory females, whereas in the post-ovulatory females, the eggs did not exceed 1.0 mm in diameter.

Social Hierarchies, Urine Volume, and Olfactory Potency of Urine In two groups of fish, males maintained a stable social hierarchy over 10 days of observation. In each group, three males were dominant (mean dominance index per day higher than 0.5), occupying a fixed territorial position in the tank, showing dark coloration and frequently displaying courtship or aggression. Among the other males of lower social rank, two in each group adopted intermittently dominant and submissive behaviors (mean dominance index per day between 0.2 and 0.4), whereas the remaining seven males in each group were clearly subordinate (mean dominance index per day lower than 0.2) and rarely or never exhibited territorial/dominant behaviors (e.g., dark coloration, courtship, or aggressive displays). Although the urine volume collected daily from each male varied, males of higher social rank consistently produced more urine than males of a lower social rank; within each group, the mean urine volume collected per male was positively correlated to the male's social rank (Fig. 4a) rather than its size either at the start (group A: length, T=0.28, P=0.206; weight, T=0.25, P=0.265; group B: length, T=0.38, P=0.084; weight, T=0.12, P=0.583) or at the end of observations (group A: length, T=0.15, P=0.486; weight, T=0.20, P=0.369; group B: length, T=0.37, P=0.107; weight, T=0.24, P=0.075). In both groups, males had a small daily growth that was not correlated with social rank (Table 1).

Univariate regression analysis used to evaluate the relationship between the mean olfactory potency of urine and fish group (fixed factor), mean urine volume per day, and mean dominance index per day provided a significant explanatory regression model (R^2_{adj}=0.61, $F_{7,16}$=7.01, P=0.001). The variation in the mean olfactory potency of male

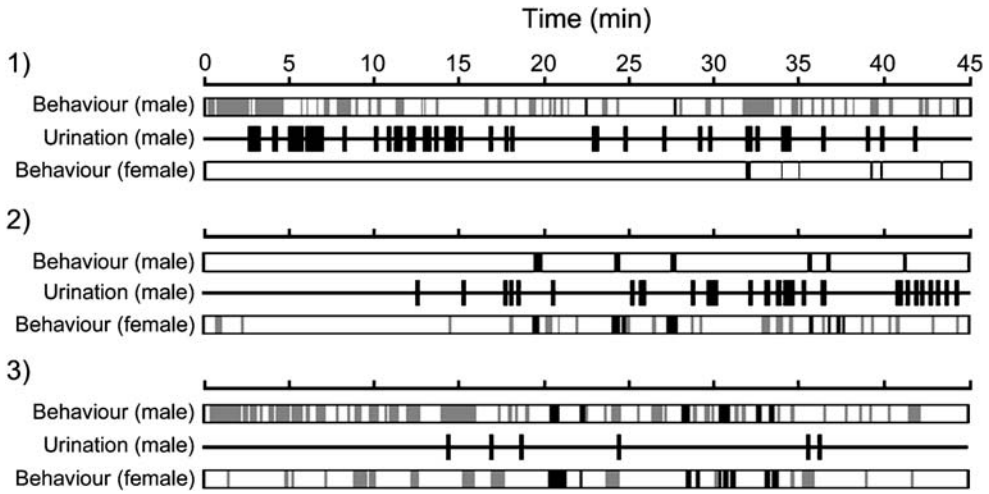

Fig. 3 Examples of male behavior, release of urine, and female behavior. Each example (*1* to *3*) shows the male's behavior (*top horizontal bar*: *white* no display, *gray* courtship displays, *black* aggressive displays), the male's urination (urine pulses of about 1 s are indicated by *vertical black bars* below the behavior of each male) and the female's behavior (*low horizontal bar*: *white* no display, *gray* receptive, *black* submissive). In examples 1 and 2, both males were in the presence of a pre-ovulatory female, and in three, the male was in the presence of a post-ovulatory female

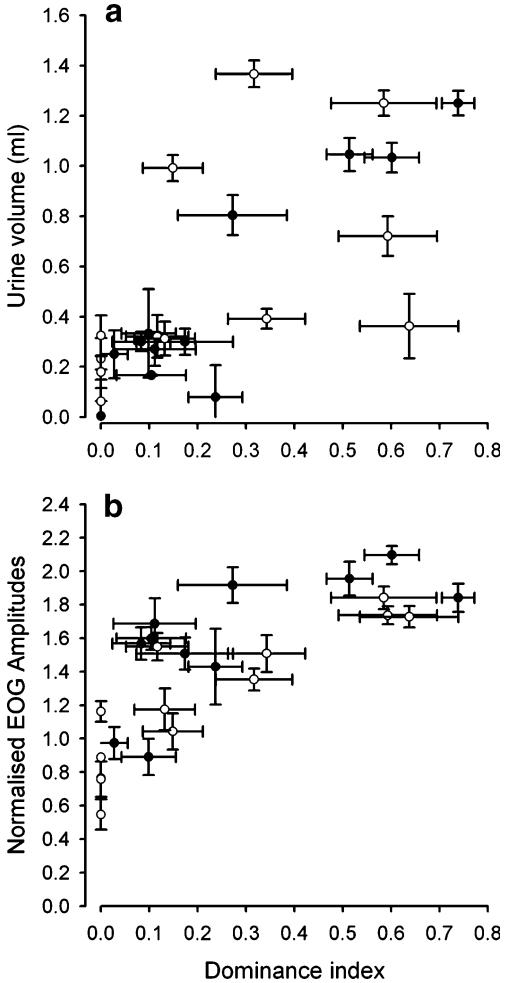

Fig. 4 Urine volume, urine olfactory potency, and dominance index of donor male tilapia in groups. Male's dominance index was calculated as the ratio of the summed frequency of dominant behaviors and the summed frequency of dominant and submissive behaviors (see "Materials and Methods"). **a** Scatter plot of mean dominance index per day (±SEM, N=10 d) and mean urine volume per day (±SEM, N=10 days) from each male in fish group A (N=12; *filled circles*) or B (N=12; *open circles*). In both groups, the urine volume was positively correlated (Kendall's correlation) to the male's dominance index (group A, T=0.60, P<0.001; group B, T=0.51, P< 0.05). **b** Relationship between dominance index (mean per day ± SEM, N=10 d) of males in fish group A (*open circles*, N=12) and B (*filled circles*, N=12), and normalized EOG amplitudes evoked by corresponding male urine (diluted 1:10,000) collected each day (mean ± SEM, number of urine samples varied between one and ten)

urine was explained by variation in the mean dominance index per day ($F_{1,18}$=4.67, P=0.044) and not by differences between the two male groups ($F_{1,18}$=1.65, P=0.215), variation in the mean urine volume collected per day ($F_{1,18}$=0.480, P=0.497), or by the interaction between the fish group and male behavior or urine volume ($F_{1,18}$= 0.502, P=0.488). The mean EOG amplitudes elicited by male urine increased with the mean dominance index per day (Fig. 4b).

Odorants in the Urine of Dominant and Subordinate Males Urine and C18 SPE eluents of urine from six dominant males evoked higher amplitude EOG responses than those of six subordinate males; matching samples of urine and SPE eluent from either group of males were not significantly different (Fig. 5). These dominant and subordinate males differed in the mean dominance index per day and mean urine volume per day; however, they did not differ in length, weight, daily growth, or GSI (Table 2).

EOG responses to liquid chromatography (LC) fractions of SPE eluent showed that fraction 15 (eluted from the LC column between 42 and 45 min) of both dominant and subordinate males evoked the largest EOG amplitudes in females, followed by fraction 16 (eluted between 45 and 48 min; Fig. 6a). The two fractions from dominant males evoked higher EOG amplitudes than the same two fractions from subordinate males (fraction 15: $F_{1,10}$=8.04, P<0.05; fraction 16: $F_{1,10}$=12.98, P<0.01; Fig. 6b). For both dominant and subordinate males, no significant difference was found between the olfactory potency of the original SPE eluent and LC fraction 15 (Student's t tests for paired samples: dominant males, t_5=0.07, P=0.946; subordinate males, t_5=2.30, P=0.07), suggesting that fraction 15 contained most of the olfactory potency found in the total SPE eluent. Control SPE eluent and methanol alone at 0.015% to 0.1% in water did not evoke EOG amplitudes higher than the water blank (data not shown).

Chromatograms of urine SPE eluent of subordinate and dominant males both had about 25 detectable peaks (Fig. 7a and b). The average retention time (RT) for CDCA (internal standard) was 68.37±0.078 min (mean ± SD). Fraction 15 corresponded to an apparent single peak (RT=43.96± 0.45 min; range from start to finish of peaks in all 12 samples = 42.5–46.5 min), which, for some samples, tailed into fraction 16. The relative area of this peak (normalized to the area of the internal standard) was higher in dominant males (11.69±1.14) than in subordinate males (6.12±0.74; Student's t test, t_{10}=4.08, P<0.005). The MS of fraction 15 was composed of two negative ions at 609.0 and 511.4 m/z for both dominant and subordinate males (Fig. 7c) with the same retention time (data not shown) strongly suggesting that they were from a single compound. The difference of m/z 98 between the two ions suggests the loss of H_2SO_4, seen for all sulfated steroids tested by Fine (2006). MS/MS on m/z 609.0 yielded a dominant negative ion of m/z 511.4, demonstrating that the two ions are from a single compound, and MS/MS on m/z 511.4 yielded a dominant ion of m/z 493.3 (loss of 18), suggesting at least one hydroxyl group in the molecule (Fig. 7d). Positive-ion MS of the peak in fraction 15 yielded three ions: m/z 535.2 $\left[M - H_2SO_4^+Na\right]$, m/z 1,047.3 [2M-2 $(H_2SO_4)^+Na$], and m/z 1,070.4 [2M-2$(H_2SO_4)^+2Na$]), consistent with fraction 15, containing a single compound. High-resolution MS analysis of the compound in fraction 15

Table 1 Daily growth (mean ± SEM, N=12) of tilapia males in two groups of fish and its correlation with dominance index

	Males in group A		Males in group B	
	Length (mm/day)	Weight (g/day)	Length (mm/day)	Weight (g/day)
Daily growth,[a] Kendall's correlation	0.41±0.04 T=0.01 P=0.666	0.33±0.07 T=0.05 P=0.836	0.22±0.04 T=0.03 P=0.881	0.27±0.05 T=0.16 P=0.472

[a] Daily growth in length and weight was significantly different from zero (Student's t test, P<0.05).

yielded ions at m/z 609.2303 and 511.2768. Although 34 potential formulae met the required criteria, only $C_{29}H_{40}N_2O_{10}S$ could explain the observed isotopic ratios that were measured (see "Materials and Methods").

Discussion

Territorial/dominant tilapia males seem to signal females by using a urinary odorant that may allow females to discriminate dominant from subordinate males. This is supported by three lines of evidence. First, territorial males urinate at higher frequency in the presence of females ready to spawn (pre-ovulatory) than they do in the presence of sexually inactive females (post-ovulatory) or in social isolation. Given that the duration of urine pulses did not change among the different social contexts, then a greater volume of urine must also be released when the male is in the presence of a spawning female. Second, a putative sulfated aminosterol-like compound with high olfactory potency is found in larger amounts in the urine of territorial/dominant males than in that of non-territorial/subordinate males. This is consistent with previous work that showed that males release sulfated steroid-like odorants in the urine (Frade et al. 2002). Third, subordinate males store less urine in the bladder than do males of higher social rank, agreeing with a previous study (Barata et al. 2007). Taken together, these results suggest that territorial/dominant males advertise social status by increasing the release of a potent urinary odorant in the presence of a spawning female; subordinate males are less capable of stimulating a female because they produce lesser quantities of the (putative sulfated aminosterol-like) odorant and are also less capable of increasing urination frequency because they store less urine.

The mixed-sex groups of fish were formed with males that previously had been kept separated from each other, all being territorial in their original tanks with four or five females, and where reproduction occurred regularly. However, when these males were placed together, a clear dominance hierarchy emerged. The dominant males were effective in defending a fixed territorial position and displayed courtship to approaching females, whereas males of lower social rank seldom or never adopted the black coloration typical of a territorial male and did not court females. This is in agreement with previous behavioral studies that showed that mating success is highly skewed toward dominant males in established groups of captive Mozambique tilapia (Oliveira and Almada 1996, 1998a). Our study also showed that dominant males store more urine than males of lower social rank, and urine of dominant males was of higher olfactory potency and related to a higher concentration of a sulfated aminosterol odorant. Therefore, we suggest that both the urine volume stored in the bladder and concentration of the sulfated aminosterol odorant reflect social dominance rather than reproductive capability (i.e., presence of mature testes).

The olfactory potency of the C18 (methanol) eluent of male urine was not significantly different from that of crude urine (Fig. 5). This indicates that the most active/important odorant(s) found in male urine are relatively non-polar (compared to substances that would have passed through

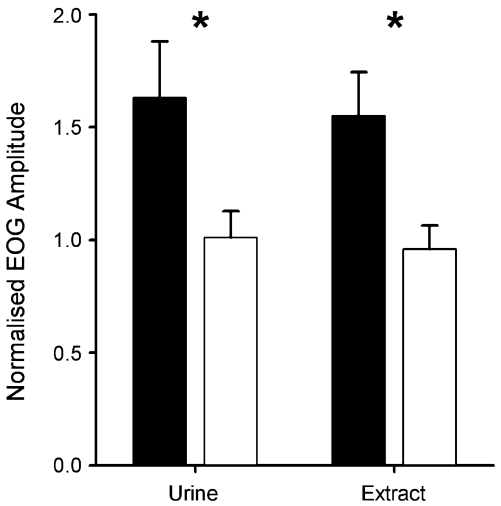

Fig. 5 Olfactory potency of male tilapia urine and extracts of urine on females. EOG amplitudes (mean ± SEM) evoked by urine and solid-phase extracts of urine (diluted 1:10,000) from subordinate (*open bars*; N=6) and dominant (*filled bars*; N=6) males. The urine and extracts of urine from dominant males elicited higher EOG amplitudes than those from subordinate males (*asterisk* between groups, $F_{1,10}$= 6.12, P<0.05); within each male type, no significant differences were found between the EOG responses elicited by the urine and the extract of urine (within subjects, $F_{1,10}$=0.58, P=0.46)

Table 2 Comparison of dominance index, urine volume, and size of dominant and subordinate tilapia males (mean ± SEM, N=6 per group)

	Dominant[a]	Subordinate[a]	t_{10} and P values
Dominance index	0.61±0.03	0.07±0.03	13.63, P<0.001
Urine volume per day (ml)	0.94±0.35	0.34±0.30	2.99, P<0.05
Length (mm)	143.5±2.8	139.7±1.7	1.17, P=0.268
Weight (g)	89.8±5.8	78.7±1.7	1.84, P=0.095
Growth in length (mm/day)	0.31±0.18	0.29±0.17	0.25, P=0.811
Growth in weight (g/day)	0.27±0.08	0.14±0.08	1.22, P=0.251
GSI (%)	1.03±0.09	0.95±0.07	0.69, P=0.505

[a] Males that were urine donors for assessment (in females) of the olfactory potency of urine, urine C18-extracts, and liquid chromatography fractions of the urine extracts

the C18 adsorbent in the aqueous urine). Furthermore, because the olfactory potency of the only EOG-active LC fraction (no. 15) was not significantly different from that of the urine extract and it contained only a single compound (as indicated by mass spectrometric analysis), it seems likely that the olfactory potency of urine extract is largely due to a single odorant (of m/z 609). Mass spectrometric analyses indicated that the odorant is a sulfated steroid derivative with a molecular weight of 610 Da. High-resolution negative-ion mass spectral data pointed to a molecular formula of $C_{29}H_{40}N_2O_{10}S$; the presence of two nitrogens in the molecule indicates that this is a sulfated aminosterol-like compound. However, further structural studies (e.g., nuclear magnetic resonance spectroscopy)

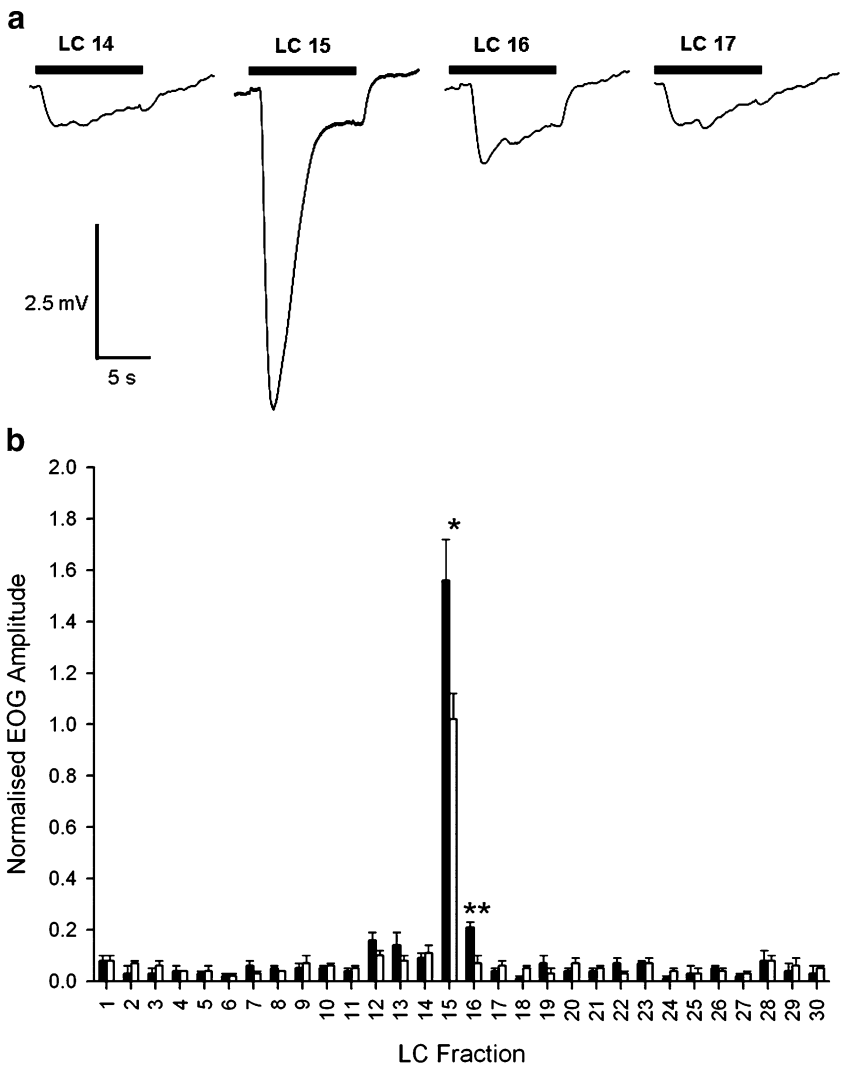

Fig. 6 Olfactory responses of female tilapia to liquid chromatography (LC) fractions of C18 solid-phase extraction eluents of male urine. **a** Typical electro-olfactograms (EOGs) recorded in response to stimuli (*black horizontal bars*) of LC fractions 14 to 17 (elution from the LC column between 40 and 51 min; each fraction corresponds to 3 min elution) diluted 1:10,000 in water. b Normalized EOG amplitudes (mean ± SEM) elicited by LC fractions (1 to 30 collected every 3 min) of extracts of urine (diluted 1:10,000) from subordinate (*open bars*; N=6) and dominant males (*filled bars*; N=6). LC fractions 15 (elution between 42 and 45 min), and 16 (elution between 45 and 48 min) from dominant males elicited higher EOG amplitudes than those from subordinate males (*P< 0.05; ** P<0.01)

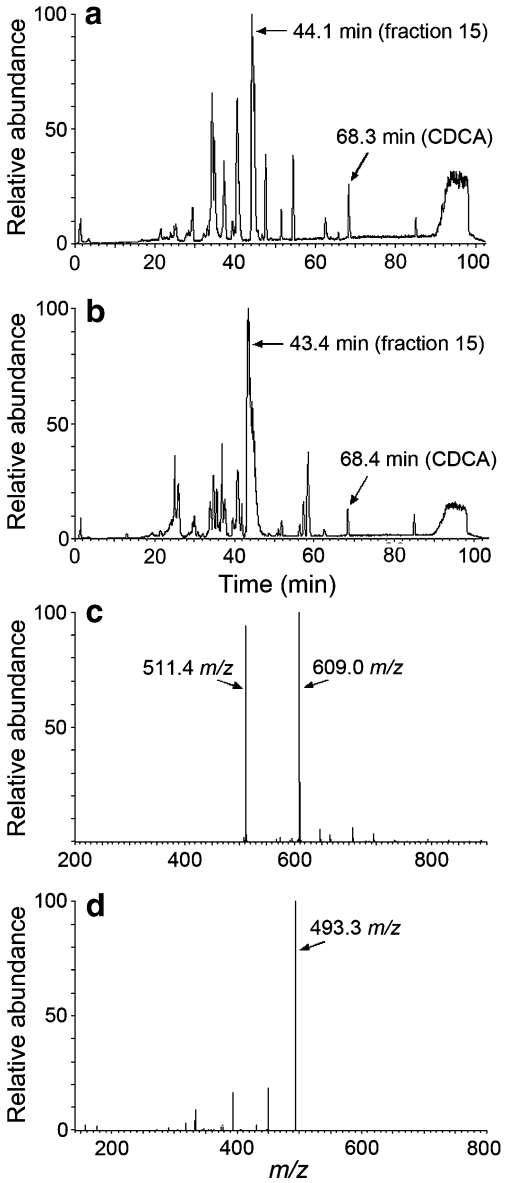

Fig. 7 Representative liquid chromatography–mass spectrometry chromatograms of male tilapia urine extracts. Subordinate male (**a**) and a dominant male (**b**). In both chromatograms, the olfactory active peak of fraction 15 (42–45 min) is indicated by the arrow and corresponding retention time; chenodeoxycholic acid (CDCA; 1 μg) was added as internal standard. **c** Mass spectrum of the peak centered at 43.4 min in (**b**). Both ions are from a single compound; the smaller shows the loss of a sulfate from the larger. The spectrum for the peak at 44.1 min in (**a**) is identical (data not shown). **d** Mass spectrometry/mass spectrometry of 511.4 *m*/*z* in **c**. The dominant ion (*m*/*z* 493.3) shows the loss of H_2O

are required for complete identification and structure assignment. Interestingly, novel sulfated aminosterols have been identified as important constituents of the migratory pheromone of the sea lamprey *Petromyzon marinus* (Sorensen et al. 2005; Hoye et al. 2007). If further studies show that the putative urinary pheromone of male tilapia is an aminosterol, it would be the second instance that such a class of compound has been shown to act as an odorant and, possibly, as pheromones in vertebrates.

The finding of a single odorant in the urine extract, the chemical identity of which does not resemble any known reproductive steroids, is intriguing. In male tilapia, the concentration of urinary steroids is modulated by social context; testosterone (T), 11-ketotestosterone (11KT), 17,20β-dihydroxy-4-pregnen-3-one (17,20β-P), and 17,20α-dihydroxy-4-pregnen-3-one (17,20α-P) are positively correlated to dominance (Oliveira et al. 1996). However, none of these steroids evokes significant EOG responses even at concentrations as high as 10^{-6} M (Frade et al. 2002), and the current study confirmed that the female olfactory system is insensitive to male urinary sex-steroids. This contrasts with the idea that pheromones are generally mixtures of compounds (Wyatt 2003) and that, in teleosts, reproductive pheromones are mixtures of sex hormones (and their metabolites) in which the ratio of components varies between species and gender and, within a sex, varies with reproductive status (Stacey and Sorensen 2006). However, we have not investigated thoroughly all possible urinary odorants. The methanol eluent from our C18 purification procedure should contain relatively low polarity compounds; more polar compounds should have passed through the column with the water of the urine. Therefore, it is possible that we missed a fraction of urinary odorants for females because we did not test the olfactory potency of the aqueous eluent.

Tilapia females have high olfactory sensitivity to male-conditioned water and to bile and intestinal fluids of males (Frade et al. 2002), suggesting that the urinary odorant found in this study may be detected as part of a mixture of odorants and that females may use the ratio, rather than the absolute amount, to discriminate between dominant and subordinate males. Possibly, dominant males but not subordinates change this ratio by increasing their urination rate in the presence of a female ready to spawn; subordinate males not only produce less of the urinary odorant but are apparently unable to store as much urine in the bladder. Subordinate males would thus be less capable of changing their profile of odorants when in the presence of a female ready to spawn. Although longer urine retention by dominant males would, at least partly, explain the higher concentration of the aminosterol odorant in their urine than in that of subordinate males, our results suggest that production of urinary odorants is higher in dominant than in subordinate males. Further research is required to determine the site of production of urinary odorants, especially the aminosterol-like compound.

Female reproductive condition affects the urination frequency of territorial males; males showed the same intensity of courtship behaviors with pre- or post-ovulatory females but urinated much more in the presence of pre-

ovulatory females. The behavior of the two groups of females was not different (perhaps due to stress because of handling before their introduction in the male's tank), suggesting that the increase in urine release by males was triggered by odors from pre-ovulatory females as previously suggested (Miranda et al. 2005). In contrast to males, females release urine in shorter pulses and at higher frequency, and this is, apparently, unaffected by the presence of a territorial male (Almeida et al. 2005). Our study appears to be the first to link the release of a urinary aminosterol-like odorant directly with both the social status of a male fish and its reproductive opportunity, which may be perceived through female odors (including urinary odorants).

Chemical signaling through urinary pheromones has been previously shown in female goldfish, which increase their release of urine containing the post-ovulatory hormonal pheromone, prostaglandin $F_{2\alpha}$ and, thereby, mark spawning sites and attract males (Appelt and Sorensen 2007). The goldfish scramble-competition mating system, floating spawning substrate, and prostaglandin pheromones in female urine are representative of many species of cyprinids (Stacey and Sorensen 2006). In other fish groups, different suites of hormonal products in the urine are used as pheromones (Colombo et al. 1980; Liley 1982; Katsel et al. 1992; Vermeirssen and Scott 2001; Stacey and Sorensen 2006) in what appears to be insipient specialization for chemical communication between the sexes. This varies taxonomically but may also be related to different life histories or reproductive strategies. In contrast, in tilapia, chemical signaling seems to be more complex occurring both between males (Barata et al. 2007) and reciprocally between males and females.

In summary, dominant/territorial tilapia males release urine in pulses of short duration, the frequency of which increases dramatically in the presence of females ready to spawn, but which does not increase in the presence of post-spawn females. Male urine is a vehicle of a putative sulfated aminosterol that is an important urinary odorant detected by the female's olfactory system. Both the concentration of this odorant and the urine volume in the bladder are higher in dominant than in subordinate males, implying that males of low social rank are less capable of stimulating females ready to spawn. We suggest that the putative aminosterol in male urine may act as a pheromonal signal of dominance and influence female spawning.

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References

ALMEIDA, O. G., MIRANDA, A., HUBBARD, P. C., FRADE, P., BARATA, E. N., and CANÁRIO, A. V. M. 2005. Urine as a social signal in the Mozambique tilapia (*Oreochromis mossambicus*). *Chem. Senses* 30:i309–i310.

APPELT, C. W., and SORENSEN, P. W. 1999. Freshwater fish release urinary pheromones in a pulsatile manner, pp. 247–256, in R. E. Johnston, D. Müller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Kluwer, New York.

APPELT, C. W., and SORENSEN, P. W. 2007. Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Anim. Behav.* 74:1329–1338.

BAERENDS, G. P., and BAERENDS VAN ROON, J. M. 1950. An introduction to the study of the ethology of cichlid fishes. *Behaviour* 1(Suppl.):1–243.

BARATA, E. N., HUBBARD, P. C., ALMEIDA, O. G., MIRANDA, A., and CANÁRIO, A. V. M. 2007. Male urine signals social rank in the Mozambique tilapia (*Oreochromis mossambicus*, Peters 1852). *BMC Biology* 5:54 DOI 10.1186/1741–7007–5–54.

BARLOW, G. W. 1991. Mating systems among cichlid fishes, pp. 173–190, in M. H. A. Keenleyside (ed.). Cichlid Fishes: Behaviour, Ecology and EvolutionChapman & Hall, London.

BRUTON, M. N., and BOLTT, R. E. 1975. Aspects of the biology of *Tilapia mossambica* Peters (Pisces: Cichlidae) in a natural freshwater lake (Lake Sibaya, South Africa). *J. Fish Biol.* 7:423–445.

COLOMBO, L., MARCONATO, A., BELVEDERE, P. C., and FRISCO, C. 1980. Endocrinology of teleost reproduction. A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* 47:355–364.

COWARD, K., and BROMAGE, N. R. 2000. Reproductive physiology of female tilapia broodstock. *Rev. Fish Biol. Fish.* 10:1–25.

FINE, J. M. 2006. Isolation, identification, and biological characterization of the Sea Lamprey (*Petromyzon marinus*) migratory pheromone. PhD dissertation. University of Minnesota, Twin Cities.

FRADE, P., HUBBARD, P. C., BARATA, E. N., and CANÁRIO, A. V. M. 2002. Olfactory sensitivity of the Mozambique tilapia to conspecific odours. *J. Fish Biol.* 61:1239–1254.

FRYER, G., and ILES, T. D. 1972. The Cichlid Fishes of the Great Lakes of Africa: Their Biology and Evolution. Oliver & Boyd, Edinburgh.

HOYE, T. E., DVORNIKOVS, V., FINE, J. M., ANDERSON, K. R., JEFFREY, C. S., MUDDIMAN, D. C., SHAO, F., SORENSEN, P. W., and WANG, J. 2007. Details of the structure determination of the sulfated steroids PSDS and PADS—new components of the sea lamprey (*Petromyzon marinus*) migratory pheromone. *J. Org. Chem.* 72:7544–7550.

KATSEL, P. L., DMITRIEVA, T. M., VALEYEV, R. B., and KOZLOV, Y. P. 1992. Sex pheromones of male yellowfin baikal sculpin (*Cottocomphorus grewingki*): isolation and chemical studies. *J. Chem. Ecol.* 18:2003–2010.

LILEY, N. R. 1982. Chemical communication in fish. *Can. J. Fish. Aquat. Sci.* 39:22–35.

MIRANDA, A., ALMEIDA, O. G., HUBBARD, P. C., BARATA, E. N., and CANÁRIO, A. V. M. 2005. Olfactory discrimination of female reproductive status by male tilapia (*Oreochromis mossambicus*). *J. Exp. Biol.* 208:2037–2043.

NELISSEN, M. H. J. 1991. Communication, pp. 225–240, in M. H. A. Keenleyside (ed.). Cichlid Fishes. Behaviour, Ecology and EvolutionChapman & Hall, London.

OLIVEIRA, R. F. 1995. Etologia social e endocrinologia comportamental da tilápia *Oreochromis mossambicus* (Teleostei, Cichlidae). PhD dissertation, Universidade de Lisboa.

OLIVEIRA, R. F., and ALMADA, V. C. 1996. Dominance hierarchies and social structure in captive groups of the Mozambique tilapia

Oreochromis mossambicus (Teleostei, Cichlidae). *Ethol., Ecol. Evol.* 8:39–55.

OLIVEIRA, R. F., and ALMADA, V. C. 1998a. Mating tactics and male-male courtship in the lek-breeding cichlid *Oreochromis mossambicus*. *J. Fish Biol.* 52:1115–1129.

OLIVEIRA, R. F., and ALMADA, V. C. 1998b. Mating tactics and male-male courtship in the lek-breeding cichlid *Oreochromis mossambicus*. *J. Fish Biol.* 52:1115–1129.

OLIVEIRA, R. F., ALMADA, V. C., and CANARIO, A. V. M. 1996. Social modulation of sex steroid concentrations in the urine of male cichlid fish *Oreochromis mossambicus*. *Horm. Behav.* 30:2–12.

SORENSEN, P. W., FINE, J. M., DVORNIKOVS, V., JEFFREY, C. S., SHAO, F., WANG, J., VRIEZE, L. A., ANDERSON, K., and HOYE, T. R. 2005. Mixture of new sulphated steroids functions as a migratory pheromone in the sea lamprey. *Nature Chem. Biol.* 1:324–328.

STACEY, N., and SORENSEN, P. 2006. Reproductive pheromones, pp. 359–412, in K. A. Sloman, R. W. Wilson, and S. Balshine (eds.). Behaviour and Physiology of Fish. Academic, London.

VERMEIRSSEN, E. L. M., and SCOTT, A. P. 2001. Male priming pheromone is present in bile, as well as in urine, of female rainbow trout. *J. Fish Biol.* 58:1039–1045.

WYATT, T. D. 2003. Pheromones and Animal Behaviour. Communication by Smell and Taste. Cambridge University Press, Cambridge.

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Metasternal Gland Volatiles and Sexual Communication in the Triatomine Bug, *Rhodnius prolixus*

Gina B. Pontes · Björn Bohman · C. Rikard Unelius · Marcelo G. Lorenzo

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Abstract Twelve compounds produced by the metasternal glands (MGs) of the triatomine bug *Rhodnius prolixus* were identified by solid phase microextraction (SPME) combined with coupled gas chromatography-mass spectrometry (GC-MS) using achiral and chiral columns. All substances were ketones or alcohols, and the same compound profile was found in the secretions produced by either sex. The most abundant compounds were 2-methyl-3-buten-2-ol, (2*S*)-pentanol, (3*E*)-2-methyl-3-penten-2-ol, and (2*R*/2*S*)-4-methyl-3-penten-2-ol. Emission of these compounds was detected more frequently from females than males, and females released them more frequently during the early hours of the scotophase, the period when sexual activity in this species is at its peak. These compounds were also detected in the headspace above mating pairs. Finally, the occlusion of the MG orifices of male or female bugs with paraffin resulted in a significant decrease in copulation frequency compared to sham-operated insects. Together, these data suggest that the MG secretions of *R. prolixus* may be involved in sexual communication.

Keywords *Rhodnius prolixus* · Sexual behavior · Metasternal glands · Pheromone · Volatiles · Identification

G. B. Pontes · M. G. Lorenzo (✉)
Laboratory of Triatomines and Chagas Disease Epidemiology,
Instituto René Rachou/FIOCRUZ,
30190002 Belo Horizonte, Minas Gerais, Brazil
e-mail: marcelo@cpqrr.fiocruz.br

B. Bohman · C. R. Unelius
School of Pure and Applied Natural Sciences,
University of Kalmar,
SE-391 82 Kalmar, Sweden

Introduction

Rhodnius prolixus Stål 1859 (Heteroptera: Reduviidae) is the main vector of Chagas disease in northern South America and in parts of Central America (Schofield 1994). This species is well adapted to live in rural houses and is considered to be of major epidemiological importance (Monteiro et al. 2003). Approximately 16–18 million people in Latin America are infected with the Chagas disease parasite, *Trypanosoma cruzi*, and another 120 million are at risk (WHO 2005).

Baldwin et al. (1971) reported that copulating pairs of *R. prolixus* emit a pheromone that is attractive to males. This phenomenon has also been observed for another vector of Chagas disease, the bug *Triatoma infestans* (Manrique and Lazzari 1995). Relatively little is known about the mechanisms of long- or short-distance orientation that mediate sexual encounters between adults of species in the subfamily Triatominae, although Baldwin et al. (1971) suggested that feeding triggers the development of sexual attraction in *R. prolixus* and that unfed males of this species do not respond to the apparent odor emitted by mating pairs.

R. prolixus adults have a pair of metasternal glands (MGs) that open to the ventral metathorax (Brindley 1930). Their function and the chemical identity of any secretions are unknown. Another set of glands of this insect, the Brindley's glands, which secrete isobutyric acid as the most abundant compound, are likely associated with alarm and defense functions (Ward 1981; Cruz López et al. 1995; Rojas et al. 2002; Manrique et al. 2006). It has been suggested that compounds produced by Brindley's glands are involved in the sexual chemical communication of triatomines (Cruz López et al. 2001; Rojas et al. 2002; Guerenstein and Guerin 2004).

In a recent study, Manrique et al. (2006) suggested that the secretions of the MGs of *T. infestans* are involved both in sexual and alarm communication, and that the secretions of Brindley's glands are restricted to alarm and defensive roles. These authors identified several highly volatile ketones and alcohols produced by the MGs and showed that the contents of these glands are emitted by adults of this species during copulation.

Our primary objective in this study was to identify volatile secretions produced by the MGs of *R. prolixus*. We further tested whether the MG compounds are emitted at different phases of the light/dark cycle by virgin males and females and examined if mating pairs of this species emit these substances. Finally, we tested whether the compounds emitted from these glands influence copulation.

Methods and Materials

Insects Insects were reared at 26±2°C and 60±10% r.h. Groups of fifth instars were sorted by sex and placed in separate flasks to keep them unmated until use in experiments. All insects used were kept under the 12:12 L:D photoperiod for at least 3 d before any experiment. Virgin insects were used for all experiments, and their nutritional status was varied according to the experiment performed. For odor-identification studies, unfed insects were dissected at d 20 after ecdysis. For the detection of emission of MG compounds, insects were fed at d 10, and used 20 d after ecdysis. For the remaining two experiments (i.e., detection of emission of MG odors during mating and evaluation of the effect of gland occlusion on mating success), insects were used at d 20 after being fed at d 10. All assays were performed at 26±2°C and 60±10% r.h.

Identification of Compounds Produced by Metasternal Glands Samples of 12 glands were obtained from six insects and stored in 2-ml vials sealed with Teflon®/silicone-lined caps. Control samples were prepared with pieces of tissue and cuticle from hind leg coxae. Female and male tissue was stored at −8°C for not more than 10 d before analysis. No change of the chemical profile was observed after storage when compared with freshly prepared samples.

Gland samples were sonicated (Thornton T14, Inpec Eletrônica, Brazil, 40 kHz) for 5 min and then heated at 50°C for 30 min. A solid phase microextraction (SPME) fiber (2 cm, DVB/CAR/PDMS-50/30 μm, Supelco, Bellefonte, PA, USA) was exposed in the headspace of the samples (in vials) for 10 min at 50°C immediately before analysis by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis was performed by using coupled Shimadzu 17A-5050A machines. Desorption time in the splitless injection port of the GC was 1 min. Helium at 30 cm s^{-1} was used as carrier gas. Transfer line and GC injector temperatures were 250 and 230°C, respectively. Analyses were performed by using a SupelcoWax-10 column (30 m×0.25 mm i.d.×0.25 μm film; Supelco), with an oven program of 40°C for 5 min, 3°C min^{-1} to 120°C, then 15°C min^{-1} to 200°C.

Tentative identification of volatile compounds was based on the comparison of retention indices (Kováts 1965) and mass spectra with data from the literature and spectral library (NIST-02). All tentative identifications were confirmed by peak enhancement in co-injections with authentic synthetic samples (Birkett et al. 2004). A typical procedure for co-injection/peak enhancement is given for 2-pentanol: A gland sample was prepared and analyzed as described. After a chromatogram was obtained, the gland sample was treated again according to the same protocol (i.e., sonicated, heated, and sampled by SPME). In parallel, a sample of synthetic standard was prepared: 1 μl of the compound was absorbed on a small piece of filter paper (1×1 cm) in a 10-ml open vial. The vial was heated at 50°C for 3 min and cooled to ambient temperature for 1 min. The same SPME fiber was exposed to the standard sample for 2 sec, and the odors were desorbed from the fiber into the GC injector. The results of both injections were compared and the identity of the compound confirmed when three criteria were fulfilled. First, the peak from the MG compound and the synthetic compound overlapped fully. Second, the peak area increased in the second injection. Third, no difference between the mass spectral profiles was observed after a scan-by-scan analysis.

The stereochemistry of chiral compounds was determined by GC with flame-ionization detection (FID; Shimadzu 17A) and GC-MS analysis. Gland samples were heated at 50°C for 30 min, and the SPME fiber was exposed in the headspace for a given time depending on the relative abundance of a compound. The method used for analysis was the same as for the GC-MS analysis, except the carrier gas velocity was 31 cm sec^{-1}, the injector and detector temperatures were both 225°C, and a CYCLOSILB column (30 m×0.25 mm i.d.×0.25 μm film, J & W Scientific) at either 80°C, for 4-methyl-3-penten-2-ol, or 30°C, for the other compounds, was used. Because a number of peaks overlapped with this column and conditions, chiral GC-MS analysis was carried out by using a GammaDex 225 column (30 m×0.25 mm i.d.×0.25 μm film) at 30°C. The retention times of compounds were compared with synthetic standards, and co-injection (peak enhancement) was carried out to confirm the identities of the enantiomers of all compounds. The configuration of 2-methyl-3-penten-2-ol was confirmed by co-injection with the synthetic (*E*)-isomer, derived from *trans*-methyl crotonate (for synthesis details, see below).

Emission of MG Compounds by Virgin Adults Groups of three virgin adults of the same sex were separated 7 d after ecdysis and transferred into 10-ml vials covered with gauze with a piece of filter paper inside as a substrate for the bugs. These vials were enclosed separately in 150-ml closed plastic containers so as to isolate each group of bugs. We worked with groups of insects to increase both the likelihood of emission and the amount of MG odors (preliminary assays with individual insects failed probably because of low levels of compounds). Each treatment included three groups of three insects. The different series of assays monitored odor emission by: (1) unfed females during the dark phase; (2) unfed females during the light phase; (3) females fed at d 9 after ecdysis, during the dark phase; (4) females fed at d 9 after ecdysis, during the light phase; (5) unfed males during the dark phase; (6) unfed males during the light phase; (7) males fed at d 9 after ecdysis, during the dark phase; and (8) males fed at d 9 after ecdysis, during the light phase. Odor sampling with a SPME fiber was carried out for 1 hr for each treatment. Volatile compounds on the fiber were desorbed immediately after sampling the headspace. This procedure was repeated every second day over a period of 12 d with all groups, i.e., giving a total of six samples per group of three insects and 18 samples for each of the eight treatments. Control samples were obtained by SPME analyses of vials containing a piece of filter paper.

The data from studies of emission of MG compounds by *R. prolixus* adults were analyzed both for individual substances and for pooled samples. This allowed the comparison of emission activity between series (treatments). Every time a MG odor was detected over the samples, this was recorded as a "detection event".

Emission of MG Compounds During Copulation One *R. prolixus* female and one male were gently transferred onto a piece of filter paper inside a 10-ml vial, so as to avoid disturbance and the consequent emission of Brindley's glands' products (Manrique et al. 2006). The vial was closed with a Teflon®/silicone-lined cap. After copulation had begun, volatiles present in the headspace were sampled for 60 min with a SPME fiber. Volatile compounds were analyzed immediately after sampling the headspace. Twenty assays were performed. Each pair of insects was used only once and then discarded.

Odors Emitted by MGs and Possible Effect on Mating in R. prolixus A pair of bugs was gently introduced into a Petri dish (10×2 cm) lined with a piece of filter paper and covered with glass to prevent escape. Whether the pair copulated or not was observed for 60 min; if the pair did not commence copulation within this time, it was considered that no copulation occurred. To evaluate the relevance of MG odors for the success of copulation, the proportion of mating pairs under different treatments was compared: (1) pairs in which males had the MG orifices occluded with paraffin (N=20), (2) pairs in which females had the MG orifices occluded with paraffin (N=20), and (3) pairs in which both males and females had the MG orifices occluded with paraffin (N=20). To test whether this treatment affected the behavior of the insects, two series of control assays were performed: (4) a group in which sham males had paraffin applied on a different area of the cuticle without covering the MG orifices (N=20), and (5) a group in which sham females had paraffin applied on a different area of the cuticle without covering the MG orifices (N=20). An additional control series (6) evaluated the mating frequency in intact pairs (N=20). All experiments were performed at 26±2°C and 60±10% r.h. The behavior of insects was studied during the first half of the dark phase of their activity cycle.

Chemicals 2-Butanone, 2-pentanone, (2*R*)-2-butanol, (2*S*)-2-butanol, 2-methyl-3-buten-2-ol, 3-pentanol, 2-pentanol, 4-methyl-2-pentanol, 3-hexanol, and 2-methyl-1-butanol were purchased from Sigma-Aldrich (Brazil). (2*S*)-3-Methyl-2-butanol, (2*S*)-2-pentanol, and (2*S*)-4-methyl-2-pentanol were purchased from Lancaster Synthesis (UK). (3*E*)-2-Methyl-3-penten-2-ol was synthesized from methyl crotonate according to Stavinoha et al. (1981), and 4-methyl-3-penten-2-ol was synthesized according to Johnson and Rickborn (1970).

(3E)-2-Methyl-3-penten-2-ol Methyl crotonate (0.96 g, 9.6 mmol) was added dropwise at <−10°C under nitrogen to an ether solution of 1.6 M methyllithium (20-ml, 32 mmol) over 30 min. The mixture was stirred for an additional 3 hr at 0°C before Baeckströms reagent (celite/Na_2SO_4, 1:1 *w*/*w*) was added. The mixture was filtered and concentrated *in vacuo*, giving (3*E*)-2-methyl-3-penten-2-ol as a colorless liquid (0.57 g, 60%). ^{1}HNMR: δ: 5.63 (m, 2H), 1.68 (d, 4.5 Hz, 3H), and 1.30 (s, 6H). ^{13}CNMR: δ: 139.37, 122.16, 70.89, 29.97, and 17.87 ppm.

4-Methyl-3-penten-2-ol Sodium borohydride (0.19 g, 5.0 mmol) was dissolved in ethanol (50%, 10-ml). 4-Methyl-3-penten-2-one (mesityl oxide, 1.0 g, 10.0 mmol) was added dropwise, while stirring at 0°C. The reaction mixture was stirred at ambient temperature overnight. K_2CO_3 was added until the solution was saturated, after which the product was extracted with diethyl ether (2× 20-ml). The ether phase was washed with brine (20-ml) and dried over $MgSO_4$. Evaporation of the solvent gave 4-methyl-3-penten-2-ol as a colorless liquid (0.88 g, 87%). ^{1}HNMR: δ: 5.20 (d, 8.6 Hz, 1H), 4.55 (dq, 8.4, 6.3 Hz, 1H), 1.71 (s, 3H), 1.68 (s, 3H), and 1.22 (d, 6.3 Hz, 3H).

^{13}CNMR: δ: 134.43, 129.57, 65.03, 25.87, 23.84, and 18.23 ppm.

A mixture enriched in (2*S*)-4-methyl-3-penten-2-ol was obtained from the racemate by a lipase-catalyzed reaction (Amano PS immobilized on diatomite, Sigma-Aldrich, Sweden; Brenna et al. 1998). Racemic 4-methyl-3-penten-2-ol (20 mg, 0.20 mmol) and vinyl acetate (100 mg, 0.86 mmol) were dissolved in dichloromethane (1-ml). Amano PS-DI (20 mg) was added to the mixture, which was left for 5 hr, with occasional shaking. Chiral GC-MS analysis showed a product enriched in the *S*-enantiomer. The assignment of the stereochemistry was based on the well-known stereochemical preference of the Amano-PS lipase (Kazlauskas et al. 1991). A mixture enriched in (3*S*)-hexanol was obtained according to the same protocol.

For all synthesized compounds, ^{1}H-NMR and ^{13}C-NMR spectra of $CDCl_3$ solutions were recorded at 500 and 125 MHz by using a Varian Unity spectrometer. Chemical shifts were expressed in ppm in relation to tetramethylsilane. The starting materials were obtained from commercial suppliers and used without further purification. NMR data corresponded well with literature data (Ando et al. 1982; Gau et al. 1999).

Statistical Analyses The results, expressed as the numbers of mating pairs per group of bugs, with or without their glands occluded, were analyzed by means of a Chi-square test followed by multiple comparisons using the Bonferroni correction. Therefore, only comparisons having $P<0.003$ were considered to show a significant difference.

Results

Identification of Compounds Produced by MGs Our results showed that the MGs of *R. prolixus* are the sources of a complex mixture of volatile substances. Twelve ketones and alcohols were identified in the MGs of *R. prolixus*, with the most abundant compound being 2-methyl-3-buten-2-ol, followed by 2-pentanol, (3*E*)-2-methyl-3-penten-2-ol, and 4-methyl-3-penten-2-ol (Table 1). The same substances were detected in the MGs of both sexes. The chiral alcohols 2-butanol, 2-pentanol, 4-methyl-2-pentanol, and 3-hexanol were found as *S*-enantiomers only, while 4-methyl-3-penten-2-ol (mesityl alcohol) was found as a mixture of the two enantiomers (Table 1). Because 3-methyl-2-butanol and 2-methyl-1-butanol were present at very low concentrations and/or co-eluted with other major compounds on both of the columns used, the chiral analysis was not unequivocal.

Emission of MG Compounds by Virgin Adults MG compounds were consistently detected in the headspace of adult bugs (Fig. 1, Table 2). Detection events (i.e., each time any MG compound was detected) were recorded more frequently in both females and males during the scotophase than the

Table 1 Compounds identified in metasternal glands of *R. prolixus*

Compound	RT[a]	Retention Index[b]	Relative Amount[c] (%) ♀	Relative Amount[d] (%) ♂	Relative Amount[e] (%) ♀ + ♂
2-Butanone	2.53	909	–[f]	–[f]	–[f]
2-Pentanone	3.68	979	–[f]	–[f]	–[f]
(2*S*)-Butanol	4.92	1,030	5.7±1.4	2.2±0.7	4.0±2.1
2-Methyl-3-buten-2-ol	5.48	1,048	61±10	62.2±7.2	61±8.6
3-Methyl-2-butanol	7.31	1,108	1.1±0.5	0.9±0.3	1.0±0.4
3-Pentanol	7.99	1,120	1.4±0.4	1.1±0.2	1.2±0.4
(2*S*)-Pentanol	8.50	1,131	20±4.9	21±5.3	20±5.0
(3*E*)-2-Methyl-3-penten-2-ol	10.07	1,166	6.1±3.0	5.3±1.5	5.7±2.3
(2*S*)-4-Methyl-2-pentanol	10.80	1,181	–[f]	–[f]	–[f]
(3*S*)-Hexanol	12.00	1,207	–[f]	–[f]	–[f]
2-Methyl-1-butanol	12.49	1,217	3.5±5.1[g]	3.3±4.1[g]	3.4±4.5[g]
(2*S*/2*R*)-4-Methyl-3-penten-2-ol	14.99	1,267	2.1±1.3	2.5±0.8	2.3±1.0

[a] Retention time (SupelcoWax-10 column)
[b] Retention indices calculated according to Kováts (1965)
[c] Relative amount (mean and SD) from eight female samples
[d] Relative amount (mean and SD) from eight male samples
[e] Relative amount (mean and standard deviation) from all 16 samples
[f] Average amount ≤0.5% of total amount of compounds in sample
[g] Three (one ♂ and two ♀) out of 16 samples contained 10.5–13.1%, and the remaining 13 samples 0.0–3.4%

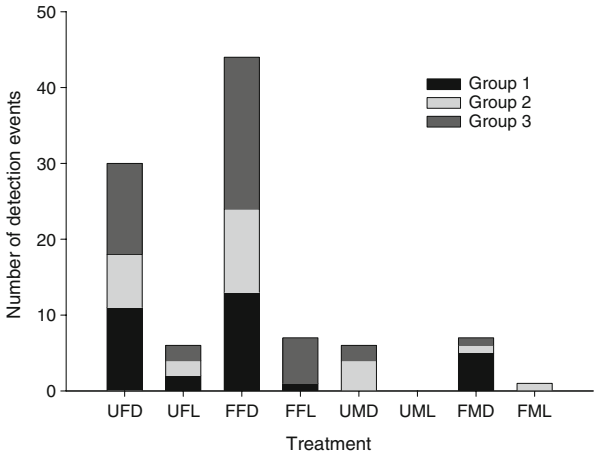

Fig. 1 Number of detection events of various *R. prolixus* metasternal gland compounds in different groups (each of three individuals) of fed/unfed males and females during the light and dark phases. *UFD* Unfed females during dark phase, *UFL* unfed females during light phase, *FFD* fed females during dark phase, *FFL* fed females during light phase, *UMD* unfed males during dark phase, *UML* unfed males during light phase, *FMD* fed males during dark phase, *FML* fed males during light phase

photophase (Fig. 1, Table 2). In general, more detection events were recorded from females than males under all experimental conditions (Fig. 1, Table 2). There was no apparent difference between unfed and fed insects.

Nine out of the 12 compounds found in the MGs were detected in the headspace over females over all the different treatments (Table 2), whereas only three of them were found in the headspace over males (Table 2). 2-Methyl-3-buten-2-ol was the most frequently detected compound in these analyses (Table 2).

Emission of MG Compounds During Copulation In 19 out of 20 assays with pairs, a successful copulation resulted. The average duration of copulation was 49.7±3.6 min. At least one of the compounds identified in the MGs was detected during 70% of the copulations. The most abundant compound produced by the MGs (2-methyl-3-buten-2-ol) was detected in 40% of the copulations. The compound most frequently found during copulation (in 60% of the samples) was 2-methyl-1-butanol. 2-Pentanone was detected in 10% of the assays.

Relevance of the Odors Emitted by MGs for the Success of Mating The percentage of copulation of untreated control pairs was 95% (Fig. 2, N=20). This was not significantly different from the percentages of copulation observed in sham-operated male and female treatments (Fig. 2). However, occlusion of female MG orifices or male MG orifices resulted in significant (P<0.003) decreases in copulation frequencies (30%, N=20 and 15%, N=20, respectively). Occlusion of both male and female orifices also resulted in a significant (P<0.003) decrease (relative to the controls) of mating percentage (15%, N=20).

Discussion

The results show that the metasternal glands of *R. prolixus* are a rich source of volatile compounds. GC-MS analysis

Table 2 The detection of *Rhodnius prolixus* metasternal gland compounds in various treatments, related to sex, feeding status, and time of day

Compound	UFD	UFL	FFD	FFL	UMD	UML	FMD	FML
2-Butanone	1	0	3	0	0	0	0	0
2-Pentanone	2	0	2	0	0	0	0	0
(2*S*)-Butanol	3	1	8	1	0	0	0	0
2-Methyl-3-buten-2-ol	7	2	11	3	4	0	6	1
3-Methyl-2-butanol	5	0	4	0	0	0	0	0
3-Pentanol	0	0	0	0	0	0	0	0
(2*S*)-Pentanol	4	1	5	1	1	0	0	0
(3*E*)-2-Methyl-3-penten-2-ol	0	1	0	1	0	0	0	0
(2*S*)-4-Methyl-2-pentanol	0	0	0	0	0	0	0	0
(3*S*)-Hexanol	0	0	0	0	0	0	0	0
2-Methyl-1-butanol	7	1	11	1	1	0	1	0
(2*S*/*R*)-4-Methyl-3-penten-2-ol	0	1	0	1	0	0	0	0
Total	29	7	44	8	6	0	7	1

Numbers indicate the detection frequency for each compound (18 SPME samples per treatment).
UFD Unfed female sampled during dark phase, *UFL* unfed female sampled during light phase, *FFD* fed female sampled during dark phase, *FFL* fed female sampled during light phase, *UMD* unfed male sampled during dark phase, *UML* unfed male sampled during light phase, *FMD* fed male sampled during dark phase, *FML* fed male sampled during light phase

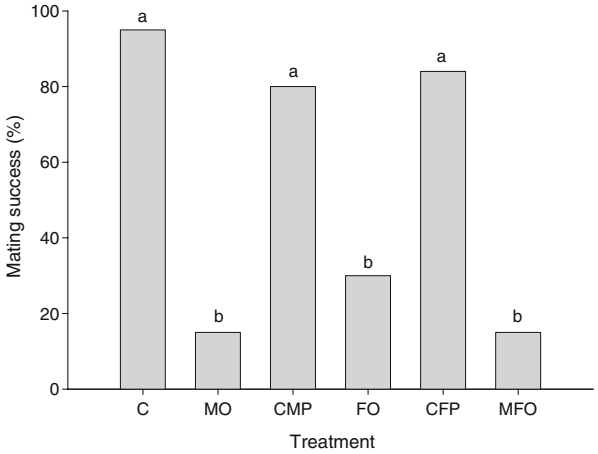

Fig. 2 Copulation (%) of *R. prolixus* pairs: *C* Control (intact) pairs, *MO* males with occluded MG orifices, *CMP* control males treated with paraffin on a different part of their body surface, *FO* females with occluded MG orifices, CFP control females treated with paraffin on a different part of their body surface, *MFO* males and females with occluded MG orifices. *Different letters atop treatments* represent significant differences (chi-square test followed by Bonferroni multiple comparisons, $P<0.003$)

revealed a mixture of 12 volatile ketones and alcohols, with the most abundant compounds being 2-methyl-3-buten-2-ol, (2*S*)-pentanol, (3*E*)-2-methyl-3-penten-2-ol, and the enantiomers of 4-methyl-3-penten-2-ol. None of these compounds had previously been reported in a triatomine species. However, 2-methyl-3-buten-2-ol has been reported as part of the aggregation pheromones of several species of bark beetles (Giesen et al. 1984; Klimetzek et al. 1989; Schlyter et al. 1992) and also as part of the alarm pheromone of the hornet wasp, *Vespa crabro* (Veith et al. 1984). 2-Pentanol has been found in the alarm pheromone of hornet wasps (Ono et al. 2003; Ono 2005), as an attractant to fruits for the coleopterans, *Carpophilus hemipterus* and *Conotrachelus nenuphar* (Phelan and Lin 1991; Prokopy et al. 2001), and as part of the defensive secretions of *Polyzosteria* and related cockroaches (Wallbank and Waterhouse 1970). To our knowledge, 2-methyl-3-penten-2-ol and 4-methyl-3-penten-2-ol have not been reported as semiochemicals for any insect species. Minor components of the secretions, 2-butanone, 2-methyl-1-butanol, and 3-hexanol have been found previously in MG secretions of *T. infestans* by Manrique et al. (2006).

Interestingly, the saturated alcohols we identified in *R. prolixus* all had an (*S*)-configuration, suggesting a common enzymatic system in their biosynthesis. In accord with what is known about the biosynthesis of 2-methyl-3-buten-2-ol in bark beetles (Lanne et al. 1989; Martin et al. 2003; Seybold et al. 2006), it is conceivable that a common allylic carbocation in the biosynthetic pathway gives rise to both (3*E*)-2-methyl-3-penten-2-ol and 4-methyl-3-penten-2-ol. Hydration at the allylic positions of the carbocation would form 2-methyl-3-penten-2-ol and 4-methyl-3-penten-2-ol. The latter addition appears not to be stereoselective as both enantiomers of 4-methyl-3-penten-2-ol are formed.

We demonstrated that the volatile compounds found in the MGs of *R. prolixus* are emitted by virgin adult bugs of both sexes. Emission of these compounds was detected more frequently from females than males. Females also released these chemicals more frequently during the early hours of the scotophase, the period when sexual activity in this species is at its peak (Manrique, personal communication). That these compounds may be involved in sexual communication is suggested by their detection, albeit in low amounts (e.g., 10–100 pg for 2-pentanol) over copulating pairs of *R. prolixus*. That only three of the MG compounds were detected over copulating pairs could have been due to the very low concentration of compounds emitted by bugs. It is worth noting that the SPME collections for analysis of MG content were of headspace above 12 glands heated to 50°C, whereas, at most, the headspace above a pair of bugs emitting volatiles consisted of the contents of four glands at 26°C. For most of the compounds identified in the glands (i.e., from two MGs), the amount was close to the detection limit of our instrument. Manrique et al. (2006) detected 3-pentanone, the main component of the MG secretions of *T. infestans*, over copulating pairs, and suggested that this species may use MG odors for communication during mating. A role for the MG odors in the sexual behavior of *R. prolixus* was further suggested by our occlusion experiments in which occlusion of the MG orifices of either males or females resulted in a significant decrease in copulation, relative to the various controls. A similar result was obtained for *T. infestans* (Crespo and Manrique 2007).

It is worth noting that we did not detect any Brindley's gland secretions during our sampling of copulating pairs. These secretions have previously been detected (Ríos Candelaria 1999; Guerenstein and Guerin 2004) over *R. prolixus* mating pairs, and it has been suggested that they may be involved in sexual communication. However, it cannot be excluded that the detection of Brindley's gland compounds in those studies may have been the result of an alarm response (Manrique et al. 2006) rather than a sexual signal. Further work is needed to clarify the role of Brindley's glands secretions in the sexual behavior of *R. prolixus*.

Overall, our data show that the release of compounds found in the MGs of adult *R. prolixus* corresponds with sexual activity of this species, and that furthermore, females

appear to release greater quantities of these compounds than males. However, whether these chemicals actually mediate sexual behavior in this species is unknown. Further work is required to determine whether the compounds are directly involved in mediating sexual behavior of adults and, if so, what is their precise role. If these chemicals are attractive to adult *R. prolixus*, they could prove useful as chemical baits in traps for monitoring or controlling *R. prolixus* populations, thereby limiting the transmission of Chagas disease to humans. The development of new methods for controlling *R. prolixus* is critical as certain populations have already developed resistance to the pyrethroid insecticides used in control programs (Zerba 1999).

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References

ANDO, W., SATO, R., YAMASHITA, M., AKASAKA, T., and MIYAZAKI, H. 1982. Quenching of singlet oxygen by 1,3,5-triaryl-2-pyrazolines. *J. Org. Chem.* 48:542–546.

BALDWIN, W. F., KNIGHT, A. G., and LYNN, K. R. 1971. A sex pheromone in the insect *Rhodnius prolixus* (Hemiptera: Reduviidae). *Can. Entomol.* 103:18–22.

BIRKETT, M. A., BRUCE, T. J. A., MARTIN, J. L., SMART, L. E., OAKLEY, J., and WADHAMS, L. J. 2004. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana*, to wheat panicle volatiles. *J. Chem. Ecol.* 30:1319–1328.

BRINDLEY, M. D. H. 1930. On the metasternal scents-glands of certain Heteroptera. *Trans. Ent. Soc. Lond.* 78:199–208.

BRENNA, E., FRONZA, G., FUGANTI, C., IGHETTI, A., and SERRA, S. 1998. Enzyme-mediated preparation of the single enantiomers of the olfactory active components of the woody odorant timberol. *Helv. Chim. Acta.* 82:1762–1773.

CRESPO, J., and MANRIQUE, G. 2007. Mating behavior of the hematophagous bug *Triatoma infestans*: Role of Brindley's and metasternal glands DOI 10.1016/j.jinsphys.2007.03.014.

CRUZ-LÓPEZ, L., MORGAN, E. D., and ONDARZA, R. N. 1995. Brindley's gland exocrine products of *Triatoma infestans*. *Med. Vet. Entomol.* 9:403–406.

CRUZ-LÓPEZ, L., MALO, E. A., OJAS, J. C., and MORGAN, E. D. 2001. Chemical ecology of triatomine bugs: vectors of Chagas disease. *Med. Vet. Entomol.* 15:351–357.

GAU, A. H., LIN, G. L., UANG, B. J., LIAO, F. L., and WANG, S. L. 1999. Regio- and diastereoselective ene reaction of 4-phenyl-1,2,4-triazoline-3,5-dione with chiral allylic alcohols and their derivatives. *J. Org. Chem.* 64:2194–2201.

GIESEN, H., OHNLE, U., VITE, J. P., PAN, M. L., and FRANCKE, W. 1984. The aggregation pheromone of the Mediterranean pine bark-beetle *Ips (Orthotomicus) erosus*. *Z. Angew. Entomol.* 98:95–97.

GUERENSTEIN, P. G., and GUERIN, P. M. 2004. A comparison of volatiles emitted by adults of three triatomine species. *Entomol. Exp. Appl.* 111:151–155.

JOHNSON, M. R., and RICKBORN, B. 1970. Sodium borohydride reduction of conjugated aldehydes and ketones. *J. Org. Chem.* 35:1041–1045.

KAZLAUSKAS, R. J., WEISSFLOCH, A. N. E., APPAPORT, A. T., and CUCCIA, L. A. 1991. A rule to predict which enantiomer of a secondary alcohol reacts faster in reactions catalyzed by cholesterol esterase, lipase from *Pseudomonas cepacia*, and lipase from *Candida rugosa*. *J. Org. Chem.* 56:2656–2665.

KLIMETZEK, D., BARTELS, J., and FRANCKE, W. 1989. The pheromone system of the elm bark beetle *Pteleobius vittatus* (F.) (Col., Scolytidae). *J. Appl. Entomol.* 107:518–523.

KOVÁTS, E. 1965. Gas chromatographic characterization of organic substances in the retention index system, pp. 229–247, in J. C. Giddings, and R. A. Keller (eds.). Advances in Chromatography, Vol1. Edward Arnold Ltd., London.

LANNE, B. S., IVARSSON, P., JOHNSSON, P., BERGSTRÖM, G., and WASSGREN, A. B. 1989. Biosynthesis of 2-methyl-3-buten-2-ol, a pheromone component of *Ips typographus* (Coleoptera: Scolytidae). *Insect Biochem.* 19:163–167.

MANRIQUE, G., and LAZZARI, C. R. 1995. Existence of sex pheromone in *Triatoma infestans* (Hemiptera: Reduviidae): I-Behavioural Evidence. *Mem. Inst. Oswaldo Cruz.* 90:645–648.

MANRIQUE, G., VITTA, A. C., FERREIRA, R. A., ZANI, C. L., UNELIUS, C. R., LAZZARI, C. R., DIOTAIUTI, L., and LORENZO, M. G. 2006. Chemical communication in Chagas disease vectors. Source, identity, and potential function of volatiles released by the metasternal and Brindley's glands of *Triatoma infestans* adults. *J. Chem. Ecol.* 32:2035–2052.

MARTIN, D., BOHLMANN, J., GERSHENZON, J., FRANCKE, W., and SEYBOLD, S. J. 2003. A novel sex-specific and inducible monoterpene synthase activity associated with a pine bark beetle, the pine engraver, *Ips pini*. *Naturwissenschaften* 90:173–179.

MONTEIRO, F. A., BARRETT, T. V., FITZPATRICK, S., CORDON-OSALES, C., FELICIANGELI, D., and BEARD, C. B. 2003. Molecular phylogeography of the Amazonian Chagas disease vectors *Rhodnius prolixus* and *R. robustus*. *Mol. Ecol* 12:997–1006.

ONO, M., TERABE, H., HORI, H., and SASAKI, M. 2003. Components of giant hornet alarm pheromone. *Nature* 424:637–638.

ONO, M. 2005. Semiochemicals that regulate social behaviour of hornets. *Aroma Res.* 6:230–236.

PHELAN, P. L., and LIN, H. 1991. Chemical characterization of fruit and fungal volatiles attractive to dried-fruit beetle, *Carpophilus hemipterus* (L.) (Coleoptera: Nitidulidae). *J. Chem. Ecol.* 17:1253–1272.

PROKOPY, R. J., PHELAN, P. L., WRIGHT, S. E., MINALGA, A. J., BARGER, R., and LESKEY, T. C. 2001. Compounds from host fruit odor attractive to adult plum curculios (Coleoptera: Curculionidae). *J. Entomol. Sci.* 36:122–134.

ROJAS, J. C., RÍOS-CANDELARIA, E., CRUZ LÓPEZ, L., SANTIESTEBAN, A., BOND-COMPEAN, J. G., BRINDIS, Y., and MALO, E. A. 2002. A reinvestigation of Brindley's gland exocrine compound of *Rhodnius prolixus* (Hemiptera: Reduviidae). *J. Med. Entomol.* 39:256–265.

RÍOS-CANDELARIA, E. 1999. Análisis químico y conductual de los compuestos volátiles emitidos por *Rhodnius prolixus* (Hemiptera: Reduviidae), vector de la enfermedad de Chagas. PhD dissertation. Universidad Autónoma de Chiapas, México.

SCHLYTER, F., BIRGERSSON, G., BYERS, J. A., and BAKKE, A. 1992. The aggregation pheromone of *Ips duplicatus* and its role in competitive interactions with *I. typographus*. *Chemoecology* 3:103–112.

SCHOFIELD, C. J. 1994. Triatominae, biología y control. Eurocommunica Publications, United Kingdom.

SEYBOLD, S. J., HUBER, D. P. W., LEE, J. C., GRAVES, A. D., and BOHLMANN, J. 2006. Pine monoterpenes and pine bark beetles: a marriage of convenience for defense and chemical communication. *Phytochem. Rev.* 5:143–178.

STAVINOHA, J. L., MARIANO, P. S., LEONE-BAY, A., SWANSON, R., and BRACKEN, C. 1981. Photocyclizations of *N*-allyliminium salts leading to the production of substituted pyrrolidines. *J. Am. Chem. Soc.* 103:3148–3160.

VEITH, H. J., KOENIGER, N., and MASCHWITZ, U. 1984. 2-Methyl-3-butene-2-ol, a major component of the alarm pheromone of the hornet *Vespa crabro*. *Naturwissenschaften* 71:328–329.

WALLBANK, B. E., and WATERHOUSE, D. F. 1970. The defense secretions of *Polyzosteria* and related cockroaches. *J. Insect Physiol.* 16:2081–2096.

WARD, J. P. 1981. A comparison of the behavioural responses of the haematophagous bug, *Triatoma infestans* to synthetic homologues of two naturally occurring chemicals (*n*- and isobutyric acid). *Physiol. Entomol.* 6:325–329.

WORLD HEALTH ORGANIZATION. 2005. Report of the Scientific Working Group on Chagas Disease. http://www.who.int/tdr/diseases/chagas/default.htm.

ZERBA, E. N. 1999. Susceptibility and resistance to insecticides of Chagas disease vectors. *Medicina-Buenos Aires* 59:41–46.

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Age-Dependent Changes in the Chemistry of Exocrine Glands of *Bombus terrestris* Queens

Klára Urbanová · Lucie Cahlíková · Oldřich Hovorka · Vladimír Ptáček · Irena Valterová

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Abstract Extracts of three different glands (mandibular, labial, and Dufour's) of virgin *Bombus terrestris* queens at ten different ages (1–8, 12, and 18 days) were analyzed for chemical composition. One hundred and twenty-seven compounds were identified in the extracts. The mandibular and labial glands contained previously reported electroantennogram-active compounds (3-hydroxydecanoic acid, fatty acids of different chain lengths, their esters, and heptacosene). These compounds reached a maximum concentration in 3- to 7-d-old queens. Geranylcitronellol was found in both labial and Dufour's glands. Its amount was inversely correlated to age of queens.

Keywords Apidae · *Bombus terrestris* · Bumblebees · Queens · Gland secretions · Sex pheromone · 3-hydroxydecanoic acid · Hymenoptera · Bombini

K. Urbanová · O. Hovorka · I. Valterová (✉)
Institute of Organic Chemistry and Biochemistry,
Academy of Sciences of the Czech Republic,
Flemingovo nám. 2,
166 10 Praha 6, Czech Republic
e-mail: irena@uochb.cas.cz

K. Urbanová · L. Cahlíková
Department of Chemistry of Natural Compounds,
Institute of Chemical Technology,
Technická 5,
166 28 Praha, Czech Republic

V. Ptáček
Department of Animal Physiology and Immunology,
Faculty of Science, Masaryk University,
Kotlářská 2,
637 11 Brno, Czech Republic

Introduction

Chemical communication in bumblebees has been studied since the late 1960s when male-marking pheromones were first described (Calam 1969; Kullenberg et al. 1970). Since then, male pheromones of a great number of species have been examined, particularly in Scandinavia (reviews Bergström et al. 1981; Morse 1982; Valterová and Urbanová 1997). These pheromones are always complex mixtures, with a species-specific composition and, as such, are useful for chemotaxonomy (Bergström and Svensson 1973; Terzo et al. 2003; Rasmont et al. 2005).

Less information is available on the chemistry of bumblebee queen sex pheromones. The experiments of Free (1971) demonstrated that the head of a bumblebee queen is important in releasing male mating behavior, suggesting that the head is a source of a pheromone. Van Honk et al. (1978) reported that the mandibular gland secretion of young virgin queens contained a sex pheromone that released mating behavior of conspecific males. Cahlíková et al. (2004) described the compositions of several exocrine gland secretions of five bumblebee species. These included secretions from mandibular, labial, and Dufour's glands of *Bombus terrestris* queens. Krieger et al. (2006) recorded electrophysiological responses of antennae of *B. terrestris* males to the queen's head and body extracts and found 21 electroantennogram (EAG)-active components, including fatty acids, their methyl and ethyl esters, ketones, hydrocarbons, and diterpenes, potentially related to sexual communication. Male mating behavior (approach, touch, and mounting) was stimulated by components of cephalic extracts, although no copulations were observed. The failure to stimulate copulation by chemicals alone suggests that a combination of different stimuli such as visual, physical (e.g., cuticular surface, hairs,

special physico-chemical or physico-mechanical properties of the surface), and chemicals may be needed (Krieger et al. 2006).

In the search for the source of the sex pheromone, we present here results on changes in quantities of the EAG-active components reported by Krieger et al. (2006) during the ontogenic development of the mandibular, labial, and Dufour's gland composition of *B. terrestris* queens. Furthermore, we correlate the quantities of some of these compounds with the mating behavior of queens as published earlier by Přidal and Hofbauer (1996) and Tasei et al. (1998).

Methods and Materials

Insects A colony of *B. terrestris* was established by the known two-queen cascade method (Ptáček et al. 2000) to stimulate queen egg laying in the laboratory. All mother queens were taken from their natural habitats during the search period to minimize the possible negative influence of artificial conditions on progeny. When the colonies turned to queen production, the queen cocoons were removed from the parental hives and allowed to mature separately. These cocoons were cared for by several bumblebee workers (Ptáček 1999). Freshly emerged queens were removed and kept separate according to age.

Virgin queens, 1 ($N=9$), 2 ($N=8$), 3 ($N=8$), 4 ($N=10$), 5 ($N=11$), 6 ($N=9$), 7 ($N=11$), 8 ($N=11$), 12 ($N=5$), and 18 ($N=5$) -d-old, were killed by freezing (−18°C) and kept frozen before dissection. Mandibular, labial, and Dufour's glands were dissected and extracted with hexane containing 1-bromodecane as internal standard (2.2 mg/ml, 30 µl per gland; purity 97%, Fluka). After 15 min of shaking and 1 hr of standing at ambient temperature, the extracts were removed and kept at −18 °C before analysis.

Chromatography Extracts were analyzed by using a gas chromatograph with a splitless injector (200°C) and a mass detector (200 °C, Fisons MD 800). A ZB-5ms column (30 m×0.25 mm, film thickness 0.25 µm, Zebron, Phenomenex, U.S.A.) and helium gas, at a constant flow of 1 ml min^{-1}, were used for analyses. The temperature program started at 70°C and, after a 2-min delay, increased at 10°C min^{-1} to 320°C.

Compound Identification and Quantification Compounds were identified by comparison of their mass spectra to spectra in the NIST library, as well as by co-chromatography with synthetic (Valterová et al. 1996) or commercially available standards.

Double bond positions of compounds were determined from the mass spectra of dimethyl disulfide (DMDS) adducts (Francis 1981; Vincenti et al. 1987). The proportions of isomeric alkenes were calculated from the relative intensities of characteristic fragments in the spectra of DMDS adducts. Acids and hydroxy acids in the mandibular gland extract were methylated with diazomethane (50 µl per gland extract; Klimetzek et al. 1989) before analysis.

Amounts of compounds of interest were expressed as proportions of the peak area of the compound to the peak area of the internal standard.

Results and Discussion

One hundred and twenty-seven compounds were identified in the extracts of three different glands (mandibular, labial, and Dufour's) of virgin *B. terrestris* queens. These compounds included hydrocarbons (saturated and unsaturated, straight-chain and methyl-branched), fatty acids and their esters, and isoprenoids. Many of the compounds were present in minor or trace quantities only. Tables 1, 2, 3 summarize the more abundant (>1%, or >0.5% for mandibular gland) components present in the extracts. Although the variability among specimens in a given age category was great, the mean and median values were almost identical, indicating a normal distribution of data.

Recently, Krieger et al. (2006) published EAG-active components from both head extracts and surface washes of *B. terrestris* queens. Although most of these compounds were present in our samples (see below), there were some differences, possibly due to the different extraction procedures (diethyl ether vs. hexane, 24 hr vs. 75 min extraction time). Components of queen *B. terrestris* head extract can originate from both mandibular and labial gland (or the hypopharyngeal gland, which was not included in our study because it does not appear to be involved in communication; Pereboom 2000). On the other hand, all exocrine glands present in the body may be present in the surface washes studied by Krieger et al. (2006). We did not detect three of the EAG-active compounds reported by Krieger et al. (2006): 2-nonanone, dodecyl hexanoate, and geranylgeraniol. In addition, we found several compounds with unknown activity. In particular, the labial gland contained substantial amounts of geranylcitronellol and its derivatives.

Mandibular Gland Mandibular gland extracts contained lower amounts of all detected compounds compared to the labial or Dufour's glands (Cahlíková et al. 2004). The extract was characterized by the prevalence (72–93%) of fatty acids. A substantial proportion (6–24%) of unbranched hydrocarbons (both unsaturated and saturated) was also identified. These extracts were methylated before analysis because of the large amounts of acids (Table 1).

Table 1 Relative abundances of compounds (>0.5% in one or more age groups) present in the mandibular gland extract of *B. terrestris* queens of different age

Compound	Age of queens in days (number of samples); relative % [mean value (standard error)]									
	1 (*N*=9)	2 (*N*=8)	3 (*N*=8)	4 (*N*=10)	5 (*N*=11)	6 (*N*=9)	7 (*N*=11)	8 (*N*=11)	12 (*N*=5)	18 (*N*=5)
Alkanes										
Tridecane	0.8 (0.2)	0.9 (0.2)	1.3 (0.2)	0.8 (0.2)	1.2 (0.3)	1.4 (0.2)	0.9 (0.1)	0.8 (0.2)	0.3 (0.1)	0.1 (<0.1)
Tetradecane	7.0 (1.9)	7.4 (1.6)	11.6 (1.6)	8.1 (2.0)	10.1 (2.9)	10.0 (2.2)	6.6 (1.1)	6.8 (1.5)	2.8 (0.3)	2.2 (0.2)
Pentadecane	0.5 (0.1)	0.5 (0.1)	1.0 (0.2)	0.6 (0.2)	0.9 (0.2)	0.9 (0.2)	0.5 (0.1)	0.7 (0.2)	0.4 (0.1)	0.1 (<0.1)
Tricosane	0.4 (0.1)	0.8 (0.1)	1.3 (0.4)	0.7 (0.2)	1.2 (0.2)	1.3 (0.2)	1.0 (0.2)	1.2 (0.2)	0.6 (0.1)	0.6 (0.1)
Pentacosane	0.3 (0.1)	0.4 (0.1)	0.7 (0.2)	0.4 (0.1)	0.9 (0.4)	0.6 (0.1)	0.7 (0.3)	0.4 (0.1)	0.2 (<0.1)	0.2 (<0.1)
Heptacosane	0.6 (0.2)	0.5 (0.1)	0.9 (0.1)	0.5 (0.1)	0.7 (0.1)	0.7 (0.2)	0.5 (0.1)	0.6 (0.2)	0.3 (0.1)	0.2 (<0.1)
Alkenes										
Undecene[a]	1.1 (0.3)	1.3 (0.4)	1.6 (0.2)	1.4 (0.3)	1.8 (0.5)	2.0 (0.6)	1.5 (0.3)	1.4 (0.3)	0.3 (<0.1)	0.3 (0.1)
Heptacos-9-ene	0.2 (0.1)	1.0 (0.6)	1.3 (0.7)	1.0 (0.2)	1.0 (0.3)	2.6 (0.6)	1.8 (0.5)	1.6 (0.4)	1.3 (0.6)	0.9 (0.2)
Heptacos-7-ene	0.3 (0.1)	0.2 (0.1)	0.2 (<0.1)	0.2 (0.1)	0.5 (0.2)	0.6 (0.2)	0.4 (0.1)	0.5 (0.2)	0.6 (0.2)	0.4 (0.1)
Nonacos-10-ene	0.5 (0.3)	0.5 (0.2)	0.6 (0.4)	0.8 (0.2)	0.5 (0.1)	1.3 (0.4)	0.5 (0.1)	0.4 (0.2)	0.9 (0.3)	0.4 (<0.1)
Nonacos-9-ene	1.7 (0.5)	1.4 (0.4)	2.6 (1.0)	2.1 (0.6)	2.4 (0.4)	2.5 (0.6)	1.4 (0.2)	1.4 (0.3)	1.8 (0.5)	0.7 (0.1)
Acids										
3-Hydroxyoctanoic acid	7.0 (1.4)	6.8 (1.7)	6.8 (1.0)	6.9 (1.4)	10.9 (1.3)	10.0 (2.3)	10.9 (1.4)	11.1 (1.8)	10.0 (1.1)	6.3 (1.5)
3-Hydroxydecanoic acid	11.7 (2.2)	13.2 (3.6)	13.6 (2.4)	12.9 (3.3)	23.9 (3.6)	18.0 (3.9)	23.7 (4.1)	25.4 (3.7)	17.6 (1.8)	15.1 (2.5)
3-Hydroxydodecanoic acid	0.3 (0.1)	0.5 (0.2)	0.3 (0.1)	0.7 (0.2)	0.8 (0.2)	0.4 (0.1)	0.7 (0.2)	0.6 (0.1)	0.3 (0.2)	0.2 (0.1)
Dodecanoic acid	0.3 (0.1)	0.3 (<0.1)	0.2 (<0.1)	0.6 (0.2)	0.5 (0.2)	0.4 (0.1)	0.4 (0.1)	0.2 (0.1)	0.3 (<0.1)	0.2 (<0.1)
Tetradecanoic acid	1.3 (0.2)	1.8 (0.2)	1.4 (0.1)	2.3 (0.4)	1.4 (0.2)	2.2 (0.3)	1.5 (0.2)	1.1 (0.2)	1.3 (0.1)	0.9 (0.1)
Hexadec-11-enoic acid	1.4 (0.2)	1.0 (0.4)	1.0 (0.2)	1.8 (0.3)	0.8 (0.2)	1.8 (0.5)	0.7 (0.2)	1.1 (0.3)	0.9 (0.1)	0.7 (0.1)
Hexadec-9-enoic acid	0.7 (0.2)	1.2 (0.4)	0.8 (0.3)	0.8 (0.2)	1.0 (0.2)	0.9 (0.3)	1.5 (0.3)	0.6 (0.2)	1.4 (0.2)	2.3 (0.2)
Hexadecanoic acid	6.2 (0.3)	6.0 (0.6)	5.2 (0.3)	6.3 (0.6)	3.8 (0.5)	6.0 (1.2)	4.4 (0.7)	3.6 (0.4)	4.0 (0.3)	3.6 (0.3)
Octadeca-9.12-dienoic acid	1.5 (0.2)	1.4 (0.2)	1.2 (0.3)	1.4 (0.3)	0.7 (0.1)	5.0 (3.6)	0.8 (0.1)	1.0 (0.2)	1.3 (0.1)	1.9 (0.2)
Octadec-9-enoic acid	41.5 (2.4)	39.5 (4.7)	37.0 (3.3)	38.2 (3.6)	25.3 (3.3)	22.3 (5.9)	30.5 (5.0)	31.3 (5.0)	43.7 (1.8)	52.4 (2.9)
Octadecanoic acid	11.2 (0.8)	8.6 (1.2)	6.6 (1.2)	7.9 (1.0)	4.9 (0.5)	5.4 (0.8)	5.4 (0.7)	5.2 (1.1)	7.5 (0.3)	8.9 (0.6)
Isoprenoids										
Squalene	2.7 (0.6)	3.7 (1.4)	2.1 (0.2)	2.5 (0.3)	3.3 (1.2)	2.4 (0.4)	2.8 (0.8)	1.9 (0.5)	1.3 (0.3)	0.9 (0.2)

[a] Double bond not determined (DMDS adducts not found)

The majority of aliphatic compounds found in the mandibular gland extracts have been previously reported by Krieger et al. (2006) as EAG-active, indicating potential pheromonal activity. However, we found that certain compounds, hexadecenoic acids (Δ^9 and Δ^{11}, while Krieger et al. 2006 reported Δ^7 and Δ^{11}) and heptacos-9-ene (Krieger et al. 2006 reported Δ^{12} and Δ^{13}), had different double bond positions compared to those reported by Krieger et al. (2006).

The composition of mandibular gland extracts of our virgin queens was similar to that reported by Hefetz et al. (1996) for egg laying queens, although a major difference was the absence of higher esters in our samples. These esters may be formed in glands of older or fertilized queens when the mandibular gland plays a potentially different role—that of producing queen pheromone (Van Honk et al. 1980; Röseler et al. 1981). Recently, Ayasse et al. (2007) reported preliminary results on the chemical basis of queen dominance. In cuticular washes, they found a fraction of esters that suppressed ovarian development in workers. Thus, the mandibular gland may not be the source of queen pheromone as reported in older literature.

The profile of the reported EAG-active components in mandibular glands of virgin queens appeared to be related to queen age. 3-Hydroxydecanoic acid and 3-hydroxyoctanoic acid showed a peak (35%) in 5-d-old queens (Fig. 1a), followed by a decline and then a second increase in queens 8 days and older. The abundances of these two compounds were strongly correlated ($R=0.97$; $P<10^{-6}$; $N=77$) with each other. Octadec-9-enoic (oleic) acid was the main component in mandibular gland extracts of females of all ages (22–52%), with the greatest amounts observed in older queens (12 and 18 d, Fig. 1b). Other fatty acids showed no clear maxima with respect to age of queens.

The amount of heptacos-9-ene was maximal in mandibular glands of 7-d-old queens (Fig. 2). This age-related maximum was also observed in labial gland extracts, but not in the Dufour's gland extracts, for which the amount of this compound was greatest in the oldest queens (18 d). The absolute amounts of this compound differed among the glands, with the labial and Dufour's gland extracts containing 10 times and 250 times, respectively, more heptacosene than the mandibular gland extracts.

A relatively high abundance of tetradecane was observed in queens of all ages (2–12%) in all three glands. Even-

Table 2 Relative abundances of compounds (>1% in one or more age groups) present in the labial gland extract of *B. terrestris* queens of different age

Compound	Age of queens in days (number of samples); relative % [mean value (standard error)]									
	1 (*N*=9)	2 (*N*=8)	3 (*N*=8)	4 (*N*=10)	5 (*N*=10)	6 (*N*=8)	7 (*N*=11)	8 (*N*=11)	12 (*N*=5)	18 (*N*=5)
Alkanes										
Tetradecane	8.9 (1.9)	2.4 (0.6)	2.2 (0.6)	2.0 (0.4)	0.9 (0.2)	1.5 (0.5)	0.7 (0.1)	1.3 (0.2)	0.8 (0.1)	4.7 (3.4)
Nonacosane	1.0 (0.2)	1.1 (0.2)	0.9 (0.1)	0.6 (0.1)	0.6 (0.1)	0.4 (0.1)	0.4 (0.1)	0.6 (0.1)	0.6 (0.1)	0.4 (0.1)
Alkenes										
Undecene[a]	0.9 (0.2)	0.3 (0.1)	0.3 (0.1)	0.2 (0.1)	0.2 (<0.1)	0.4 (0.2)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)	0.7 (0.5)
Heptacos-9-ene	1.1 (0.2)	1.8 (0.3)	1.2 (0.2)	2.4 (0.8)	0.8 (0.2)	1.1 (0.4)	1.3 (0.2)	1.0 (0.3)	1.2 (0.1)	4.1 (1.2)
Nonacosadiene[a]	1.2 (0.4)	0.8 (0.2)	0.1 (<0.1)	0.7 (0.1)	0.4 (0.2)	3.1 (1.8)	0.2 (<0.1)	0.3 (0.1)	0.2 (0.1)	2.2 (0.4)
Nonacos-10-ene	2.1 (0.3)	4.2 (1.2)	2.6 (0.3)	3.2 (0.4)	2.3 (0.2)	2.0 (0.5)	2.0 (0.2)	2.8 (0.3)	2.1 (0.1)	4.7 (0.5)
Nonacos-9-ene	8.7 (0.9)	7.3 (1.4)	5.9 (0.4)	6.7 (0.4)	4.6 (0.6)	6.4 (2.0)	4.0 (0.2)	5.4 (0.5)	5.2 (0.5)	5.9 (1.3)
Hentriacont-9-ene	0.5 (0.1)	1.0 (0.3)	1.0 (0.1)	1.0 (0.1)	0.5 (0.1)	0.9 (0.2)	0.5 (0.1)	1.2 (0.2)	0.4 (0.1)	0.7 (0.2)
Acids										
Hexadecanoic acid	1.4 (0.5)	0.7 (0.2)	0.2 (0.1)	0.2 (0.1)	0.1 (<0.1)	0.2 (0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (<0.1)	0.1 (0.1)
Unseparated C_{18} acids[b]	5.2 (1.8)	3.1 (0.8)	1.6 (0.6)	0.9 (0.4)	0.3 (0.1)	0.3 (0.2)	0.3 (<0.1)	0.5 (0.3)	0.1 (<0.1)	<0.1 (<0.1)
Esters										
Methyl hexadecanoate	2.3 (0.7)	2.6 (0.6)	1.9 (0.3)	1.7 (0.4)	1.2 (0.2)	1.8 (0.9)	0.7 (0.1)	0.7 (0.2)	0.1 (<0.1)	<0.1 (<0.1)
Methyl octadec-11-enoate + octadecatrienoate[c]	6.2 (1.9)	5.9 (1.3)	3.8 (0.9)	4.2 (1.4)	2.4 (0.4)	4.1 (2.0)	1.8 (0.2)	1.9 (0.5)	0.8 (0.1)	0.1 (<0.1)
Methyl octadec-9-enoate	1.6 (0.5)	1.6 (0.3)	1.3 (0.2)	1.4 (0.3)	1.1 (0.2)	1.8 (0.6)	1.1 (0.1)	0.9 (0.2)	0.5 (0.1)	0.1 (<0.1)
Ethyl dodecanoate	<0.1 (<0.1)	0.2 (0.1)	1.2 (0.4)	0.5 (0.2)	0.4 (0.1)	0.8 (0.3)	0.6 (0.2)	0.2 (0.1)	<0.1 (<0.1)	<0.1 (<0.1)
Ethyl octadec-11-enoate	1.1 (0.3)	0.9 (0.1)	0.5 (0.1)	0.5 (0.1)	0.3 (0.1)	0.8 (0.2)	0.4 (0.1)	0.3 (0.1)	0.9 (0.2)	0.1 (<0.1)
Dodecyl dodecanoate	0.2 (0.1)	1.8 (0.6)	4.5 (0.5)	3.9 (0.7)	6.7 (0.7)	3.5 (0.8)	7.5 (0.6)	3.9 (0.6)	5.2 (0.7)	1.1 (0.3)
Dodecyl tetradecanoate	0.4 (<0.1)	2.1 (0.5)	3.6 (0.5)	3.5 (0.6)	6.7 (0.5)	2.8 (0.8)	7.4 (0.7)	4.5 (0.5)	4.2 (0.4)	1.2 (0.2)
Dodecyl hexadec-11-enoate	1.4 (0.5)	0.6 (0.1)	0.2 (0.1)	0.2 (0.1)	0.4 (0.1)	0.3 (0.1)	0.5 (0.1)	0.7 (0.2)	0.4 (0.1)	0.2 (0.1)
Dodecyl hexadec-9-enoate + hexadecanoate[c]	1.2 (0.4)	4.5 (1.4)	8.5 (0.7)	9.4 (1.2)	14.4 (0.9)	11.2 (2.1)	15.6 (0.5)	14.0 (1.2)	18.6 (1.1)	13.5 (2.6)
Dodecyl octadec-11-enoate	5.6 (1.0)	8.1 (0.9)	10.3 (1.0)	10.6 (1.5)	12.8 (1.5)	10.6 (2.7)	11.2 (1.4)	12.0 (1.2)	12.0 (0.9)	14.1 (1.8)
Dodecyl octadecatrienoate[a]	10.3 (1.4)	17.2 (2.5)	21.5 (2.0)	20.7 (1.6)	24.6 (1.9)	19.0 (4.1)	22.9 (2.1)	26.7 (2.7)	34.3 (2.2)	35.2 (2.6)
Isoprenoids										
Geranylcitronellol	11.2 (1.5)	10.6 (2.7)	6.5 (1.5)	4.3 (1.0)	2.9 (1.0)	1.8 (1.2)	3.6 (1.6)	1.5 (1.1)	0.1 (<0.1)	<0.1 (<0.1)
GC[d] dodecanoate	0.4 (0.1)	1.3 (0.5)	2.9 (0.5)	2.6 (0.5)	1.7 (0.4)	3.9 (0.7)	4.3 (0.4)	1.9 (0.6)	1.1 (0.2)	1.4 (0.4)
GC[d] octadecenoate[a]	3.2 (0.8)	1.6 (0.3)	1.3 (0.3)	1.5 (0.5)	0.4 (0.1)	1.1 (0.2)	0.9 (0.1)	0.7 (0.2)	0.2 (<0.1)	0.1 (<0.1)
GC[d] octadecenoate + GC[d] octadecadienoate[a,c]	3.5 (1.2)	1.6 (0.3)	1.5 (0.2)	1.6 (0.5)	0.5 (0.1)	1.5 (0.3)	1.0 (0.1)	1.0 (0.3)	0.3 (<0.1)	0.2 (0.1)
Squalene	2.7 (2.2)	1.2 (0.4)	0.4 (0.1)	0.3 (0.1)	0.2 (0.1)	1.2 (0.7)	0.2 (<0.1)	0.4 (0.1)	0.8 (0.2)	0.6 (0.2)

[a] Double bond positions not determined (DMDS adducts not found)
[b] Unseparated mixture of octadecenoic, octadecatrienoic, and octadecanoic acids
[c] Unseparated mixture
[d] *GC* Geranylcitronellyl

numbered chain hydrocarbons are generally uncommon in bumblebees. The relatively high amounts of this compound suggest that its biological significance may be worth studying.

According to Tasei et al. (1998), the majority of *B. terrestris* queens mate at age 6.1±0.4 days, although this varied between colonies (7.8±0.6 days in one, and 3.0± 0.3 days in another). Přidal and Hofbauer (1996) observed that the greatest percentage (30%) of mating took place in 6- to 7-d-old queens, although a relatively high percentage of queens (15%) also mated at 5-d-old. Thus, the EAG-active compounds in the mandibular glands that peak in 5-d-old queens may be important in sexual communication in *B. terrestris*.

Labial Gland Several compounds reported by Krieger et al. (2006) as EAG-active were found in the labial gland extracts (Table 2). The extracts consisted mostly of esters (30–70%, increasing with age), acids (0–7%, decreasing with age), isoprenoids (2–22%, decreasing with age), and hydrocarbons (10–25%, no pattern with age).

Table 3 Relative abundances of compounds (>1% in one or more age groups) present in the Dufour's gland extract of *Bombus terrestris* queens of different age

Compound	Age of queens in days (number of samples); relative % [mean value (standard error)]									
	1 (*N*=9)	2 (*N*=8)	3 (*N*=8)	4 (*N*=10)	5 (*N*=11)	6 (*N*=7)	7 (*N*=11)	8 (*N*=11)	12 (*N*=5)	18 (*N*=5)
Alkanes										
Tetradecane	11.1 (5.8)	4.1 (2.4)	1.3 (0.6)	0.4 (0.1)	2.5 (1.6)	0.8 (0.2)	0.4 (0.1)	1.5 (0.8)	0.4 (0.1)	0.2 (<0.1)
Henicosane	1.6 (0.5)	1.1 (0.5)	0.9 (0.2)	1.2 (0.2)	1.8 (0.2)	0.9 (0.1)	2.0 (0.3)	1.5 (0.2)	1.5 (0.2)	1.5 (0.1)
Tricosane	2.9 (0.6)	4.5 (0.7)	3.2 (0.3)	3.2 (0.3)	6.3 (0.6)	3.8 (0.4)	5.2 (0.7)	6.3 (0.8)	5.4 (0.5)	6.0 (0.7)
Pentacosane	1.8 (0.2)	2.2 (0.2)	1.7 (0.1)	1.7 (0.2)	2.4 (0.3)	1.9 (0.2)	1.8 (0.2)	2.0 (0.2)	2.0 (0.1)	2.0 (0.2)
Heptacosane	5.9 (1.4)	1.0 (0.6)	3.9 (0.4)	2.4 (0.5)	1.1 (0.5)	2.7 (0.5)	1.6 (0.5)	0.8 (0.4)	<0.1 (<0.1)	<0.1 (<0.1)
Octacosane	0.3 (0.1)	0.1 (<0.1)	0.9 (0.5)	0.3 (0.1)	0.4 (0.2)	0.1 (<0.1)	0.1 (<0.1)	0.3 (0.1)	0.1 (<0.1)	0.1 (<0.1)
Nonacosane	6.4 (1.9)	2.3 (0.6)	2.5 (0.3)	1.7 (0.4)	2.1 (0.5)	4.3 (1.9)	1.0 (0.4)	2.0 (0.8)	0.6 (0.4)	1.2 (0.2)
Hentriacontane	0.1 (<0.1)	0.2 (0.1)	0.1 (<0.1)	0.4 (0.2)	0.9 (0.7)	0.3 (0.1)	0.5 (0.4)	0.2 (<0.1)	0.3 (0.1)	0.4 (0.1)
Alkenes										
Undecene[a]	1.2 (0.6)	0.6 (0.3)	0.2 (0.1)	0.1 (<0.1)	0.3 (0.2)	0.1 (<0.1)	<0.1 (<0.1)	0.2 (0.1)	<0.1 (<0.1)	<0.1 (<0.1)
Pentacos-11-ene	0.8 (0.2)	0.7 (0.1)	0.6 (0.1)	0.8 (0.1)	1.4 (0.2)	1.3 (0.1)	2.0 (0.3)	1.9 (0.3)	3.0 (0.4)	3.4 (0.3)
Hexacos-9-ene	0.1 (<0.1)	0.2 (0.1)	0.1 (<0.1)	0.3 (0.1)	0.4 (0.1)	0.2 (<0.1)	0.3 (<0.1)	1.3 (0.9)	0.4 (<0.1)	0.3 (<0.1)
Heptacos-10-ene	2.9 (0.7)	6.5 (1.5)	7.4 (1.2)	4.6 (1.7)	1.0 (0.6)	1.2 (0.9)	<0.1 (<0.1)	13.3 (3.0)	0.2 (0.1)	0.8 (0.3)
Heptacos-9-ene	2.9 (0.3)	2.6 (0.6)	4.1 (1.2)	6.7 (1.4)	11.1 (1.5)	13.8 (1.9)	15.1 (2.8)	5.4 (2.3)	12.7 (4.1)	23.2 (1.1)
Heptacos-8-ene	3.1 (0.8)	2.3 (0.5)	1.0 (0.5)	2.4 (0.5)	3.2 (0.5)	1.4 (0.7)	3.0 (0.9)	1.0 (0.4)	2.8 (1.2)	1.6 (0.4)
Heptacos-7-ene	<0.1 (<0.1)	3.5 (1.1)	<0.1 (<0.1)	1.2 (0.6)	3.4 (0.7)	1.9 (1.0)	1.1 (0.4)	0.9 (0.4)	0.6 (0.5)	1.5 (0.5)
Nonacos-9-ene	19.6 (4.0)	21.6 (3.1)	26.7 (1.7)	32.7 (1.6)	29.5 (2.2)	23.6 (2.4)	29.5 (2.7)	24.8 (1.2)	26.8 (1.0)	23.2 (0.8)
Hentriacont-11-ene	1.7 (0.3)	2.1 (0.5)	1.6 (0.5)	2.0 (0.5)	2.3 (0.4)	2.3 (0.6)	1.7 (0.3)	3.4 (0.8)	1.2 (0.4)	1.9 (0.1)
Hentriacont-9-ene	1.4 (0.3)	1.4 (0.3)	3.1 (0.9)	3.0 (0.5)	1.0 (0.3)	1.5 (0.4)	0.8 (0.5)	1.5 (0.8)	1.5 (0.3)	0.5 (0.1)
Alkadienes										
Heptacosadiene[a]	0.1 (<0.1)	0.1 (<0.1)	0.2 (<0.1)	0.3 (0.1)	0.5 (0.1)	0.7 (0.2)	1.4 (0.4)	0.8 (0.2)	2.4 (0.2)	0.7 (0.3)
Heptacosa7,17-diene	0.1 (<0.1)	0.1 (0.1)	0.1 (<0.1)	0.1 (<0.1)	0.3 (<0.1)	0.8 (0.4)	4.0 (2.2)	0.8 (0.3)	9.7 (4.7)	2.5 (0.8)
Octacosadiene[a]	0.2 (0.1)	0.2 (0.1)	0.2 (<0.1)	0.8 (0.2)	0.8 (0.3)	0.2 (0.1)	0.5 (0.1)	1.5 (0.8)	0.1 (<0.1)	0.3 (0.1)
Nonacosa-12,16-diene	0.7 (0.1)	2.8 (0.8)	2.4 (0.3)	3.2 (0.2)	3.5 (0.3)	3.9 (0.6)	4.1 (0.3)	4.4 (0.5)	5.1 (0.5)	6.0 (0.5)
Nonacosa-9,19-diene	3.6 (1.3)	3.9 (1.5)	1.7 (0.5)	1.2 (0.1)	2.5 (0.8)	3.0 (1.2)	2.2 (0.7)	1.9 (0.7)	2.1 (0.3)	2.1 (0.1)
Hentriacontadiene[a]	0.2 (0.1)	0.5 (0.2)	0.8 (0.2)	1.0 (0.2)	0.9 (0.1)	1.2 (0.2)	1.0 (0.1)	1.3 (0.2)	0.7 (0.2)	1.1 (0.1)
Acids										
Unseparated C_{18} acids[b]	4.2 (1.5)	4.8 (3.3)	3.1 (2.5)	0.2 (0.1)	0.6 (0.2)	0.8 (0.3)	0.2 (0.1)	0.2 (0.1)	0.1 (0.1)	0.3 (0.1)
Esters										
Ethyl octadec-9-enoate	0.7 (0.3)	1.6 (1.1)	1.1 (0.3)	0.4 (0.1)	0.8 (0.2)	1.0 (0.4)	1.0 (0.3)	0.3 (0.2)	0.5 (0.1)	0.2 (0.1)
Decyl octadec-9-enoate	0.8 (0.5)	0.2 (0.1)	0.4 (0.1)	0.3 (0.1)	0.2 (<0.1)	0.4 (0.1)	0.2 (0.1)	0.2 (<0.1)	0.2 (<0.1)	0.2 (<0.1)
Hexadecyl octadecenoate[a]	7.2 (1.8)	5.4 (1.7)	7.4 (1.1)	6.0 (1.4)	1.7 (0.5)	3.4 (0.6)	1.7 (0.3)	1.1 (0.3)	1.0 (0.3)	0.7 (0.1)
Hexadecyl octadecanoate[a]	0.2 (0.1)	1.7 (1.1)	0.1 (0.1)	0.6 (0.5)	0.2 (0.1)	0.1 (<0.1)	0.2 (0.1)	0.9 (0.4)	<0.1 (<0.1)	<0.1 (<0.1)
Octadecenyl octadecadienoate[a]	1.2 (0.4)	0.5 (0.2)	1.4 (0.6)	1.6 (0.5)	0.4 (0.1)	0.2 (0.1)	0.8 (0.2)	0.4 (0.2)	0.6 (0.1)	0.6 (0.1)
Unseparated C_{18} esters[c]	2.1 (0.5)	5.2 (1.8)	6.2 (1.5)	4.5 (1.1)	2.5 (0.7)	5.1 (1.2)	2.5 (0.5)	1.7 (0.5)	1.3 (0.1)	1.3 (0.1)
Icosyl octadecenoate[a]	0.3 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)	0.7 (0.1)	0.7 (0.2)	0.6 (0.1)	0.9 (<0.1)	1.1 (<0.1)
Isoprenoids										
Geranylcitronellol	0.6 (0.3)	2.7 (2.2)	0.5 (0.2)	0.2 (<0.1)	0.4 (0.1)	0.2 (<0.1)	1.3 (0.5)	0.7 (0.5)	0.9 (0.3)	0.4 (0.1)
GC[d] octadecenoate[a]	1.2 (0.4)	1.3 (0.6)	5.7 (1.3)	3.8 (1.0)	2.2 (0.4)	3.5 (0.6)	2.5 (0.4)	2.7 (0.7)	3.8 (0.4)	1.9 (0.1)
GC[d] octadecenoate[a]	0.5 (0.2)	0.1 (0.1)	0.9 (0.4)	1.3 (0.4)	1.2 (0.3)	4.3 (1.7)	1.2 (0.3)	1.8 (0.5)	2.2 (0.4)	4.2 (0.5)
GC[d] octadecanoate	0.3 (0.2)	1.3 (0.6)	0.5 (0.2)	0.4 (0.1)	0.4 (0.2)	0.7 (0.3)	0.3 (0.1)	0.5 (0.1)	0.5 (0.1)	0.5 (0.1)
Squalene	3.3 (1.9)	1.9 (1.7)	0.3 (0.1)	0.2 (<0.1)	0.8 (0.4)	0.4 (0.2)	0.1 (<0.1)	0.2 (<0.1)	0.1 (<0.1)	0.1 (<0.1)

[a] Double bond position not determined (DMDS adducts not found)
[b] Unseparated mixture of octadecenoic, octadecadienoic, and octadecatrienoic acids
[c] Unseparated mixture of octadecatrienyl octadecenoate, octadecadienoyl octadecenoate, and octadecyl octadecanoate
[d] *GC* Geranylcitronellyl

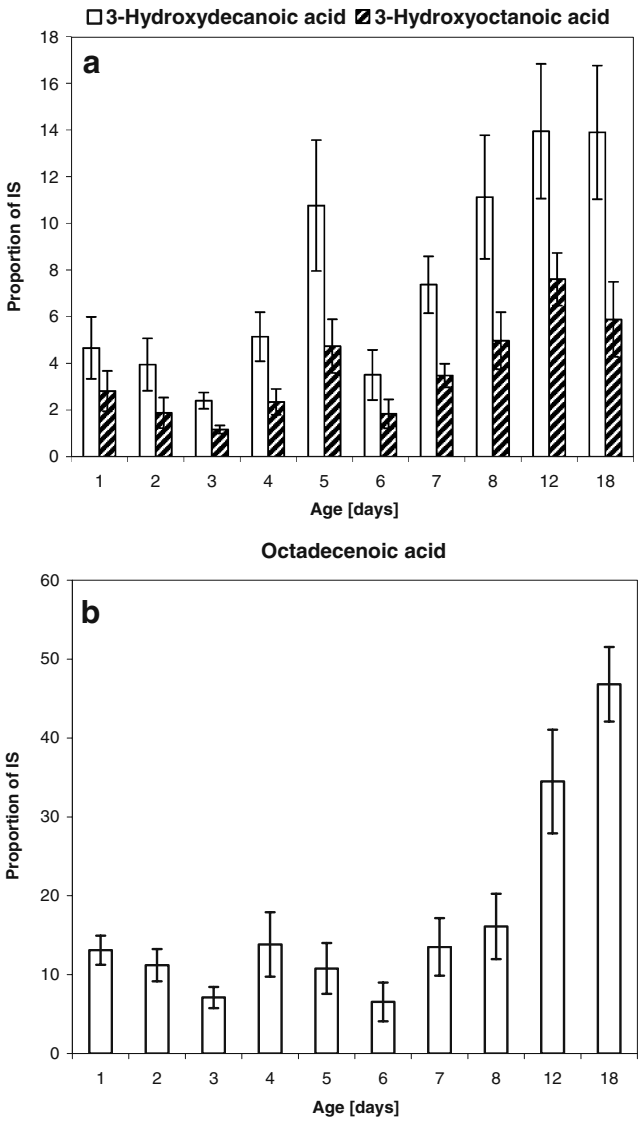

Fig. 1 Content (percentage of area of a gas chromatograph peak to the internal standard, IS) of hydroxy acids (**a**) and octadec-9-enoic acid (**b**) in the mandibular glands of different age *B. terrestris* queens. *Bars* represent mean±SE

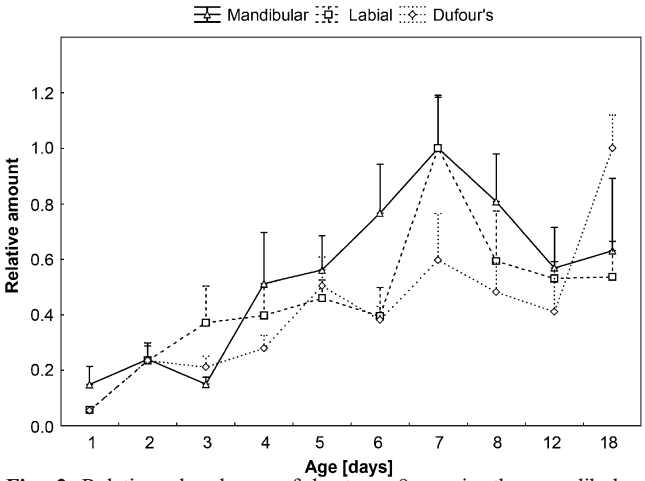

Fig. 2 Relative abundance of heptacos-9-ene in the mandibular, labial, and Dufour's glands of different aged *B. terrestris* queens. Data are mean±SE

The amounts of methyl and ethyl esters of fatty acids reached a maximum in 3- to 7-d-old queens (Fig. 3). Hexadec-9-enoic (palmitoleic) and hexadecanoic (palmitic) acids were maximal in the labial glands of 6-d-old queens (Fig. 4a). The amount of fatty acids was about ten times lower in the labial gland than in the mandibular gland. The amounts of higher esters increased with age as shown in the example of dodecyl octadecenoate (Fig. 4b).

As found for mandibular gland compounds, some compounds in the labial glands had different double bond positions than those reported by Krieger et al. (2006). Hexadec-9-enoic acid and its methyl ester were found in our samples, while Krieger et al. 2006 reported Δ^7 and Δ^{11}. Seven heptacosene isomers (Δ^7 to Δ^{13}) were detected in our samples, whereas Krieger et al. 2006 reported only Δ^{12} and Δ^{13} isomers as EAG-active compounds.

Hydroxy acids were not found in the labial gland extracts. Although Krieger et al. (2006) detected geranylgeraniol in head extracts, geranylcitronellol was the most abundant (2–11%) isoprenoid detected in our labial gland extracts. We found geranylcitronellol in both labial and Dufour's gland extracts, but not in the extracts of the mandibular gland. In the labial gland, its amount peaked in 3-d-old queens (Fig. 5). The difference in the presence of geranylgeraniol (Krieger et al. 2006) and geranylcitronellol (our work) is interesting. Krieger et al. (2006) failed to obtain good separation of geranylgeraniol from linoleic

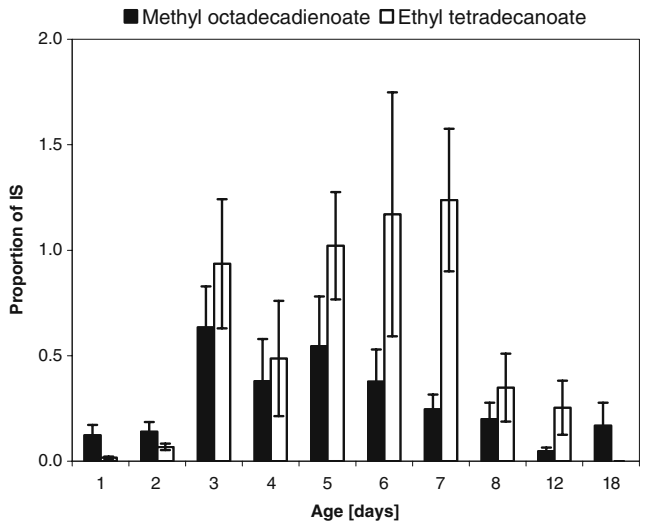

Fig. 3 Content (percentage of area of a gas chromatograph peak to the internal standard, IS) of selected fatty acid esters in the labial glands of different aged *B. terrestris* queens. *Bars* represent mean±SE

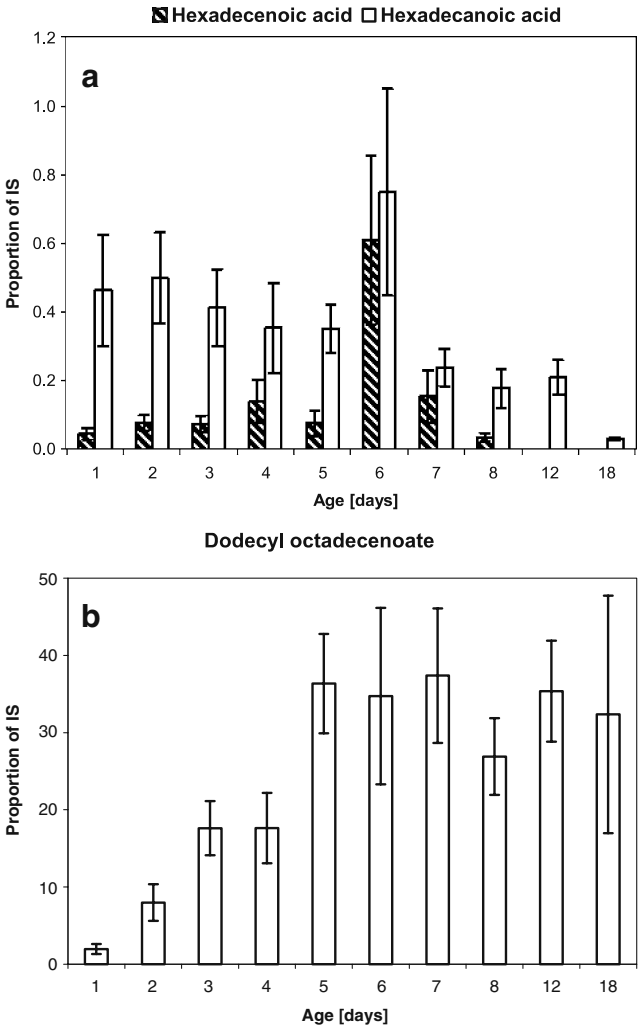

Fig. 4 Content (percentage of area of a gas chromatograph peak to the internal standard, IS) of selected fatty acids (**a**) and dodecyl octadec-11-enoate (**b**) in the labial glands of different aged *B. terrestris* queens. *Bars* represent mean±SE

acid, thereby precluding them from determining which component actually elicited an EAG response. In preliminary EAG tests, we found geranylcitronellol elicited responses from both males' and workers' antennae (Kalinová, unpublished results), suggesting this compound may play a role in communication.

Reports on the chemical composition of the labial gland in bumblebee queens are scarce. Preliminary analyses of *Bombus hypnorum* were reported by Genin et al. (1984). Hefetz et al. (1996) studied the chemistry of several glands of egg laying *B. terrestris* queens. Our data on labial gland composition of young virgin queens is similar to theirs (Hefetz et al. 1996) for egg laying queens, except we found large amounts of geranylcitronellol, whereas they detected esters of this alcohol with fatty acids of different chain lengths. As the relative proportion, as well as the quantity, of geranylcitronellol in the labial gland decreases with queen age (Table 2, Fig. 5), it seems likely that increasing esterification in older females consumes this alcohol.

Dufour's Gland The majority of compounds present in the Dufour's gland extracts were hydrocarbons (65–80%), with alkanes (10–32%) and alkenes (32–56%) being the major constituents. Tricosane and nonacos-9-ene were the most abundant hydrocarbons, with alkadienes of medium abundance (5–20%) and branched alkanes present in trace amounts only (<0.5%). Beside hydrocarbons, acids (0.1–5%), methyl esters (4–17%), and isoprenoids (5–9%) were detected in the Dufour's gland extracts. Among all the compounds we identified in the Dufour's gland, only five (heptacos-12-ene, heptacos-13-ene, oleic acid, linoleic acid, and linolenic acid) were reported by Krieger et al. (2006) as EAG active. All five of these compounds were present in only minor or trace amounts (< 5%) in our Dufour's gland extracts and did not show any specific pattern in amount with respect to queen age.

Geranylcitronellol was produced in low quantities in Dufour's glands of young queens, and its amount increased with age (7 d and older, Fig. 5). Geranylcitronellol was detected previously in the Dufour's gland of *B. terrestris* workers (Tengo et al. 1991), but its function was not determined. However, it has been described in several bumblebee species as a component of the male marking pheromone (see review by Valterová and Urbanová 1997). Geranylcitronellol appears to be a common compound in different bumblebee species, and it may play different roles in different contexts.

The Dufour's gland is one of the best studied glands, with regard to chemistry, in bees. Its primary function is the

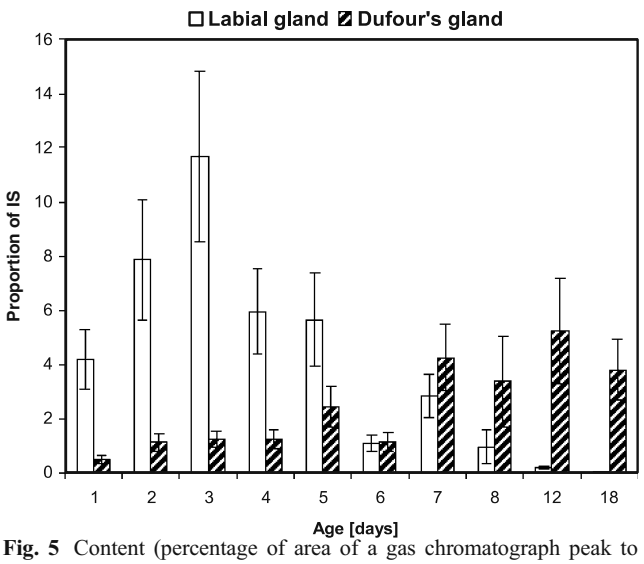

Fig. 5 Content (percentage of area of a gas chromatograph peak to the internal standard, IS) of geranylcitronellol in the labial and Dufour's glands of different age *B. terrestris* queens. *Bars* represent mean±SE

production of a hydrophobic secretion for the isolation of brood cells (Hefetz 1987). In bumblebees, the Dufour's gland has several functions, not all of which are fully understood. Three main functions have been determined: (1) marking of the nest entrance or construction of an odor trail from the nest entrance to the core of the nest in underground nesting species (Cederberg 1977; Pouvreau 1996), (2) nest-mate (kin) recognition (Tengo et al. 1991; Hefetz et al. 1993), and (3) egg marking (Ayasse et al. 1999). Social nest parasites (*Psithyrus*) are known to recognize and prefer the nest odor of host bumblebee species (Cederberg 1979; Fisher 1984; Foster and Gamboa 1989).

With regard to the findings of Krieger et al. (2006), the sex pheromone of queens is likely to be produced in some of the cephalic glands. Thus, a role for the Dufour's gland secretion in sexual communication seems unlikely. Furthermore, most of the compounds found in this gland were not reported as EAG-active by Krieger et al. (2006). Our results suggest that the sexual pheromone of *B. terrestris* will likely be present in either the mandibular or the labial glands, possibly in the mandibular gland where the amounts of active compounds correlate with the receptivity of queens. We suggest that hydroxy acids, with their high abundance and timing of maximum quantities, are the most likely candidates for constituents of the sex pheromone of *B. terrestris* queens and should be studied further in behavioral tests.

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References

AYASSE, M., BIRNBAUM, J., TENGÖ, J., VAN DOORN, A., TAGHIZADEH, T., and FRANCKE, W. 1999. Caste- and colony-specific chemical signals on eggs of the bumble bee, *Bombus terrestris* L. (Hymenoptera: Apidae). *Chemoecology* 9:119–126.

AYASSE, M., SRAMKOVA, A., KUCHLER, M., VAN DOORN, A., SCHULZ, C., and FRANCKE, W. 2007. Chemical ecology and dominance signals in the bumble bee *Bombus terrestris*. 23rd ISCE Annual Meeting, Jena, Germany. Book of Abstracts, p. 37.

BERGSTRÖM, G., and SVENSSON, B. G. 1973. Studies on natural odoriferous compounds. *Chem. Scr.* 4:231–238.

BERGSTRÖM, G., SVENSSON, B. G., APPELGREN, M., and GROTH, I. 1981. Complexity of bumble bee marking pheromones: biochemical, ecological and systematical interpretations, pp. 175–183, in P. J. Howse, and J.-L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London, New York.

CAHLÍKOVÁ, L., HOVORKA, O., PTÁČEK, V., and VALTEROVÁ, I. 2004. Exocrine gland secretions of virgin queens of five bumblebee species (Hymenoptera: Apidae, Bombini). *Z. Naturforsch., C:Biosci.* 59:582–589.

CALAM, D. H. 1969. Species and sex-specific compounds from the heads of male bumblebees (*Bombus* spp.). *Nature* 221:856–857.

CEDERBERG, B. 1977. Evidence for trail marking in *Bombus terrestris* workers (Hymenoptera, Apidae). *Zoon* 5:143–146.

CEDERBERG, B. 1979. Odour guided host selection in *Psithyrus* (Hym., Apidae). *Ent. Tidskr.* 100:128–129.

FISHER, R. M. 1984. Recognition of host nest odour by the bumblebee parasite *Psithyrus ashtoni* (Hymenoptera: Apidae). *J. N. Y. Entomol. Soc.* 9:503–507.

FRANCIS, G. W. 1981. Alkylthiolation for the determination of double-bond positions in unsaturated fatty-acid esters. *Chem. Phys. Lipids* 29:369–374.

FREE, J. B. 1971. Stimuli eliciting mating behaviour of bumblebee (*Bombus pratorum* L.) males. *Behaviour* 40:55–61.

FOSTER, R. L., and GAMBOA, G. J. 1989. Nest entrance marking with colony specific odors by the bumble bee *Bombus occidentalis* (Hymenoptera: Apidae). *Ethology* 81:273–278.

GENIN, E., JULLIEN, R., PEREZ, F., FONTA, C., and MASSON, C. 1984. Données préliminaires sur les médiateurs chimiques du Bourdon *Bombus hypnorum* (Hyménoptère, Apoïdea, Bombinae). *C. R. Séances Acad. Sci., Sér. 3* 299:297–302.

HEFETZ, A. 1987. The role of Dufour's gland secretions in bees. *Physiol. Entomol.* 12:243–253.

HEFETZ, A., TENGÖ, J., LÜBKE, G., and FRANCKE, W. 1993. Inter-colonial and intra-colonial variation in Dufour's gland secretion in the bumble bee species *Bombus hypnorum* (Hymenoptera: Apidae), pp. 469–480, in K. Wiese, S. Kapitsky, and G. Benninger (eds.). Arthropod sensory systems. Birkhäuser Verlag, Basel, Boston, Berlin.

HEFETZ, A., TAGHIZADEH, T., and FRANCKE, W. 1996. The exocrinology of the queen bumble bee *Bombus terrestris*. *Z. Naturforsch, C:Biosci.* 51:409–422.

KLIMETZEK, D., KÖHLER, J., KRIHN, S., and FRANCKE, W. 1989. Das Pheromon-System des Waldreben-Borkenkfers, *Xylocleptes bispinus* Duft (Col. Scolytidae). *J. Appl. Entomol.* 107:304–309.

KRIEGER, G. M., DUCHATEAU, M.-J., VAN DOORN, A., IBARRA, F., FRANCKE, W., and AYASSE, M. 2006. Identification of queen sex pheromone components of the bumblebee *Bombus terrestris*. *J. Chem. Ecol.* 32:453–471.

KULLENBERG, B., BERGSTRÖM, G., and STÄLLBERG-STENHAGEN, S. 1970. Volatile components of the cephalic marking secretion of male bumblebees. *Acta Chem. Scand.* 24:1481–1483.

Morse, D. H. 1982. Behavior and ecology of bumble bees, pp. 245–322, in H. R. Hermann (ed.). Social Insects, vol. III. Academic Press, New York.

PEREBOOM, J. J. M. 2000. The composition of larval food and the significance of exocrine secretions in the bumblebee *Bombus terrestris*. *Insectes Soc.* 47:11–20.

POUVREAU, A. 1996. Le marquage de l' entrée du nid de bourdons. *Entomol. Exp. Appl.* 80:355–364.

PŘIDAL, A., and HOFBAUER, J. 1996. Laboratory rearing and nutrition of young queens of bumblebee (*Bombus terrestris* L.) from emergence to diapause. *Sci. Studies Res. Inst. Fodder Plants Troubsko* 14:125–131.

PTÁČEK, V. 1999. Obtaining and overwintering young bumble bee (Hymenoptera Bombinae) queens. In Insect pollination in greenhouses. Proceedings of the specialist's meeting held in Soesterbeg, The Netherlands, 30 Sept.–2 Oct. 1999. Utrecht, The Netherlands: Apimondia, ICPBR-Pollination Section., 2000. 55–57.

PTÁČEK, V., PERNOVÁ, E., and BOROVEC, R. 2000. The two-queen cascade method as an alternative technique for starting bumble bee (*Bombus,* Hymenoptera, Apidae) colonies in laboratory (Preliminary study). *Pszczel. Zesz. Nauk.* 44:305–309.

RASMONT, P., TERZO, M., AYTEKIN, M., HINES, H., URBANOVÁ, K., CAHLÍKOVÁ, L., and VALTEROVÁ, I. 2005. Cephalic secretions of

the bumblebee subgenus *Sibiricobombus* Vogt suggests *Bombus niveatus* Kriechbaumer and B*ombus vorticosus* Gerstaecker are conspecific (Hymenoptera, Apidae, *Bombus*). *Apidologie* 36:571–584.

RÖSELER, P.-F., RÖSELER, I., and VAN HONK, C. G. J. 1981. Evidence for inhibition of corpora allata activity in workers of *Bombus terrestris* by a pheromone from the queen's mandibular glands. *Experientia* 37:348–351.

TASEI, J.-N., MOINARD, C., MOREAU, L., HIMPENS, B., and GUYONNAUD, S. 1998. Relationship between aging, mating and sperm production in captive *Bombus terrestris*. *J. Apicultural Res.* 37:107–113.

TERZO, M., VALTEROVÁ, I., URBANOVÁ, K., and RASMONT, P. 2003. De la nécessité de redécrire les phéromones sexuelles des mâles de bourdons [Hymenoptera: Apidae, Bombini] publiées avant 1996 pour leur utilisation en analyse phylogénétique. *Phytoprotection* 83:39–49.

TENGÖ, J., HEFETZ, A., BERTSCH, A., SCHMITT, U., LÜBKE, G., and FRANCKE, W. 1991. Species specificity and complexity of Dufour's gland secretion of bumble bees. *Comp. Biochem. Physiol.* 99B:641–646.

VALTEROVÁ, I., SVATOŠ, A., and HOVORKA, O. 1996. Analysis of the labial gland secretion of the cuckoo-bumblebee (*Psithyrus vestalis*) males and synthesis of abundant geranylcitronellol. *Collect. Czech. Chem. Commun.* 61:1501–1508.

VALTEROVÁ, I., and URBANOVÁ, K. 1997. Chemical signals of bumblebees. *Chem. Listy* 91:846–857(in Czech).

VAN HONK, C. G. J., VELTHIUS, H. H. W., RÖSELER, P.-F., and MALOTAUX, M. E. 1980. The mandibular glands of *Bombus terrestris* queens as a source of queen pheromones. *Entomol. Exp. Appl.* 28:191–198.

VAN HONK, C. G. J., VELTHIUS, H. H. W., RÖSELER, P.-F. 1978. A sex pheromone from the mandibular glands in bumblebee queens. *Experientia* 34:838–839.

VINCENTI, M., GUGLIEMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double bonds position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. *Anal. Chem.* 59:694–699.

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Homofarnesals: Female Sex Attractant Pheromone Components of the Southern Cowpea Weevil, *Callosobruchus chinensis*

Kenji Shimomura · Satoshi Nojima · Shunsuke Yajima · Kanju Ohsawa

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Abstract The southern cowpea weevil, *Callosobruchus chinensis* (Coleoptera: Bruchidae), is a major pest of stored legumes in warm temperate and tropical climates. The female sex attractant pheromone was extracted from filter-paper shelters taken from containers that housed virgin females. The extracts were purified by various chromatographic techniques, and the biologically active components in the fractions were screened by gas chromatographic–electroantennographic detection analysis with male antennae. Two compounds that elicited electrophysiological responses were isolated, and gas chromatography–mass spectrometry, nuclear magnetic resonance, and micro-chemical analyses suggested that the active compounds were homofarnesals, (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienals. Males of *C. chinensis* were significantly attracted to filter paper discs loaded with the synthetic compounds at 0.01–0.1 ng compared to solvent control in a Y-tube olfactometer assay. These pheromone components represent unique chemical structures within the genus *Callosobruchus*.

Keywords Bruchidae · *Callosobruchus chinensis* · (2*Z*,6*E*)- and (2*E*,6*E*)-7-Ethyl-3,11-dimethyl-2,6,10-dodecatrienal · GC-EAD · Homofarnesal · Seed beetle · Sex attractant pheromone · Southern cowpea weevil

K. Shimomura (✉) · S. Yajima · K. Ohsawa
Department of Biosciences, Faculty of Applied Bioscience,
Tokyo University of Agriculture,
Tokyo 156-8502, Japan
e-mail: k3shimom@nodai.ac.jp

S. Nojima
Department of Entomology and W. M. Keck Center
for Behavioral Biology, North Carolina State University,
Raleigh, NC 27695, USA

Introduction

Seed beetles of the genus *Callosobruchus* (Coleoptera: Bruchidae), especially *Callosobruchus chinensis* and *Callosobruchus maculatus*, are economically important pests of stored legumes (Labeyrie 1981; Fujii et al. 1990). Females of these species lay their eggs on seeds, and newly emerged larvae immediately bore into the seeds. They spend their entire larval stage in the seeds, excavating the inside to feed, and before they pupate, larvae mine escape routes for adult emergence. Each larva consumes a considerable amount of the seed content (Hariri 1981; Rees 1995). These species are polyvoltine and can reproduce continuously under suitable conditions (Watanabe 1990; Rees 1995), resulting in rapid population increases when compared to other stored products pests (Imura 1990; Rees 1995). Thus, seed damage by these beetles can be devastating.

The host ranges and distributions of *C. chinensis* and *C. maculatus* are relatively wide when compared with congeneric pest species (reviewed in Tuda et al. 2005), and they are expanding their habitats *via* international trade in legumes (Yoneda et al. 1990). However, the life histories of these two species are different. While *C. maculatus* has been reported only in cultivated and stored legumes (reviewed in Tuda et al. 2005), *C. chinensis* has the capability to infest not only cultivated host plants in the field and stored legumes but also a few wild legumes (Shinoda and Yoshida 1985, 1990; Shinoda et al. 1991, 1992; Tuda et al. 2005). Shinoda and Yoshida (1990) suggested that populations that complete their life cycle on wild hosts could serve as reservoirs and a source of both field and storage infestation, making *C. chinensis* a potentially more threatening and persistent pest than *C. maculatus*.

Synthetic pesticides and fumigants have been employed to control seed beetles and other stored product pests (White and Leesch 1996; Heaps 2006). However, because these chemicals are not environmentally safe and pose human health concerns, they need to be replaced with alternative tools (Subramanyam and Hagstrum 2000).

Chemical signals are indispensable for almost all insects for communication between conspecific individuals (e.g., mate finding, social organization, and defense) and for the recognition of specific features of the environment (e.g., for finding food sources, preferred hosts, and suitable oviposition habitats) (Cardé and Bell 1995). These chemical cues, i.e., semiochemicals, can be an alternative tool for pest control. In fact, some semiochemicals, e.g., female sex attractant pheromones of moths, have been used directly to suppress pest populations (Minks and Cardé 1997). These agents generally act on only the target pest species and are harmless to natural enemies and other beneficial insects and humans (Minks and Cardé 1997).

Several classes of semiochemicals have been studied in *C. chinensis*, including oviposition stimulants from host seeds (Ueno et al. 1990; Matsumoto et al. 1994; Tebayashi et al. 1995; Ignacimuthu et al. 2000), an oviposition-marking pheromone (Oshima et al. 1973; Sakai et al. 1986; Honda and Ohsawa 1990), and a female sex pheromone (Tanaka et al. 1981). The process of mate location and mating in *C. chinensis* is mediated by two distinct pheromonal signals: a sex attractant pheromone that attracts males from a distance and a contact sex pheromone that elicits copulation behavior from males (Tanaka et al. 1981). The contact sex pheromone has been characterized as a mixture of (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid and C_{25}–C_{35} straight chain and methyl branched hydrocarbons (Tanaka et al. 1981, 1982), but the sex attractant pheromone has not been identified despite its high potential for pest control applications (Burkholder and Ma 1985; Phillips 1997).

In this paper, we report the isolation and identification of the female sex attractant pheromone of *C. chinensis* and discuss the unique structural properties of its components relative to previously identified pheromones of congeneric species.

Methods and Materials

Insects A laboratory colony was used in the experiment. Insects were reared on *Vigna angularis* in a dark incubator at 27°C and 50% relative humidity. Newly emerged adults were sexed and held apart until they were used.

Pheromone Collection: 1. Volatile Trapping A glass chamber (500 ml) with a charcoal filter tube in the inlet was used for the collection of airborne female sex attractant pheromone. The pheromone in the headspace was drawn (approximately 1.0 l/min) by a vacuum pump through a Tenax TA tube (1.5 g, 80/100 mesh, 12 mm i.d., 90 mm length; GL Science, Inc., Tokyo, Japan). The airborne collections were made for 7 d from 1,000 virgin females, and then, the pheromone was eluted from the adsorbent with 10 ml pentane (Sigma-Aldrich Inc., St. Louis, USA). The extracts were combined and concentrated in a nitrogen stream in an ice bath and kept in a freezer (−30°C) until they were used.

Pheromone Collection: 2. Filter Paper Extraction Preliminary gas chromatographic–mass spectrometric (GC-MS) analysis of volatile pheromones obtained by air-borne collections suggested that the pheromone components are novel compounds and that they occur in small amounts in the volatile collection. Therefore, pheromone collection was scaled up by using the filter paper collection method (Tanaka et al. 1981) to facilitate the isolation of sufficient amounts of the pheromone components for nuclear magnetic resonance (NMR) analysis. Virgin females were kept in a group in glass containers (90 mm i.d., 100 mm height) with corrugated filter paper shelters (90 mm i.d., Advantec No.2, Toyo Roshi Kaisha, Ltd., Tokyo, Japan) for 14–20 d. Filter paper shelters were collected from the containers and Soxhlet-extracted with pentane for 24 hr. The solvent was evaporated under reduced pressure, and the extracts were kept at −30°C until they were used. Extracts were collected from approximately 32,000 virgin females.

Column Chromatography Crude pheromone extracts were fractionated by column chromatography with 1 g of silica gel (Wakogel C-200, Wako Pure Chemical Industries Ltd., Osaka, Japan). The column was eluted sequentially with 10 ml each of pentane–diethyl ether in ratios of 10:0 (*v*/*v*), 9.5:0.5, 9:1, 8:2, 5:5, and 0:10.

High Performance Liquid Chromatography Active fractions were further purified by high performance liquid chromatography (HPLC) with LC-5A (Shimadzu, Kyoto, Japan) and a silica gel column (Shim-pack HRC-SIL, 5 µm, 4.6×250 mm, Shimadzu). The solvent system comprised of a pentane–diethyl ether gradient, programmed as follows: 0% ether in pentane for 1 min, then increased linearly to 50% diethyl ether at 1%/min. Flow rate was set at 1 ml/min, and the effluent was monitored with a SPD-2A (Shimadzu) variable wavelength detector at 254 nm, a wavelength providing high sensitivity for conjugated chemicals, and collected every 1 min.

Preparative Gas Chromatography Electroantennographic detection (EAD) active compounds were isolated by a

micro-preparative GC integrated with an NMR sample preparation technique (Nojima et al. 2004). An HP 5890 series II GC (Palo Alto, CA, USA) equipped with a ZB-1 wide bore capillary column (30 m×0.53 mm i.d., 1.50 μm film thickness; Phenomenex, Torrance, CA, USA) was operated in splitless mode. Nitrogen was used as a carrier gas at a head pressure of 21 kPa and a flow rate of 4 ml/min. Oven temperature was set initially at 60°C for 3 min, then increased at 10°C/min to 280°C, and held for 10 min. The injector and preparative collection port temperatures were set at 250°C and 150°C, respectively. A pre-cleaned collection trap, consisting of a 40-cm long deactivated wide bore capillary tube (Agilent, Palo Alto, CA, USA), was connected to the end of the separation column with a press fit connecter (Alltech Associates, Inc., Deerfield, IL, USA). The other end of the trap was set into the preparative GC outlet port so its end protruded a few centimeters above the port.

To collect an active component, the trap was drawn out of the port just before the retention time of a target component, and a preconditioned cooling sheath (Nojima et al. 2004) was set on the exposed collection trap. Retention times of active components were established before preparative GC work by flame ionization detector (FID) under the same analytical conditions. After the sample collection window was over, the sheath was removed and the collection trap pulled out to detach it from the press fit connector. The collection trap was immediately set up for NMR sample preparation and eluted with $CDCl_3$ (99.996%, Isotec, Miamisburg, OH, USA) directly into a 5 mm i.d. NMR micro tube (Shigemi, Tokyo, Japan).

Coupled Gas Chromatographic–Electroantennographic Detection Analysis GC-EAD technique was used for a high throughput screening tool to check male antennal active compounds in fractions to facilitate purification processes. An HP 5890 series II GC was modified for GC-EAD analyses (Nojima et al. 2003). A DB-5 or DB-WAX capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness; J&W scientific, Folsom, CA, USA) was used for the analyses. Nitrogen was used as the carrier gas at a head pressure of 135 kPa and a flow rate of 2 ml/min. Samples were injected in the GC in splitless mode, and the purge valve was off for 1 min. Oven temperature was set initially at 60°C for 3 min, then increased at 10°C/min to 220°C for DB-WAX or 280°C for DB-5, and held for 10 min. Injector temperature was set at 250°C for DB-5 and 230°C for DB-WAX, and FID and EAD outlet temperatures were set at 280°C. The column effluent was combined with nitrogen make-up gas (30 ml/min) and then split 1:1 to the FID and EAD. The EAD outlet was secured in a charcoal-filtered and humidified air stream flowing at approximately 300 ml/min over the antennal preparation in a sample delivery glass tube (12 mm i.d., 100 mm length).

A male antenna was plucked from the head of the beetle, and the tip of the antenna was cut with scissors on moistened filter paper. Then, the antenna was bridged between Ag–AgCl electrodes bathed in 0.5% NaCl solution. The antennal preparation was placed inside of the sample delivery tube. The output signal from the antenna was amplified 10-fold by a customized high-input impedance DC amplifier (Nojima et al. 2003). The outlet signal was filtered by a resistance/capacitor high-pass filter with the cut-off frequency of about 0.5 Hz. A dual tracking constant voltage power supply (SK Electronics CO. LTD., Fukuoka, Japan) was used for the amplifier at ±15 V. The EAD signals were recorded on a D-2500 Chromatography integrator (Hitachi, Tokyo, Japan) synchronized with the GC integrator.

Gas Chromatographic–Mass Spectrometric Analyses Gas chromatography–electron impact mass spectrometry (GC-EI-MS) was carried out with a Shimadzu GC 17A equipped with a DB-5 capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness) in splitless mode, coupled to a Shimadzu QP-5000 quadrupole MS in EI mode. Helium was used as the carrier gas at a head pressure of 100 kPa and a flow rate of 1.6 ml/min. Oven temperature was set initially at 60°C for 3 min, increased at 10°C/min to 280°C, and held for 10 min. The injector and interface temperatures were set at 250°C and 280°C, respectively. Gas chromatography–chemical ionization mass spectrometry (GC-CI-MS) was performed on a Shimadzu GCMS-QP2010. Iso-butane was used as the reagent gas at 50 kPa, and other analytical conditions were the same as those of GC-EI-MS analysis.

Nuclear Magnetic Resonance Analysis ^{1}H-NMR analysis of an isolated EAD active compound was performed on a JNM-ECA 400 MHz (JEOL, Tokyo, Japan), while all NMR spectra of synthetic compounds were recorded on a JNM-A400 (JEOL; 400 MHz for ^{1}H and 100 MHz for ^{13}C) in $CDCl_3$. Chemical shifts were expressed in parts per million relative to TMS.

Microchemical Analysis: 1. N,N-Dimethylhydrazone Derivatization An aliquot of the isolated EAD active compounds in hexane (approximately 300 female equivalents) was mixed with a solution of *N*,*N*-dimethylhydrazine in hexane (0.1%, 50 μl) in a 600 μl glass vial. The solution was vigorously mixed, and then the reaction mixture was subjected to GC-EI-MS.

Microchemical Analysis: 2. Ozonolysis A microgenerator of ozone was prepared (Beroza and Bierl 1966, 1967) with oxygen (3 ml/min) passing through a high-voltage spark generated by Tesla lamp checker HF-20 (Shinko Electric &

Instrumentation Co. Ltd., Osaka, Japan). A slow stream of ozone was bubbled in a vial containing an aliquot of the isolated EAD active compound in hexane. To prevent the sample from evaporating, the vial was cooled in a dry ice/acetone bath. After bubbling for 20 sec, a few microliters of a solution of triphenylphosphine in dichloromethane were added to the reaction mixture to reduce the ozonides to carbonyl compounds, and the reactants were analyzed by GC-MS.

Synthesis: 1. Nonstereospecific Preparation of 7-Ethyl-3,11-Dimethyl-2,6,10-Dodecatrienal Isomers A mixture of (2*Z*,6*Z*)-, (2*Z*,6*E*)-, (2*E*,6*Z*)-, and (2*E*,6*E*)-methyl 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienoates was prepared (Mori et al. 1971) and converted to the corresponding aldehydes via alcohols as follows. One molar diisobutylaluminium hydride in hexane solution (16 ml, 16 mmol) was added dropwise to a solution of the mixture of methyl dodecatrienoates (1.6 g, 6.1 mmol) in dry hexane (20 ml) at −78°C under argon. After 10 min stirring, methanol and dilute HCl solution were added, and the reaction mixture was allowed to warm to room temperature. Then, the mixture was extracted with ether, washed with H_2O, saturated $NaHCO_3$ solution and saturated NaCl solution, dried with $MgSO_4$, and evaporated. The residue was purified by column chromatography on silica gel eluted with hexane–EtOAc (3:1) to give a mixture of 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienols (1.4 g, 5.9 mmol, 97%). An activated MnO_2 (16.5 g) was added to the mixture of the dodecatrienols (1.4 g, 5.9 mmol) in hexane solution (100 ml) at 0°C and stirred for 8 hr. After removing the MnO_2, the residue was concentrated and purified by column chromatography on silica gel eluted with hexane–EtOAc (3:1) to give an isomeric mixture of 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienals (1.2 g, 5.1 mmol, 86%). MS: (2*Z*,6*Z*)-isomer: 234 (M^+, 0.6), 205 (2), 150 (6), 137 (5), 109 (12), 107 (12), 95 (39), 84 (36), 81 (32), 69 (71), 55 (32), 41 (100). MS: (2*Z*,6*E*)-isomer: 234 (M^+, 0.4), 205 (2), 150 (7), 137 (6), 109 (16), 107 (12), 95 (52), 84 (38), 81 (49), 69 (69), 55 (44), 41 (100). MS: (2*E*,6*Z*)-isomer: 234 (M^+, 2), 205 (5), 151 (11), 137 (7), 109 (19), 107 (18), 95 (55), 84 (72), 81 (29), 69 (84), 55 (42), 41 (100). MS: (2*E*,6*E*)-isomer: 234 (M^+, 1), 205 (2), 150 (10), 137 (12), 109 (17), 107 (17), 95 (50), 84 (80), 81 (33), 69 (86), 55 (47), 41 (100).

Synthesis: 2. (2Z/E,6E)-7-Ethyl-3,11-Dimethyl-2,6,10-Dodecatrienals A mixture of (2*Z*,6*E*)- and (2*E*,6*E*)-methyl 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienoate was prepared (Mori 1972) and converted to the corresponding aldehydes in the same way as described in nonstereospecific preparation of the isomers to give a mixture of (2*Z*,6*E*/*Z*)-7-ethyl-3,11-dimethyl-2,6,10-dodecanals (585 mg, 2.5 mmol, 83% from methyl ester). The geometrical isomers were separated and purified by preparative silica HPLC (isocratic solvent system at 3% EtOAc in hexane) resulting in (2*Z*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal (>95% chemical purity, and >99% isomeric purity by GC and HPLC analyses, respectively) and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal (>95% chemical purity and >97% isomeric purity by GC and HPLC analyses, respectively). (2*Z*,6*E*)-7-Ethyl-3,11-dimethyl-2,6,10-dodecatrienal; δH (ppm) 9.93 (1H, d), 5.88 (1H, d), 5.06 (2H, m), 2.60 (2H, t), 2.26 (2H, q, *J*=7.3 Hz), 1.94–2.05 (6H, br), 1.68 (3H, s), 1.60 (3H, s), 1.56 (3H, s), 0.96(3H, t, *J*=7.3 Hz), δC (ppm) 191.18, 164.32, 143.36, 131.84, 128.91, 124.49, 121.94, 36.70, 31.90, 27.06, 25.99, 25.45, 23.49, 22.96, 18.01, 13.54.

(2*E*,6*E*)-7-Ethyl-3,11-dimethyl-2,6,10-dodecatrienal; δH (ppm) 10.00 (1H, d), 5.89 (1H, d), 5.06 (2H, m), 2.24 (2H, m), 2.18 (2H), 1.97–2.06 (6H, br), 1.68 (3H, s), 1.60 (3H, s), 1.56 (3H, s), 0.96(3H, t), δC (ppm) 191.95, 164.33, 142.91, 131.81, 127.77, 124.58, 122.30, 40.85, 36.30, 26.66, 25.47, 25.19, 23.00, 17.44, 17.39, 12.88.

Behavioral Assays A Y-tube olfactometer (15 mm i.d. glass Y-tube, 70 mm long arms connected to a 100-mm long stem at 120° angle) was used for behavioral assays. The assay was carried out at 27°C and 55% relative humidity. Newly emerged males were kept singly in a glass tube (8 mm i.d. × 30 mm long) both ends of which were covered with nylon mesh. Males were conditioned in an assay room in the dark overnight. The lights in the assay room were turned on 3 hr before assays to acclimatize the test insects to the light conditions. A test sample in hexane was applied to a filter paper disc (6 mm i.d.). The disc was inserted in the end of one arm of the olfactometer after the solvent had evaporated, while a control disc treated with hexane alone was inserted in the end of the other arm. Then, the nylon mesh cover of the glass tube containing the male was removed, and the tube was introduced into the end of the Y-tube olfactometer. Immediately, a vacuum pump was connected to the stem, and air filtered through an activated-charcoal cartridge was drawn through the Y-tube from the stem at approximately 1 l/min.

The number of males that reached the discs within 10 min was recorded. Attraction to the individual synthetic pheromone components was tested against the solvent-only control at various doses, whereas attraction to mixtures of synthetic pheromone components at various ratios was tested against a pure synthetic pheromone component [either the (2*Z*,6*E*)- or the (2*E*,6*E*)-isomer] at one dose (10 ng). Thirty males were tested in for each comparison. The distribution of males between the two stimuli in the comparison was subjected to a binomial test (two-tailed test with test proportion set at 0.5) (Siegel 1956). Males that did not reach either stimulus during the test were not included in the data analysis.

Results

In a preliminary experiment, GC-EAD analyses of headspace volatiles collected from virgin female *C. chinensis* consistently revealed three EAD active compounds A, B, and C (Fig. 1). Since GC-MS analysis of headspace collection suggested that pheromone components are novel compounds and occur small amounts in the collection, pheromone collection was scaled up by using the filter paper collection method (Tanaka et al. 1981). Crude pheromone extracts were accumulated from approximately 32,000 virgin females and fractionated by silica gel column chromatography, and active compounds were monitored by GC-EAD analysis. All EAD active compounds were found in the 5% ether fraction, and the fraction was further purified by normal-phase HPLC. The EAD active compounds were found in the 20–21 min (compound A) and 22–23 min (compounds B and C) fractions, respectively. Compounds A and B were further purified by preparative GC, and a relatively concentrated sample of compound A (approximately 27,000 female equivalent, approximately 20 μg/$CDCl_3$ 0.4 ml) was subjected to NMR analysis. We did not pursue the purification and chemical identification of compound C, which gave relatively weak EAD responses and was present in relatively small amounts in the extracts, less than 10% of compounds A and B, in this study.

GC-EI-mass spectra of compounds A (Fig. 2a) and B (Fig. 2b) showed a base peak at *m/z* 41 and almost identical characteristic fragments at *m/z* (percent intensity relative to the base peak) 81 (46), 84 (35), and 150 (9) for compound A, and *m/z* 81 (31), 84 (66), and 150 (9) for compound B, suggesting that these are closely related compounds. In GC-CI-MS, compounds A and B showed an identical set of characteristic fragments at *m/z* 217 $[M + 1\text{-}H_2O]^+$ (100), 235 $[M + 1]^+$ (40), 277 $[M + 43]^+$ (3) and 291 $[M + 57]^+$ (5), indicating a molecular weight of 234.

The ^{1}H-NMR spectrum of compound A was difficult to interpret due to signals that likely originated from contaminants, especially in the high-field region. However, verifiable and clearly distinguishable signals were observed at δ 9.90 (d, 1H, *J*=8.2 Hz), 5.88 (d, 1H, *J*=8.2 Hz), 5.06 (m, 2H) 2.59 (t, 2H), 2.26 (q, 2H, *J*=7.4 Hz), and 0.95 (t, 3H, *J*=7.4 Hz). Obscured signals that overlapped with contaminant signals were observed around δ 1.56–2.08. The presence of an aldehyde group is evident from one proton doublet at δ 9.90, which is coupled to an olefinic proton at δ 5.88, indicating α,β-unsaturated aldehyde moiety. The presence of an ethyl group is also evident in the spectrum from the three-proton triplet at δ 0.95 and two-proton quartet at δ 2.26 with an appropriate coupling constant, *J*=7.4 Hz.

The mass spectra of compounds A and B gave little useful information to elucidate the chemical structures. The compounds were converted into *N*,*N*-dimethylhydrazones, which often give important fragments for structural elucidation of aldehyde compounds (Attygalle 1998). The mass spectra of the *N*,*N*-dimethylhydrazone of both compounds A and B showed a molecular ion peak at *m/z* 276 M^+ and an intense peak at *m/z* 125 in the higher mass region. *N*,*N*-dimethylhydrazone derivatives of α,β-unsaturated straight chain aldehydes give an intense ion fragment at *m/z* 111 from an allylic fission of the molecular ion (Attygalle 1998). Thus, it was suggested that compounds A and B could be α,β-unsaturated aldehydes with a methyl branch either in the β or γ positions.

To obtain more structural information, compounds A and B were subjected to ozonolysis. The resultant products were characterized as 4-oxo-pentanal and 4-oxo-hexanal by GC-MS analysis. Compound A gave 4-oxo-hexanal as the

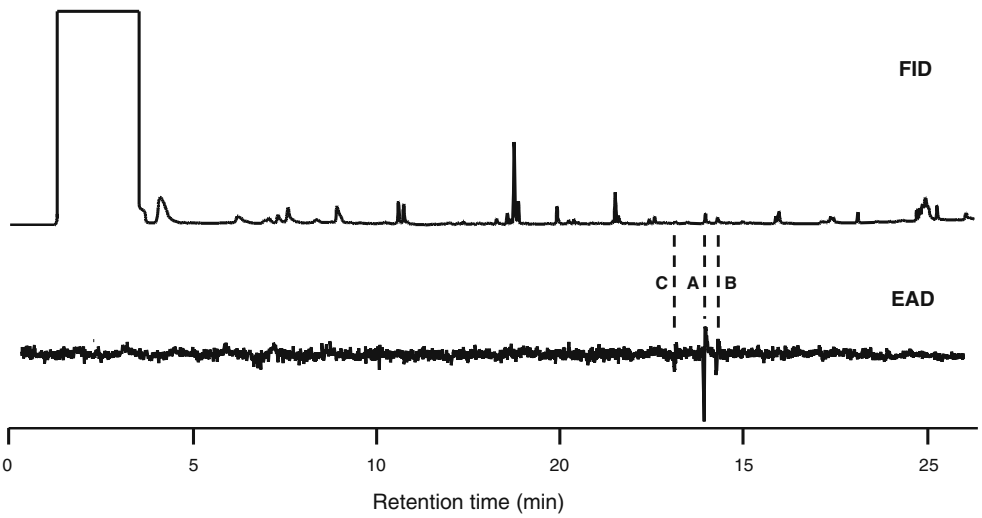

Fig. 1 Coupled gas chromatographic–electroantennographic detection analysis of antenna of male *Callosobruchus chinensis* to headspace volatile collections from virgin female *C. chinensis* (DB-5 column)

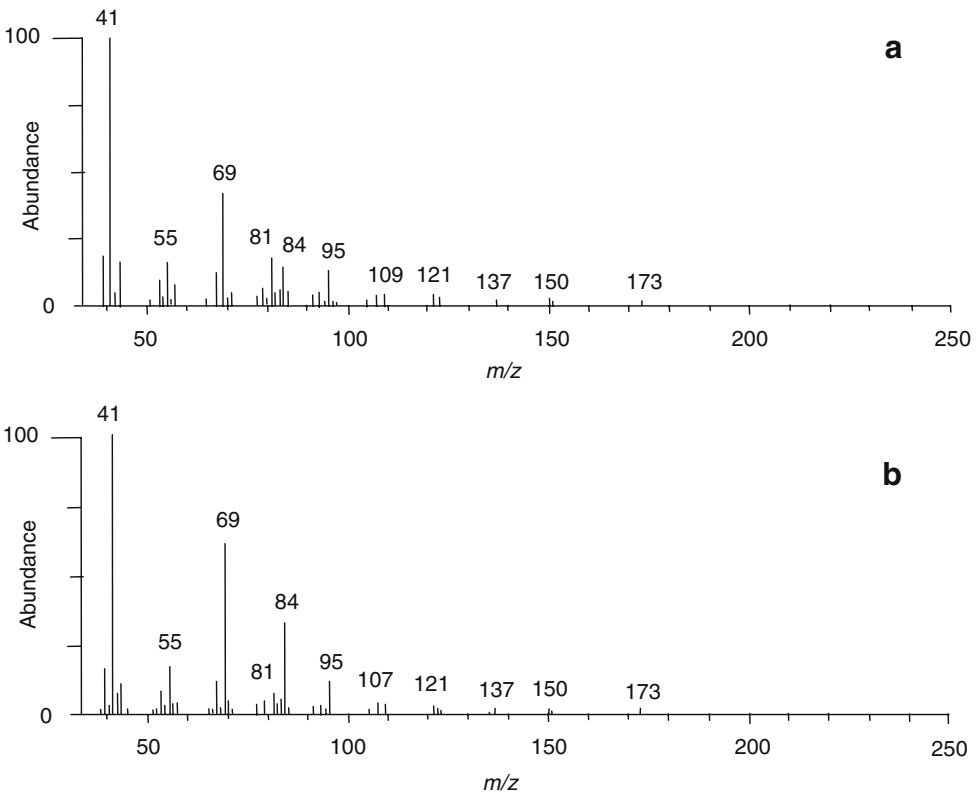

Fig. 2 EI-mass spectra of EAD-active compounds A (**a**) and B (**b**) from female *Callosobruchus chinensis*

only detectable product. However, we deduced the chemical structure of compound A by referring to the ozonolysis results of compound B because compound A was likely an isomer of compound B.

The pieces of the structure for 4-oxo-pentanal, 4-oxo-hexanal, and α,β-unsaturated aldehyde use C_5H_8, C_6H_{10}, and C_2H_2O, respectively (Fig. 3b–d), and these pieces make $C_{13}H_{20}O$. A molecular weight of 234 can take a number of likely molecular formulas, which contain at least one oxygen atom for the aldehyde moiety, including $C_{12}H_{26}O_4$, $C_{13}H_{14}O_4$, $C_{14}H_{18}O_3$, $C_{15}H_{22}O_2$, and $C_{16}H_{26}O$. Of these molecular formulas, only $C_{15}H_{22}O_2$ or $C_{16}H_{26}O$

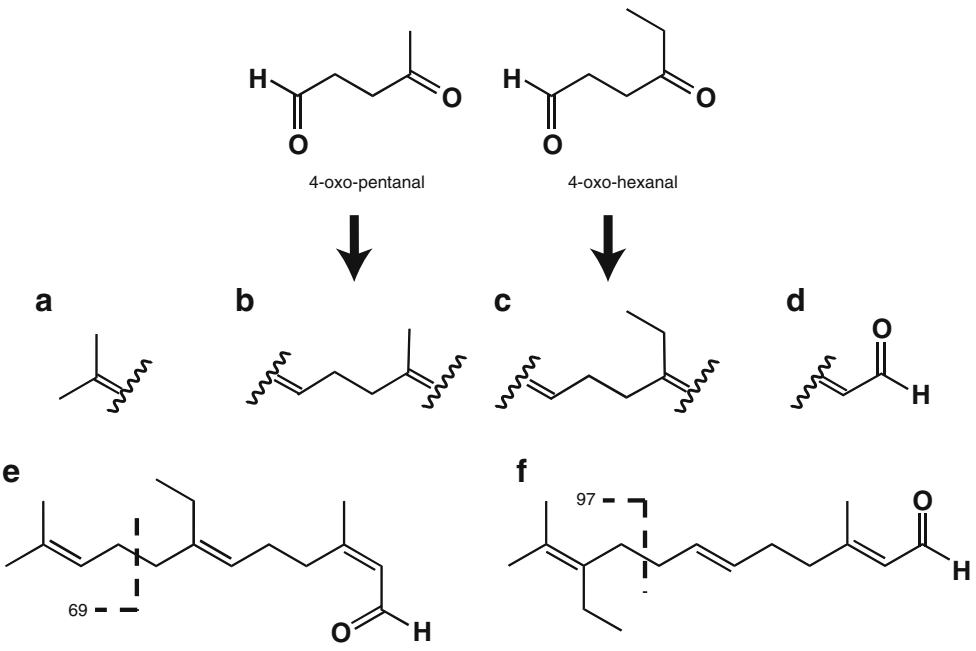

Fig. 3 Structural elucidation of EAD active compound A from female *Callosobruchus chinensis*. Partial structures of compound A, *a*, *b*, *c*, and *d*, were elucidated based on the results of GC-MS, microchemical, and ^{1}H-NMR analyses. Structures *e* and *f* are candidate structures of compound A, showing expected fragments at a diallylic fission in GC-MS analysis

satisfy the partial formula $C_{13}H_{20}O$. Then, the remaining pieces of the structure are calculated as C_2H_2O or C_3H_6. The first piece can be either of an α,β-unsaturated aldehyde, CHO–CH=, or a distorted epoxyside, O<CH_2–C=, while the second piece can be either of $(CH_3)_2C$= or an ethyl group, CH_3–CH_2–CH=. Taking account of the ^{1}H-NMR spectrum of compound A, in which one aldehydic proton and three olefinic protons were observed, together with ozonolysis results in which three olefinic protons were already assigned (Fig. 3b–d), the most likely partial structure accounting for the remaining piece was $(CH_3)_2C$= (Fig. 3a). Thus, the molecular formula of compound A was elucidated as $C_{16}H_{26}O$.

Considering compound A has a methyl branch at either the β or γ positions of the aldehyde group, the structure of compound A was deduced to be either 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal or 10-ethyl-3,11-dimethyl-2,6,10-dodecatrienal (Fig. 3e,f). However, the mass spectra of compound A showed a fragment at *m/z* 69 (Fig. 2a), suggesting a diallylic fission between C-8 and C-9 of structure e, not between C-8 and C-9 of structure f, which would give a fragment at *m/z* 97 (Fig. 3f). Therefore, the structure of compound A was elucidated as a homofarnesal, 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal, and compound B was deduced as a stereoisomer of compound A.

There are four possible geometrical isomers for 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal. To verify the structural elucidations, the four isomers were synthesized as a mixture. The GC-EAD analysis of the mixture showed that two of the four isomers elicited responses in male antennae (Fig. 4), and the retention times and the fragment patterns of these two isomers matched those of the natural compounds A and B.

7-Ethyl-3,11-dimethyl-2,6,10-dodecatrienal is a homolog of farnesal, 3,7,11-trimethyl-2,6,10-dodecatrienal, in which a C-7 ethyl group is substituted to methyl group. Farnesal also has four geometrical isomers, and those isomers can be easily resolved and determined by GC retention times and MS fragmentation patterns (Zagatti et al. 1987; Leal et al. 1989). By referring to the retention orders on GC columns and MS patterns of farnesals, compounds A and B were elucidated to be (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienals, respectively.

To confirm the geometrical configurations of compounds A and B, (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal were synthesized as a mixture. Then, each isomer was purified and isolated by HPLC, and the geometrical configurations of the isomers were unambiguously determined by GC and spectroscopic analyses. Consequently, compounds A and B were identified as (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienals, respectively. The ratio of the 2*Z*,6*E*- and 2*E*,6*E*-isomers in the pheromone extracts was estimated at 1:1 by GC analysis.

Y-tube olfactometer assays with synthetic pheromones showed that males responded dose-dependently to both synthetic pheromone components (Fig. 5). The attractive threshold of the (2*Z*,6*E*)-isomer was lower, and the number of males attracted to the (2*Z*,6*E*)-isomer (Fig. 5a) was higher than to the (2*E*,6*E*)-isomer (Fig. 5b) at the same doses. The activities of mixtures of the isomers in various ratios were compared with either the (2*Z*,6*E*)- or (2*E*,6*E*)-isomer alone. The attraction of the (2*Z*,6*E*)-isomer (Fig. 6a) was not significantly different from the mixtures in various ratios except for a 1:9 ratio of (2*Z*,6*E*):(2*E*,6*E*), which was less attractive. The attraction of the (2*E*,6*E*)-isomer (Fig. 6b) was significantly lower than the mixtures of (2*Z*,6*E*) and (2*E*,6*E*) in the ratios 9:1, 7.5:2.5, and 5:5. More than 87% of males reached either discs in all comparative trials between the mixtures and pure isomers,

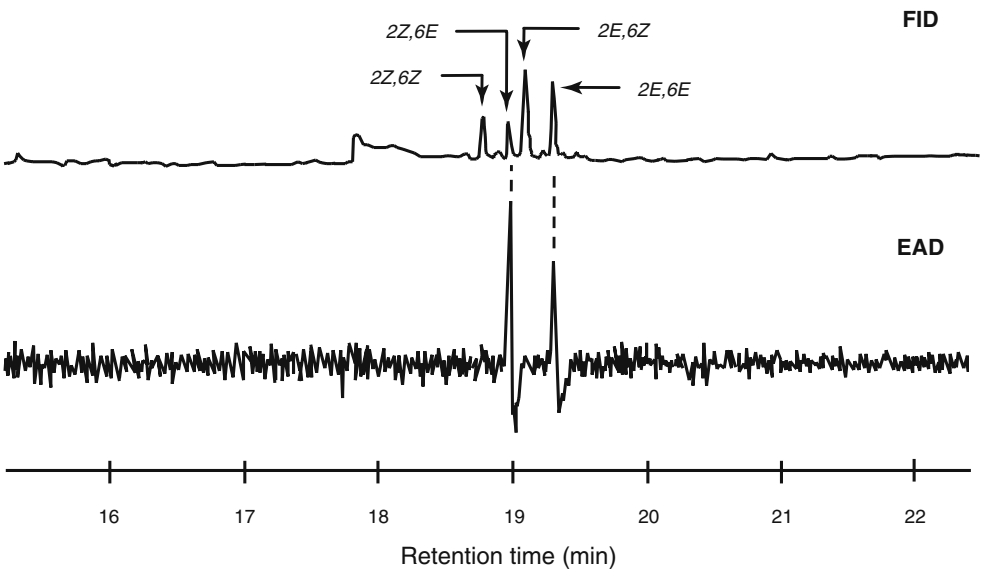

Fig. 4 GC-EAD responses of male *Callosobruchus chinensis* antennae to synthetic isomers of 7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal

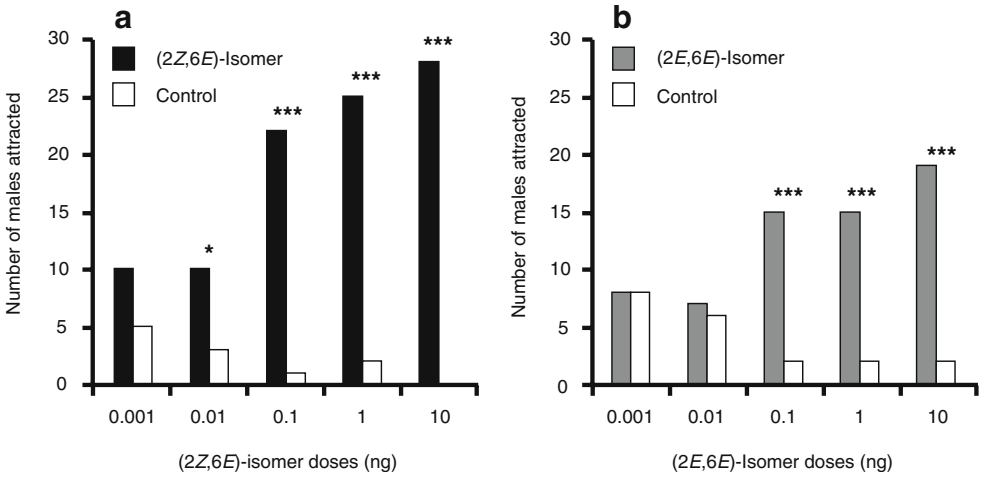

Fig. 5 Responses of male *Callosobruchus chinensis* to **a** (2*Z*,6*E*)- and **b** (*2E,6E*)-isomers in a Y-tube olfactometer. Thirty males were tested at each dose. The distribution of males between a sample and control in each treatment was subjected to a binomial test (two-tailed test with the test proportion set at 0.5). *Asterisks above bars* indicate significant differences from the test proportion (*$P<0.1$, **$P<0.05$, ***$P<0.01$). Males that did not reach either stimulus in each comparison were not included in the data analysis

which was similar to or more than those of (2*Z*,6*E*)-isomer alone vs. control trials at the dose–response study.

Discussion

Two homofarnesals, (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienal, were identified from the solvent extracts of filter paper-shelters taken from virgin female containers of *C. chinensis*. Both compounds elicited consistent EAD responses from the antennae of conspecific males. Males were attracted to 0.01–10 ng of the synthetic homofarnesals in Y-tube olfactometer assays. The (2*Z*,6*E*)-isomer showed equal or better activity than various mixtures of the (2*Z*,6*E*)- and (2*E*,6*E*)-isomers, and the responses to either mixtures or pure isomers in each trial were similar to those to the pure (2*Z*,6*E*)-isomer, which suggests that there is neither synergism nor antagonism between the two isomers.

Some bioactive natural products have been mischaracterized due to unexpected factors, such as thermal and chemical lability of the compounds during purification and/or analytical procedures (Mori 2005). The controversy over the periplanone-A, a component of the female sex attractant pheromone of the American cockroach, *Periplaneta americana*, is a case in point. Thermal lability of the genuine pheromone at the GC purification step misled the structural identification (Mori 2005). The pheromone components of *C. chinensis* have three double bonds: two isolated and one conjugating to the aldehyde group. These double bonds can be isomerized to other forms by physical or chemical factors. In fact, farnesal, which has almost the same chemical and geometrical structure as the pheromone components, was reported to isomerize spontaneously to other forms, i.e.,

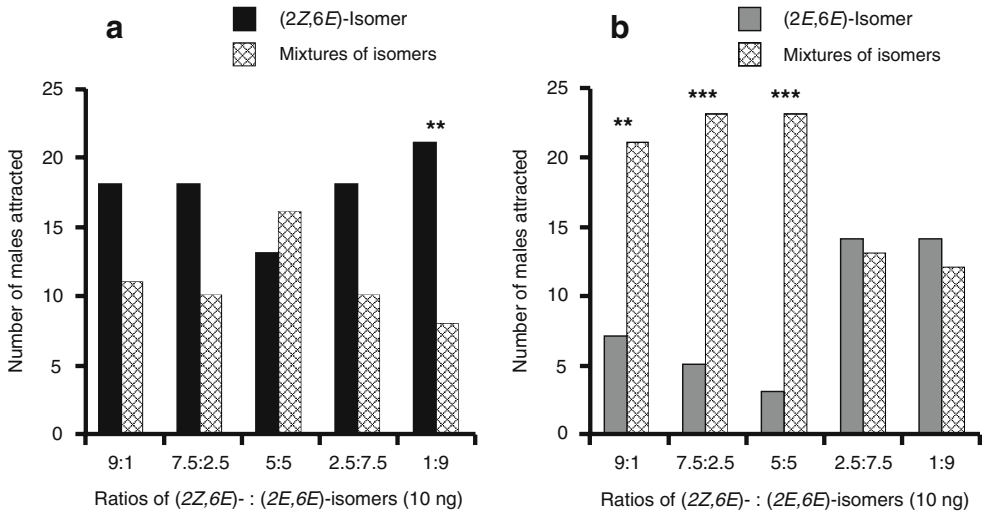

Fig. 6 A comparison of responses of male *Callosobruchus chinensis* to mixtures of (*2Z,6E*)- and (2*E*,6*E*)-isomers at various ratios and either a the (2*Z*,6*E*)-isomer or **b** the (2*E*,6*E*)-isomer at 10 ng. Thirty males were tested in each treatment. The distribution of males between samples in each treatment was subjected to a binomial test (two-tailed test with the test proportion set at 0.5). *Asterisks above bars* indicate significant differences from the test proportion (*$P<0.1$, **$P<0.05$, ***$P<0.01$). Males that did not reach either stimulus in each comparison were not included in the data analysis

(*E*,*E*)-farnesal isomerizes to (*Z*,*E*)-farnesal (Zagatti et al. 1987). Indeed, we found that the conjugated double bond of the synthetic pheromones was thermally isomerized to other configurations under our GC conditions. The percentages of isomerization of (2*Z*,6*E*) to (2*E*,6*E*)-isomers and (2*E*,6*E*) to (2*Z*,6*E*)-isomers were approximately 60% and 30%, respectively. Thus, although the ratio of the isomers in the pheromone extracts was estimated at 1:1 by GC analysis, the naturally occurring ratio probably tends to the (2*Z*,6*E*)-isomer. Therefore, together with the results of behavioral assays, (2*Z*,6*E*)-isomer appears to be more biologically significant than the (2*E*,6*E*)-isomer in the pheromonal communication system of *C. chinensis*. However, this needs to be clarified, and a field evaluation using synthetic compounds is under way.

The genus *Callosobruchus* is comprised of about 20 species (Borowiec 1987), and the distributions and habitats of some overlap considerably (Tuda et al. 2005). To date, female sex attractant pheromones have been identified from three species, *Callosobruchus analis*, *Callosobruchus subinnotatus*, and *C. maculatus*, as homologs of short chain fatty acids, including (*Z*)-3-methyl-2-heptenoic acid for *C. analis* (Cork et al. 1991), a mixture of (*Z*)-3-methyl-2-heptenoic acid and (*E*)-3-methyl-2-heptenoic acid for *C. subinnotatus* (Shu et al. 1998, 1999), and a mixture of (*Z*)-3-methyl-2-heptenoic acid, (*E*)-3-methyl-2-heptenoic acid, (*Z*)-3-methyl-3-heptenoic acid, (*E*)-3-methyl-3-heptenoic acid, and 3-methyleneheptanoic acid for *C. maculatus* (Phillips et al. 1996). The pheromone composition and the ratio of the mixture seem to play important roles in conspecific discrimination among these species (Phillips et al. 1996; Shu et al. 1999; Mbata et al. 2000). Interestingly, the chemical structure of the female sex attractant pheromone of *C. chinensis* is quite different from those of other species in the genus.

Recently, a molecular-based phylogenic study of the genus *Callosobruchus* separated this genus group into two distinct clades; one contains *C. chinensis*, which was designated the *chinensis* group, whereas the other contains *C. analis*, *C. subinnotatus*, and *C. maculatus*, which was designated the *maculatus* group (Tuda et al. 2006). This topology is also congruent with morphological character states of male genitalia and primary locality (Tuda et al. 2006). The divergence of pheromone compounds between the two clades is another corroboration of the results of the molecular phylogenic analysis.

Besides the female sex attractant pheromone, females of *C. chinensis* employ a contact sex pheromone that elicits copulation behavior from males (Tanaka et al. 1981). The contact sex pheromone of *C. chinensis* was characterized as a mixture of a monoterpene dicarboxylic acid, (*E*)-3,7-dimethyl-2-octene-1,8-dioic acid, and C_{25}–C_{35} straight chain and methyl-branched hydrocarbons (Tanaka et al. 1981, 1982). *C. maculatus* has a similar contact sex pheromone system, whose composition has recently been characterized as a mixture of dicarboxylic acids and hydrocarbons, 2,6-dimethyloctane-1,8-dioic acid and nonanedioic acid, and a mixture of C_{27}–C_{35} straight chain and methyl-branched hydrocarbons (Nojima et al. 2007). Interestingly, they reported that both *C. chinensis* and *C. maculatus* could elicit copulation behavior from heterospecific males in laboratory observations, suggesting that the contact sex pheromones lack species specificity.

Males of *C. chinensis* and *C. maculatus* are attracted to females by species-specific sex attractant pheromones. In this behavioral context, the contact sex pheromone need not be species-specific. A systematic study of the sex pheromones of *Callosobruchus* spp. could provide a key to understanding the evolution of the attractant and contact sex pheromone communication systems of this genus.

The female sex attractant pheromone of *C. chinensis*, (2*Z*,6*E*)- and (2*E*,6*E*)-7-ethyl-3,11-dimethyl-2,6,10-dodecatrienals, are homosesquiterpenoid, in which one extra carbon unit is added to the C-7 methyl group of a sesquiterpenoid, farnesal. Some homosesquiterpenes have been reported from insects, and those are synthesized *via* the mevalonate pathway from mevalonate and homomevalonate (Morgan 1999). The pheromone components of *C. chinensis* are likely to be biosynthesized *via* a similar pathway to that of other homosesquiterpenoids, but this remains to be clarified.

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References

ATTYGALLE, A. B. 1998. Microchemical techniques, pp. 207–294, in J. G. Millar, and K. F. Haynes (eds.). Methods in Chemical Ecology: Vol. 1, Chemical Methods. Kluwer, Norwell, MA.

BEROZA, M., and BIERL, B. A. 1966. Apparatus for ozonolysis of microgram to milligram amounts of compound. *Anal. Chem.* 38:1976–1977.

BEROZA, M., and BIERL, B. A. 1967. Rapid determination of olefin position in organic compounds in microgram range by ozonolysis and gas chromatography. *Anal. Chem.* 39:1131–1135.

BOROWIEC, L. 1987. The genera of seed-beetles (Coleoptera: Bruchidae). *Pol. Pis. Entomol.* 57:3–207.

BURKHOLDER, W. E., and MA, M. 1985. Pheromones for monitoring and control of stored-product insects. *Annu. Rev. Entomol.* 30:257–272.

CARDÉ, R. T., and BELL, W. J. (eds.). 1995. Chemical Ecology of Insects. Chapman & Hall, New York.

CORK, A., HALL, D. R., BLANEY, W. M., and SIMMONDS, M. S. J. 1991. Identification of a component of the female sex pheromone of *Callosobruchus analis* (Coleoptera: Bruchidae). *Tetrahedron Lett.* 32:129–132.

FUJII, K., GATEHOUSE, A. M. R., JOHNSON, C. D., MITCHELL, R., and YOSHIDA, T. (eds.). 1990. Bruchids and Legumes: Economics, Ecology and Coevolution. Kluwer, Dordrecht.

HARIRI, G. 1981. Distribution and importance of bruchid attacks on different species of pulses consumed in the Near East, pp. 215–221, in V. Labeyrie (ed.). The Ecology of Bruchids Attacking Legumes (Pulses). Junk, The Hague.

HEAPS, J. W. (ed.). 2006. Insect Management for Food Storage and Processing, 2nd Edition. AACC International, St. Paul, MN.

HONDA, H., and OHSAWA, K. 1990. Chemical ecology for stored product insects. *J. Pesticide Sci.* 15:263–270 (in Japanese with English abstract).

IGNACIMUTHU, S., WÄCKERS, F. L., and DORN, S. 2000. The role of chemical cues in host finding and acceptance by *Callosobruchus chinensis*. *Entomol. Exp. Appl.* 96:213–219.

IMURA, O. 1990. Life histories of stored-products insects, pp. 257–270, in K. Fujii, A. M. R. Gatehouse, C. D. Johnson, R. Mitchell, and T. Yoshida (eds.). Bruchids and Legumes: Economics, Ecology and Coevolution. Kluwer, Dordrecht.

LABEYRIE, V. (ed.). 1981. The Ecology of Bruchids Attacking Legumes (Pulses). Junk, The Hague.

LEAL, W. S., KUWAHARA, Y., SUZUKI, T., and KUROSA, K. 1989. The alarm pheromone of the mite *Suidasia medanensis* Oudemans, 1924 (Acariformes, Suidasiidae). *Agric. Biol. Chem.* 10:2703–2709.

MATSUMOTO, H., TEBAYASHI, S., KUWAHARA, Y., MATSUYAMA, S., SUZUKI, T., and FUJII, K. 1994. Identification of taxifolin present in the azuki bean as an oviposition stimulant of the azuki bean weevil. *J. Pesticide Sci.* 19:181–186.

MBATA, G. N., SHU, S., and AMASWAMY, S. B. 2000. Sex pheromones of *Callosobruchus subinnotatus* and *C. maculatus* (Coleoptera: Bruchidae): congeneric responses and role of air movement. *Bull. Entomol. Res.* 90:147–154.

MINKS, A. K., and CARDÉ, R. T. 1997. Use of pheromones in direct control, pp. 333–459, in R. T. Cardé, and A. K. Minks (eds.). Insect Pheromone Research, New Directions. Chapman & Hall, New York.

MORGAN, E. D. 1999. Terpenes, pp. 85–103, in E. D. Morgan (ed.). Biosynthesis in InsectsThe Royal Society of Chemistry. Milton Road, Cambridge.

MORI, K. 1972. Synthesis of compounds with juvenile hormone activity—XII. A stereoselective synthesis of 6-ethyl-10-methyldodeca-5-*trans*, 9-*cis*-dien-2-one, a key intermediate in the synthesis of C_{18}-*Cecropia* juvenile hormone. *Tetrahedron* 28:3747–3756.

MORI, K. 2005. Synthetic examination of incorrectly proposed structures of biomolecules. *Chem. Rec*. 5:1–16.

MORI, K., MITSUI, T., FUKAMI, J., and OHTAKI, T. 1971. Synthesis of compounds with juvenile hormone activity part VII. A convenient non-stereoselective synthesis of the C_{18}-*Cecropia* juvenile hormone and its analogues; effect of the terminal alkyl substituents on biological activity. *Agri. Biol. Chem.* 35:1116–1127.

NOJIMA, S., KIEMLE, D. J., WEBSTER, F. X., and ROELOFS, W. L. 2004. Submicro scale NMR sample preparation for volatile chemicals. *J. Chem. Ecol.* 30:2153–2161.

NOJIMA, S., LINN, C. JR., MORRIS, B., ZHANG, A., and ROELOFS, W. L. 2003. Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol.* 29:321–336.

NOJIMA, S., SHIMOMURA, K., HONDA, H., YAMAMOTO, I., and OHSAWA, K. 2007. Contact sex pheromone components of the cowpea weevil, *Callosobruchus maculatus*. *J. Chem. Ecol.* 33:923–933.

OSHIMA, K., HONDA, H., and YAMAMOTO, I. 1973. Isolation of an oviposition marker from azuki bean weevil, *Callosobruchus chinensis* (L.). *Agri. Biol. Chem.* 37:2679–2680.

PHILLIPS, T. W. 1997. Semiochemicals of stored-product insects: research and applications. *J. Stored Prod. Res.* 33:17–30.

PHILLIPS, T. W., PHILLIPS, J. K., WEBSTER, F. X., TANG, R., and BURKHOLDER, W. E. 1996. Identification of sex pheromones from cowpea weevil, *Callosobruchus maculatus*, and related studies with *C. analis* (Coleoptera: Bruchidae). *J. Chem. Ecol.* 22:2233–2249.

REES, D. P. 1995. Coleoptera, pp. 1–40, in B. Subramanyam, and D. W. Hagstrum (eds.). Integrated Management of Insects in Stored Products. Marcel Dekker, Madison Avenue, New York.

SAKAI, A., HONDA, H., OSHIMA, K., and YAMAMOTO, I. 1986. Oviposition marking pheromone of two bean weevils, *Callosobruchus chinensis* and *Callosobruchus maculatus*. *J. Pesticide Sci.* 11:163–168.

SHINODA, K., and YOSHIDA, T. 1985. Ecology of azuki weevil in azuki field. I. Emergent prevalence and damage of azuki in azuki field on autumn. *Jpn*. *Appl. Entmol. Zool.* 29:14–20 (in Japanese with English summary).

SHINODA, K., and YOSHIDA, T. 1990. Life history of the azuki bean weevil, *Callosobruchus chinensis* (Coleoptera: Bruchidae), in the field, pp. 149–159, in K. Fujii, A. M. R. Gatehouse, C. D. Johnson, R. Mitchell, and T. Yoshida (eds.). Bruchids and Legumes: Economics, Ecology and Coevolution. Kluwer, Dordrecht.

SHINODA, K., YOSHIDA, T., and IGARASHI, H. 1992. Population ecology of the azuki bean beetle, *Callosobruchus chinensis* (L.), (Coleoptera: Bruchidae) on two wild leguminous hosts, *Vigna angularis* var. *nipponensis* and *Dunbaria villosa*. *Appl. Entmol. Zool.* 27:311–318.

SHINODA, K., YOSHIDA, T., and OKAMOTO, T. 1991. Two wild leguminous host plants of the azuki bean weevil, *Callosobruchus chinensis* (L.) (Coleoptera: Bruchidae). *Appl. Entmol. Zool.* 26:91–98.

SHU, S., MBATA, G. N., CORK, A., and RAMASWAMY, S. B. 1998. Female sex pheromone in *Callosobruchus subinnotatus* (Coleoptera: Bruchidae): Production and male responses. *Ann. Entomol. Soc. Am.* 91:840–844.

SHU, S., MBATA, G. N., CORK, A., and RAMASWAMY, S. B. 1999. Sex pheromone of *Callosobruchus subinnotatus*. *J. Chem. Ecol.* 25:2715–2727.

SIEGEL, S. 1956. The one-sample case, pp. 35–60, in S. Siegel (ed.). Nonparametric Statistics for the Behavioral Science. McGraw-Hill, New York.

SUBRAMANYAM, B., and HAGSTRUM, D. W. (eds.). 2000. Alternatives to Pesticides in Stored-product IPM. Kluwer, Boston, MA.

TANAKA, K., OHSAWA, K., HONDA, H., and YAMAMOTO, I. 1981. Copulation release pheromone, Erectin, from the azuki bean weevil (*Callosobruchus chinensis* L.). *J. Pesticide Sci.* 6:75–82.

TANAKA, K., OHSAWA, K., HONDA, H., and YAMAMOTO, I. 1982. Synthesis of Eectin, a copulation release pheromone of the azuki bean weevil, *Callosobruchus chinensis* L. *J. Pesticide Sci.* 7:535–537.

TEBAYASHI, S., MATSUYAMA, S., SUZUKI, T., KUWAHARA, Y., NEMOTO, T., and FUJII, K. 1995. Quercimertrin: the third oviposition stimulant of the azuki bean weevil from the host azuki bean. *J. Pesticide Sci.* 20:299–305.

TUDA, M., CHOU, L.-Y., NIYOMDHAM, C., BURANAPANICHPAN, S., and TATEISHI, Y. 2005. Ecological factors associated with pest status in *Callosobruchus* (Coleoptera: Bruchidae): high host specificity of non-pests to Cajaninae (Fabaceae). *J. Stored Pro. Res.* 41:31–45.

TUDA, M., RÖNN, J., BURANAPANICHPAN, S., WASANO, N., and ARNQVIST, G. 2006. Evolutionary diversification of the bean beetle genus *Callosobruchus* (Coleoptera: Bruchidae): traits associated with stored-product pest status. *Mol. Ecol.* 15:3541–3551.

UENO, T., KUWAHARA, Y., FUJII, K., TAPER, M. L., TOQUENAGA, Y., and SUZUKI, T. 1990. D-Catechin: an oviposition stimulant of azuki bean weevil *Callosobruchus chinensis* in the host azuki bean. *J. Pesticide Sci.* 15:573–578.

WATANABE, N. 1990. Diversity in life cycle patterns of bruchids occurring in Japan (Coleoptera: Bruchidae), pp. 141–147, in K. Fujii, A. M. R. Gatehouse, C. D. Johnson, R. Mitchell, and T. Yoshida (eds.). Bruchids and Legumes: Economics, Ecology and Coevolution. Kluwer, Dordrecht.

WHITE, N. D. G., and LEESCH, J. G. 1996. Chemical Control, pp. 287–330, in B. Subramanyam, and D. W. Hagstrum (eds.). Integrated Management of Insects in Stored Products. Marcel Dekker, Madison Avenue, New York.

YONEDA, M., KANEDA, M., and AKIYAMA, H. 1990. Plant quarantine and fumigation of imported grain legumes in Japan, pp. 115–120, in K. Fujii, A. M. R. Gatehouse, C. D. Johnson, R. Mitchell, and T. Yoshida (eds.). Bruchids and Legumes: Economics, Ecology and Coevolution. Kluwer, Dordrecht.

ZAGATTI, P., KUNESCH, G., AMIANDRASOA, F., MALOSSE, C., HALL, D. R., LESTER, R., and NESBITT, B. F. 1987. Sex pheromones of rice moth, *Corcyra cephalonica* Staomtpm I. Identification of male pheromone. *J. Chem. Ecol.* 13:1561–1573.

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(*E*,*E*)-α-Farnesene, an Alarm Pheromone of the Termite *Prorhinotermes canalifrons*

Jan Šobotník · Robert Hanus · Blanka Kalinová · Rafal Piskorski · Josef Cvačka · Thomas Bourguignon · Yves Roisin

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Abstract The behavioral and electroantennographic responses of *Prorhinotermes canalifrons* to its soldier frontal gland secretion, and two separated major components of the secretion, (*E*)-1-nitropentadec-1-ene and (*E*,*E*)-α-farnesene, were studied in laboratory experiments. Behavioral experiments showed that both the frontal gland secretion and (*E*,*E*)-α-farnesene triggered alarm reactions in *P. canalifrons*, whereas (*E*)-1-nitropentadec-1-ene did not affect the behavior of termite groups. The alarm reactions were characterized by rapid walking of activated termites and efforts to alert and activate other members of the group. Behavioral responses to alarm pheromone differed between homogeneous and mixed groups, suggesting complex interactions. Antennae of both soldiers and pseudergates were sensitive to the frontal gland secretion and to (*E*,*E*)-α-farnesene, but soldiers showed stronger responses. The dose responses to (*E*,*E*)-α-farnesene were identical for both soldiers and pseudergates, suggesting that both castes use similar receptors to perceive (*E*,*E*)-α-farnesene. Our data confirm (*E*,*E*)-α-farnesene as an alarm pheromone of *P. canalifrons*.

J. Šobotník · R. Hanus (✉) · B. Kalinová · R. Piskorski · J. Cvačka
Institute of Organic Chemistry and Biochemistry,
Czech Academy of Sciences, v.v.i.,
Flemingovo nám. 2,
166 10 Praha 6, Czech Republic
e-mail: robert@uochb.cas.cz

J. Šobotník · T. Bourguignon · Y. Roisin
Behavioural and Evolutionary Ecology,
Université Libre de Bruxelles-CP 160/12,
avenue F.D. Roosevelt 50,
1050 Brussels, Belgium

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Introduction

Termites, as creatures generally living in permanent darkness, largely rely upon chemical communication during intraspecific interactions. Trail-following pheromones produced by the sternal gland (Matsumura et al. 1968; Bordereau et al. 1991; Laduguie et al. 1994; Peppuy et al. 2001), or the food-marking pheromone produced by the labial glands (Reinhard et al. 2002) are well known. Other means of chemical communication include alarm pheromones produced by the frontal gland of soldiers (Vrkoč et al. 1978; Roisin et al. 1990; Reinhard and Clément 2002), and sexual pheromones produced by tergal (Bordereau et al. 2002), posterior sternal (Peppuy et al. 2004), or sternal glands in alate imagoes (McDowell and Oloo 1984; Bordereau et al. 1991; Laduguie et al. 1994). In addition, there are several other glands whose functions are still unknown or hypothetical, such as the mandibular glands (Cassier et al. 1977; Šobotník and Hubert 2003), tarsal glands (Bacchus 1979), or epidermal glands (Šobotník et al. 2003).

When disturbed, termites communicate alarm mechanically via vibrations (Howse 1964, 1965; Connétable et al. 1999; Röhrig et al. 1999), or with alarm pheromones. With some exceptions (Maschwitz and Mülhenberg 1972; Traniello et al. 1984), pheromones mediate alarm communication in termite species with a developed frontal gland, i.e., in the families Serritermitidae, Rhinotermitidae, and Termitidae (Noirot 1969; Costa-Leonardo and Kitayama 1991). Although the involvement of the frontal gland

secretion in alarm communication has been proven for many species (Kriston et al. 1977; Kaib 1990; Roisin et al. 1990; Pasteels and Bordereau 1998; Reinhard and Clément 2002), a striking gap exists in our knowledge of the chemicals actually involved in alarm communication. Alarm pheromones have been identified only in three *Nasutitermes* species, including α-pinene and limonene in *N. rippertii,* carene and limonene in *N. costalis* (Vrkoč et al. 1978), and α-pinene in *N. princeps* (Roisin et al. 1990). The production of alarm pheromones may also be linked with soldier polymorphism: alarm pheromones are probably produced solely by small soldiers in *Schedorhinotermes lamanianus* (Kaib 1990) or by large soldiers in *Nasutitermes exitiosus* (Kriston et al. 1977).

The genus *Prorhinotermes* (Isoptera: Rhinotermitidae) is the only genus in the subfamily Prorhinotermitinae (Quennedey and Deligne 1975). Its crucial phylogenetic position within Rhinotermitidae as well as a peculiar combination of primitive and advanced characteristics makes it an attractive model to study. *Prorhinotermes* species have a linear ontogenetic scheme lacking a true worker caste; the work tasks are performed by temporarily specialized helpers, pseudergates, which retain capacity to become either alate imagos (through a single nymphal instar) or neotenic reproductives (by a single molt; Roisin 1988). Soldiers in *Prorhinotermes* originate from pseudergates or larvae through an intermediary presoldier instar (Roisin 1988; Hanus et al. 2006). They are abundant (up to 22%), they guard inside the nest and on its peripheries, but also act as defenders when the nest is being moved (Haverty 1977, Roisin et al. 2001). Soldiers' defense includes their piercing mandibles and a powerful frontal chemical weapon; they also perform vibratory alarm movements in response to disturbance (Deligne et al. 1981; Hanus et al. 2005).

The frontal gland secretion in soldiers of the genus *Prorhinotermes* contains predominantly (*E*)-1-nitropentadec-1-ene, with small amounts of sesquiterpenes (Vrkoč and Ubik 1974; Hanus et al. 2006; Piskorski et al. 2007). On average, the frontal gland of *P. canalifrons* soldiers contains 153 μg of (*E*)-1-nitropentadec-1-ene and 1.8 μg of (*E*,*E*)-α-farnesene, the latter representing about 65% of its sesquiterpenoid content (Piskorski et al. 2007). Whereas the defensive function of the frontal gland secretion, due to the large amounts of toxic (*E*)-1-nitropentadec-1-ene, is well established (Kuldová et al. 1999), its role in alarm communication and the contribution of (*E*,*E*)-α-farnesene remain hypothetical. The aims of the experiments described in this paper were as follows: (1) to determine whether the soldier frontal gland secretion is responsible for alarm communication in *P. canalifrons*; (2) to determine if (*E*,*E*)-α-farnesene mediates the alarm reaction; (3) to evaluate potential differences in alarm reaction among castes; (4) to verify the function of the putative alarm pheromone in conditions closer to natural ones, i.e., at the level of a whole colony. Thus, we studied the alarm reactions in homogeneous (formed by either pseudergates or soldiers) and heterogeneous termite groups (formed by pseudergates and soldiers), and subsequently in small complete colonies. As experimental stimuli, the whole frontal gland secretion, and solutions of synthesized (*E*)-1-nitropentadec-1-ene and (*E*,*E*)-α-farnesene were used. Electrophysiological responses of termite antennae were also recorded to investigate possible differences between castes in sensitivity to gland secretion, and (*E*,*E*)-α-farnesene.

Materials and Methods

Termites The colonies of *Prorhinotermes canalifrons* (Sjöstedt 1904) used for experiments originated from Saint-Denis (Réunion). The termites were collected in 2001 and since then were reared in containers in permanent darkness at 27°C and elevated humidity. Wet fine sand served as substratum. Colonies were continuously provided with decayed birch wood. The experimental design described below is summarized in Table 1.

Experimental Groups Termite groups of 4 different caste compositions were tested: (A) 40 pseudergates +10 soldiers (close to natural proportions, Haverty 1977), (B) 50

Table 1 The design of experiments: caste composition, stimuli used, number of repetitions, and number of individuals measured in experiments A–D

Experiment	Caste Composition	Stimuli	Individuals Measured per Repetition	No. of Repetitions per Stimulus	Individuals Measured per Stimulus	Individuals Measured
A	40 Ps+10 sold	bl, hex, npd, fg, far	10 Ps+5 sold	5	50 Ps+25 sold	250 Ps+125 sold
B	50 Ps	bl, hex, npd, fg, far	10 Ps	2	20	100 Ps
C	20 sold	bl, hex, npd, fg, far	10 sold	2	20	100 sold
D	Ps+sold+neo+pres+L	fg, far	Not evaluated	4	0	0

Ps pseudergate, *sold* soldier, *neo* neotenic, *pres* presoldier, *L* larva, *bl* blank control, *hex* hexane, *npd* (E)-1-nitropentadec-1-ene, *fg* frontal gland secretion, *far* (*E*,*E*)-α-farnesene

pseudergates, (C) 20 soldiers, and (D) incipient colonies. In experiments A and B, termites were transferred from the original colonies into experimental arenas 20–24 h prior to observation to allow to acclimatize after manipulation. This delay was reduced to 4–6 h only in experiment C because soldiers are care-dependent. The incipient colonies (experiment D) originated from groups of 60 pseudergates isolated and left to develop for 8 months. At the time of experimentation, these groups consisted of 25–35 pseudergates, 6–10 soldiers, 5–15 neotenics, 0–2 presoldiers, 0–5 larvae from the first to the third instar, and 0–8 eggs. The complete population of each small colony was carefully extracted from its original box and transferred to the experimental chamber inside the birch wood (see Fig. 1). The corridor connecting the chamber with the exterior was initially closed with a piece of filter paper, and was opened after 2 days. Behavioral tests were performed from the next day on.

Experimental Design All experiments were performed at 27°C under red light in Petri dishes (85 mm diameter) with wet filter paper as substratum (Fig. 1). A piece of birch wood served as a food and natural substrate. The test substance was loaded onto a piece of filter paper (7×3 mm) and introduced immediately into the Petri dish through a slit in the dish cap. The filter paper was hung out of reach of termites by a pin bridged over the slit.

The following stimuli were tested in experiments A–C: (1) untreated paper (blank control), (2) 1 μl of pure hexane (solvent control), (3) 31 μg of (*E*)-1-nitropentadec-1-ene (~0.2 frontal gland equivalent, FGE) in 1 μl of hexane, (4) one soldier frontal gland secretion (1 FGE), (5) 1.5 μg of (*E*,*E*)-α-farnesene (~1 FGE) in 1 μl of hexane. Each group was tested once for one stimulus and once for the control in random order. In experiment D, only two stimuli were tested: (4) frontal gland secretion, and (5) (*E*,*E*)-α-farnesene in random order with at least 2-day intervals between exposures (each stimulus tested once in every group).

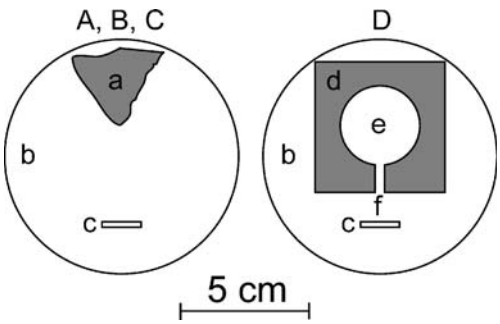

Fig. 1 Schematic drawing of the experimental design of behavioral bioassays: *left*, experiments A–C; *right*, experiment D. The arenas are drawn to scale; *a*, piece of birch wood; *b*, filter paper; *c*, slit in the lid of the Petri dish through which filter paper loaded with test solution was hung; *d*, birch wood test chamber; *e*, central arena where whole incipient colonies were placed, reaching from bottom to top of the dish; *f*, narrow corridor, the only connection between the central arena and the exterior

The frontal gland secretion of a soldier was obtained by decapitation, evacuation of the thoracic and abdominal part of the frontal gland reservoir with a slight pressure by forceps tip against the body, and then smashing the head on the test filter paper. For synthesis of (*E*)-1-nitropentadec-1-ene (>99%) (see Kuldová et al. 1999). (*E*,*E*)-α-farnesene (68%; from plant material; minor components incl. α-caryophyllene, hydrocarbon and sesquiterpene oxidation products) was provided by Anna-Karin Borg-Karlson, KTH, Sweden. Minor components included α-caryophyllene, sesquiterpene, and hydrocarbon oxidation products.

Recording The behavior of all termite groups was recorded with a Panasonic WV-CL920 camera and a Panasonic DVD hard drive recorder DMR-HS2. Each recording started 2 min before introduction of the stimulus and lasted for 7 min. The parameter evaluated in behavioral experiments was the speed of termite walking. New software, MouseTracer, was developed to track the position of the mouse cursor. The cursor was controlled by the experimenter, and the selected termite was followed on the screen. The cursor position, recorded 20 times per second of observation, was initially expressed in pixels and subsequently converted into millimeters.

The selection of termites to track in all experiments was designed to include all possible termite statuses at the beginning of the experiment: individuals close and far from the introduction slit, in the center and at the periphery of a termite group, individuals resting or walking in the arena, etc.

Statistics Experiment A was repeated five times for each stimulus, B and C twice, and then the data were merged for a particular stimulus. In each repetition of experiment A, the tracks of ten pseudergates and five soldiers were analyzed; ten individuals were tracked in experiments B and C. Thus, 50 pseudergates and 25 soldiers were analyzed in A, 20 pseudergates in B, and 20 soldiers in C for each stimulus. Experiment D was performed with four colonies, but the data were not evaluated statistically because termites often walked on the vertical walls of the chamber, which prevented them from being tracked.

Kruskal–Wallis rank tests and multiple comparisons of non-parametric data were used to compare the speed of particular castes before and after insertion of the stimulus, as well as the antennal responses in electroantennography (EAG) experiments compared with controls. The walking speeds between A, B, and C and the latency in response to olfactory stimuli were compared by means of nonparametric Mann–Whitney *U* tests. The curves in Fig. 2 were

obtained by using the moving average of the distances walked by the individual every 0.05 s, expressed as the average for every 5 s. Statistica® 7.1 was used for all calculations.

Electroantennography EAG recordings were performed by using individual antennae of pseudergates and soldiers with the antennal tip cut off. Glass Ag/AgCl microelectrodes filled with insect saline solution were used. Electrodes were connected to an amplifier (Syntech), and the tenfold amplified signal was fed to a PC via an IDAC 2 PC board (Syntech). (*E*,*E*)-α-farnesene was diluted in hexane in decadic steps (10 pg–10 μg/μl). From each concentration, 1 μl of stimulus solution was loaded onto a 1×0.5 cm piece of filter paper inserted in a Pasteur pipette. After solvent evaporation, the loaded odorant cartridges were sealed with parafilm and stored at −20°C until used. Prior to each experiment, the cartridges were allowed to warm up for 1 h at room temperature. Stimuli were delivered by injection of an air pulse (1 s, air flow 0.8 l/min) through an odorant cartridge into a continual air stream directed towards the antenna. Standard stimuli, i.e. cartridges with filter paper loaded with 100 ng of (*E*,*E*)-α-farnesene, were applied at the beginning and the end of each experiment to determine

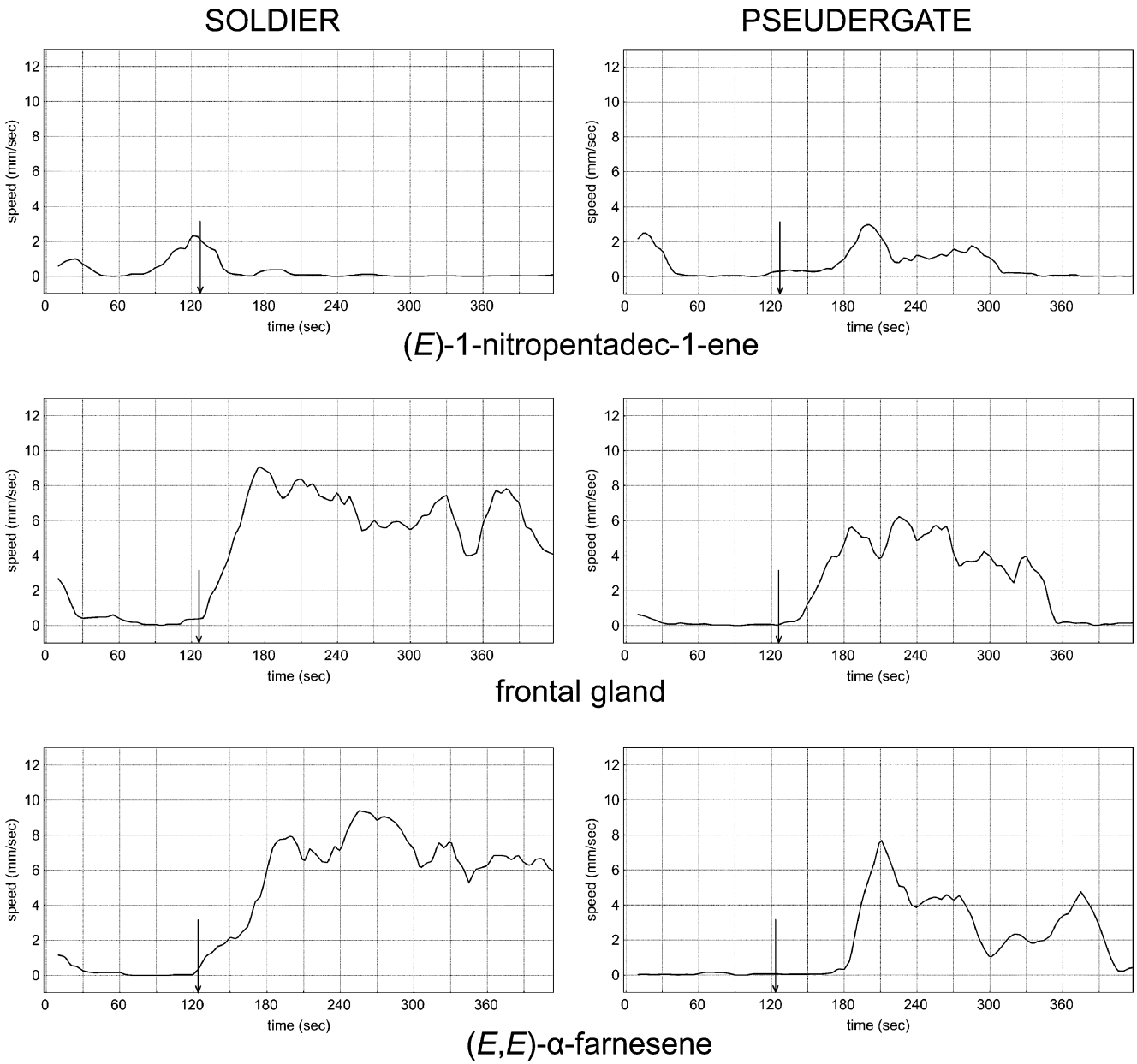

Fig. 2 Typical dynamic recordings of walking speed of soldiers and pseudergates exposed to different olfactory stimuli (experiment A). The curves represent a moving average of distances walked scored second by second. *Arrows* mark the insertion of the stimulus

possible changes in antennal sensitivity during EAG recordings and to allow normalization between experiments. The antennal responses (maximal negative deflection during stimulation in mV) were normalized to these standard stimuli and expressed in percent. Each stimulus was tested once on each antenna, with 19 different antennae per stimulus used; thus, the data analyzed represent 19 independent measurements for each stimulus.

Results

General Features of Alarm Behavior At the beginning of each experiment, most termites were quietly clustered near the piece of wood, and usually only a few individuals, predominantly soldiers, would slowly explore the arena. After insertion of an untreated filter paper, termite activity increased slightly for a while due to the disturbance, but decreased within a few seconds. Similar responses were elicited by hexane and (*E*)-1-nitropentadec-1-ene solution. On the other hand, insertion of a paper treated with frontal gland secretion or (*E*,*E*)-α-farnesene solution led to a long-lasting activation characterized by fast walking, scanning of the space with straightened antennae, zigzag searching for the odor source, mandibular opening and closing (in soldiers), and nestmate alerting. Activation led to marked change in termite distribution within the arena, namely the accumulation of termites below the odor source.

During nestmate activation, the alerting termites (usually soldiers, less commonly also pseudergates) searched for quiescent termites, touched them with their antennae, and subsequently performed a series of longitudinal vibrations. This behavior was usually repeated several times (most frequently 2–4 times during 3–10 s) until the alerting termite calmed down or until all nestmates were alerted. Soldiers reacted to alerting stimulation more easily than pseudergates: one alerting signal was usually enough to activate a soldier, whereas repeated alerting was often necessary to activate a pseudergate. The behavioral responses elicited by the frontal gland secretion did not differ qualitatively from those elicited by (*E*,*E*)-α-farnesene. Typical time courses of activation of soldiers and pseudergates are shown in Fig. 2.

Heterogeneous Groups of 40 Pseudergates +10 Soldiers—Experiment A The results of Experiment A are summarized in Fig. 3. Exposure to frontal gland secretion increased the walking speed in both pseudergates and soldiers ($P<10^{-6}$ for both): the average speed increased from 0.53 to 2.15 mm/s in pseudergates, and from 0.58 to 3.39 mm/s in soldiers. The same was observed for (*E*,*E*)-α-farnesene, which increased the speed of walking to 2.9 mm/s in pseudergates, and to 4.87 mm/s in soldiers ($P<10^{-6}$ for both). (*E*,*E*)-α-farnesene at the dose used apparently represented a more effective stimulus than the frontal gland secretion for both pseudergates and soldiers.

Soldiers generally reacted more quickly than pseudergates, as documented by individual measurements of 12 soldiers and 14 pseudergates exposed to (*E*,*E*)-α-farnesene: median value of the first maximum in walking speed was 20 s in soldiers and 40 s in pseudergates ($P<7\times10^{-3}$). With the exception of differences in reaction times, the responses of pseudergates and soldiers were similar. About 1 min after the introduction of the paper treated with (*E*,*E*)-α-farnesene or frontal gland secretion, all termites ran vigorously in the arena.

Homogeneous Groups of 50 Pseudergates or 20 Soldiers—Experiments B and C The responses of homogeneous groups of either pseudergates or soldiers were qualitatively similar compared to the mixed groups in experiment A: hexane and nontreated paper elicited no significant responses (Fig. 4). In contrast, the locomotion of soldiers and pseudergates was significantly faster after exposure to the frontal gland secretion or (*E*,*E*)-α-farnesene. The increase in speed in pseudergates did not differ from that in mixed groups. In soldiers, on the other hand, the increase was lower for the frontal gland secretion (1.51 vs. 3.39 mm/s in mixed groups; $P<6\times10^{-4}$) as well as for (*E*,*E*)-α-farnesene (2.63 vs. 4.87 mm/s in mixed groups; $P<10^{-6}$) when compared to observations in heterogeneous groups.

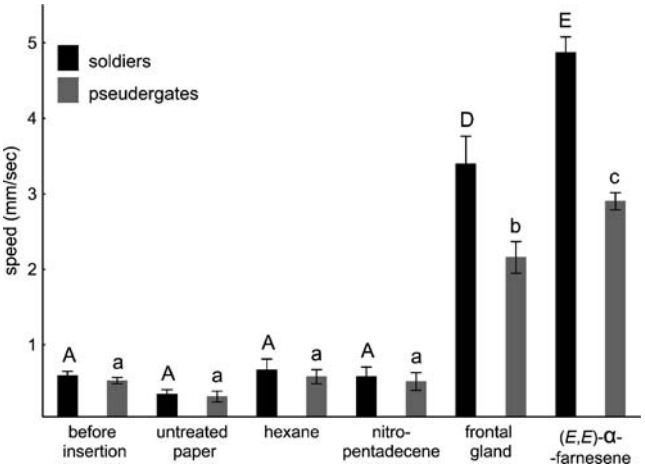

Fig. 3 Average walking speed of soldiers and pseudergates during behavioral bioassays with mixed groups (experiment A) exposed to various olfactory stimuli before (*the two columns on the left*, $n=125$ soldiers and 250 pseudergates) and after the insertion of the stimulus ($n=25$ soldiers and 50 pseudergates for each stimulus). *Columns* represent means, whiskers represent SEM. The *columns marked with different letters* are significantly different (Kruskal–Wallis test; $P<0.05$; multiple non-parametric comparison for unequal number of observations)

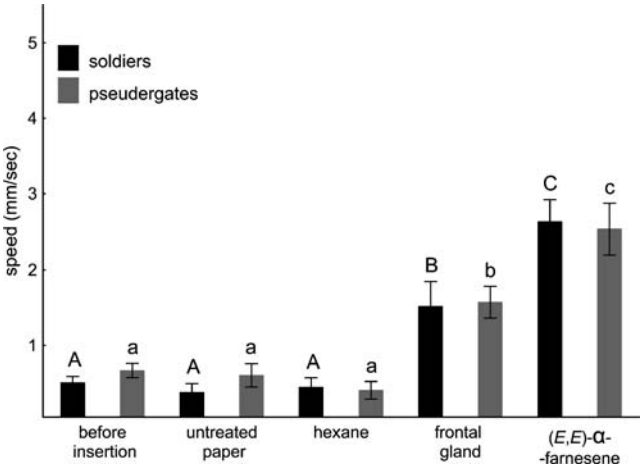

Fig. 4 Average speed of soldiers and pseudergates from single-caste groups (experiments B or C) exposed to different olfactory stimuli during behavioral bioassays. *Columns* represent means, whiskers represent SEM. The *columns marked with different letters* are significantly different (Kruskal–Wallis test; $P<0.05$; multiple non-parametric comparison for unequal number of observations)

Incipient Colonies—Experiment D Prior to the introduction of the test substances, members of all castes of incipient colonies were randomly distributed within the chamber with at least one soldier patrolling the corridor that connected the chamber with the outside. Introduction of (*E*,*E*)-α-farnesene or frontal gland secretion strikingly affected the walking speed of termites. The alerting behavior was also performed, predominantly by soldiers. The most prominent effect was the accumulation of the soldiers in the narrow corridor or in its vicinity, whereas members of other castes revealed a tendency to move to the parts of the chamber located farthest from the entrance or to climb up the chamber walls. Soldiers (and never a member of any other caste) were often seen to leave the corridor and perform zigzag searching; one to three soldiers were observed out of the chamber in four out of eight experiments recorded.

Electroantennography Antennae of both pseudergates and soldiers responded to frontal gland secretion and to (*E*,*E*)-α-farnesene (Fig. 5). The responses of soldiers to particular stimuli were slightly but significantly higher than that of pseudergates: the mean values for the standard stimulus were 0.253 mV in pseudergates and 0.327 mV in soldiers ($P<0.04$).

On the other hand, the responses of the two castes did not differ qualitatively: the dose response curves expressed as percent of the standard were identical for both castes (Fig. 5). The EAG response threshold to (*E*,*E*)-α-farnesene (compared to air control) was 100 ng for both castes. Above this threshold, the EAG response increased with the doses of (*E*,*E*)-α-farnesene: no EAG saturation was observed for the biologically relevant concentrations tested. The EAG response to the frontal gland secretion from a single soldier was approximately equivalent to that elicited by 1 μg of (*E*,*E*)-α-farnesene.

Discussion

Our results show that the frontal gland secretion is involved in alarm signaling in *P. canalifrons*. However, the major constituent of the frontal gland secretion, (*E*)-1-nitropentadec-1-ene, has no alarm effect. The function of this highly toxic compound (see Kuldová et al. 1999) can, therefore, be considered as purely defensive. In contrast, (*E*,*E*)-α-farnesene triggered several changes in behavior including increase in walking speed, searching for the odor source (zigzag running), attraction of both soldiers and pseudergates to the source (experiments A, B, and C), or attraction of soldiers and withdrawal of members of other castes from it (experiment D), and alerting of nestmates, performed predominantly by soldiers. Thus, (*E*,*E*)-α-farnesene clearly functions as an alarm pheromone in *P. canalifrons*. Together with the dominant (*E*)-1-nitropentadec-1-ene, isomers of α-farnesene also have been documented in species-specific mixtures in the frontal gland secretions of other *Prorhinotermes* species, *P. simplex* and *P. inopinatus* (Piskorski et al. 2007). As in *P. canalifrons*, we predict that

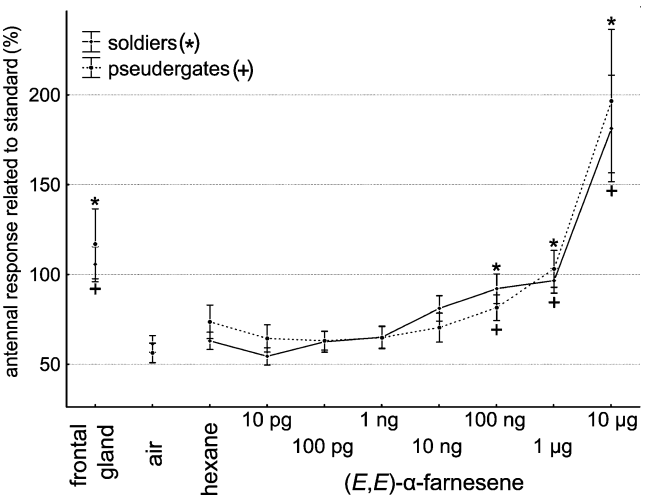

Fig. 5 EAG responses recorded from antennae of soldiers and pseudergates exposed to control stimuli (air and hexane), to the content of the soldier frontal gland, and to (*E*,*E*)-α-farnesene in seven doses. The responses are related to the value of response to a standard stimulus, i.e., 100 ng of (*E*,*E*)-α-farnesene. Nineteen measurements were performed for each stimulus. Points represent means, whiskers represent SEM. The values marked with *asterisks* (for soldiers) or *crosses* (for pseudergates) are significantly different from the air control (Kruskal–Wallis test; $P<0.05$; multiple non-parametric comparison)

these compounds will mediate alarm communication in these two species.

Behavioral effects of farnesene isomers have been recorded from numerous insect species. For example, (*E*,*E*) and/or (*Z*,*E*) isomers of α-farnesene act as sexual or trail pheromones in several insect orders, including many ant genera (e.g., Cammaerts 1973; Attygalle and Morgan 1983; Van der Meer 1983; Detrain et al. 1987). (*E*)-β-farnesene has been shown to be an alarm pheromone of aphids (Dawson et al. 1990) and a kairomone for their predators (ladybirds; Al Abassi et al. 2000; Francis et al. 2004). It has also been found in soldiers of *Reticulitermes* species (Quintana et al. 2003).

Although (*E*,*E*)-α-farnesene mediated an alarm response in both soldiers and pseudergates of *P. canalifrons*, soldiers were more sensitive, showing a stronger response in both behavioral tests and EAG. Similar EAG dose response curves suggests that both castes may use the same type of receptors. Thus, the difference in antennal sensitivity is probably based on differences in numbers of sensillae rather than on differences in sensitivity of olfactory receptor neurons tuned to (*E*,*E*)-α-farnesene. The numbers of sensillae between soldiers and pseudergates were not compared, but the soldiers' antennae are longer due to an increase in the number of antennal segments during soldier development from pseudergates (see Hanus et al. 2006).

The fact that soldiers are more sensitive to the alarm pheromone than pseudergates reflects their different social roles within termite societies. In this respect, the higher latency of response to chemical alarm in pseudergates can also be understood as caste specialization. The lower sensitivity and longer latency may ensure that pseudergates are not disturbed by stimuli that eventually appear as not dangerous, and rely upon soldiers to be alerted. The lower sensitivity of pseudergates is compensated by soldier nestmate alerting behavior mediated by mechanical means. Interestingly, the behavioral responses of soldiers to the alarm pheromone were more pronounced in heterogeneous groups containing both soldiers and pseudergates. In the absence of pseudergates, soldiers may exhibit lower activity simply because there are no other castes to alert.

A curious phenomenon that has been repeatedly observed in experiments with alarm pheromones is the accumulation of termites near the source of odor (Roisin et al. 1990, Reinhard and Clément 2002). We suggest that this response may be an artifact of the situation during testing because this was never observed when more natural conditions were used, i.e., in experiment D.

In both behavioral and EAG tests, the responses of both castes to the frontal gland content of one soldier were lower than to the equivalent dose of (*E*,*E*)-α-farnesene in hexane solution. This is probably due to (1) incomplete emptying of the gland on the tested paper or/and (2) different physical properties of the two solutions: hexane, being more volatile than (*E*)-1-nitropentadec-1-ene (which constitutes over 90% of the secretion), may enhance the evaporation of (*E*,*E*)-α-farnesene and thus volatilize higher amounts of alarm pheromone during the experiment. When considering the lower threshold dose of the alarm pheromone for sensorial and behavioral responses of pseudergates and soldiers, approximately 10% of the total amount of (*E*,*E*)-α-farnesene in one soldier frontal gland, one has to keep in mind that many soldiers participate in the alarm communication in a colony. It can thus be initiated and mediated by only a partial emptying of the frontal gland reservoir of a number of soldiers.

Termite soldiers are specialized for fulfilling defensive tasks to such a degree that they are not able to feed themselves and are fully dependent on the pseudergates' care. Their fundamental importance in *Prorhinotermes*, documented by their high proportion in the colony compared to other termite species (7–22%; Haverty 1977), comprises a wide variety of functions that include guarding the nest and foraging groups, direct physical defense, initiation and mediation of alarm by both vibratory and chemical means, and egg evacuation after an attack on the nest (Roisin et al. 2001; Hanus et al. 2005). Nevertheless, the role of pseudergates (or true workers in other termite species) in colony defense should not be overlooked because in many cases, they play a crucial role in defense (Thorne and Haverty 1991; Shelton and Grace 1996; Clément and Bagnères 1998).

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References

Al Abassi, S., Birkett, M. A., Pattersson, J., Pickett, J. A., Wadhams, L. J., and Woodcock, C. M. 2000. Response of the seven-spot ladybird to an aphid alarm pheromone and an alarm pheromone inhibitor is mediated by paired olfactory cells. *J. Chem. Ecol.* 26:1765–1771.

Attygalle, A. B., and Morgan, E. D. 1983. Trail pheromone of the ant *Tetramorium caespitum* L. *Naturwissenschaften* 70:364–365.

Bacchus, S. 1979. New exocrine gland on the legs of some Rhinotermitidae (Isoptera). *Int. J. Insect Morphol. Embryol.* 8:135–142.

Bordereau, C., Robert, A., Bonnard, O., and Le Quéré, J.-L. 1991. (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol: sex pheromone in a

higher fungus-growing termite, *Pseudacanthotermes spiniger* (Isoptera, Macrotermitinae). *J. Chem. Ecol.* 17:2177–2191.

BORDEREAU, C., CANCELLO, E. M., SÉMON, E., COURRENT, A., and QUENNEDEY,, B. 2002. Sex pheromone identified after solid phase microextraction from tergal glands of female alates in *Cornitermes bequaerti* (Isoptera, Nasutitermitinae). *Insect. Soc.* 49:209–215.

CAMMAERTS, M.–C. 1973. Aggregation pheromones of the workers of *Myrmica rubra*. *J. Insect Physiol.* 19:299–1315.

CASSIER, P., FAIN-MAUREL, M. A., and LEBRUN, D. 1977. Electron microscopic study of the mandibular glands of *Kalotermes flavicollis* Fabr. (Isoptera; Calotermitidae). *Cell Tiss. Res.* 182:327–339.

CLÉMENT, J.–L., and BAGNÈRES, A.–G. 1998. Nestmate recognition in termites, pp. 126–155, in R. K. Van der Meer, M. D. Breed, K. E. Espelie, and M. L. Winston (eds.). Pheromone Communication in Social Insects: Ants, Wasps, Bees and TermitesWestview, Boulder.

CONNÉTABLE, S., ROBERT, A., BOUFFAULT, F., and BORDEREAU, C. 1999. Vibratory alarm signals in two sympatric higher termite species: *Pseudacanthotermes spiniger* and *P. militaris* (Termitidae, Macrotermitinae). *J. Insect Behav.* 12:329–342.

COSTA-LEONARDO, A. M., and KITAYAMA, K. 1991. Frontal gland dehiscence in the Brazilian termite *Serritermes serrifer* (Isoptera: Serritermitidae). *Sociobiology* 19:333–338.

DAWSON, G. W., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid semiochemicals—a review, and recent advances on the sex-pheromone. *J. Chem. Ecol.* 16:3019–3030.

DELIGNE, J., QUENNEDEY, A., and BLUM, M. S. 1981. The enemies and defense mechanisms of termites, pp. 1–76, in H. R. Hermann (ed.). Social Insects, Vol. 2. Academic Press, New York.

DETRAIN, C., PASTEELS, J. M., BRAEKMAN, J. C., and DALOZE, D. 1987. (*Z*,*E*)-alpha-farnesene, main constituent of the hypertrophied Dufour's gland of the major workers of *Pheidole pallidula* (Formicidae). *Experientia* 43:345–346.

FRANCIS, F., LOGNAY, G., and HAUBRUGE, E. 2004. Olfactory responses to aphid and host plant volatile releases: (*E*)-β-farnesene, an effective kairomone for the predator *Adalia bipunctata*. *J. Chem. Ecol.* 30:741–755.

HANUS, R., ŠOBOTNÍK, J., and CIZEK,, L. 2005. Egg care by termite soldiers. *Insect. Soc.* 52:357–359.

HANUS, R., ŠOBOTNÍK, J., VALTEROVÁ, I., and LUKÁŠ,, J. 2006. The ontogeny of soldiers in *Prorhinotermes simplex* (Isoptera, Rhinotermitidae). *Insect. Soc.* 53:249–257.

HAVERTY, M. I. 1977. The proportion of soldiers in termite colonies: a list and a bibliography (Isoptera). *Sociobiology* 2:199–216.

HOWSE, P. E. 1964. The significance of the sound produced by the termite *Zootermopsis angusticollis* (Hagen). *Animal Behav.* 12:284–300.

HOWSE, P. E. 1965. On the significance of certain oscillatory movements of termites. *Insect. Soc.* 12:335–346.

KAIB, M. 1990. Intra- and interspecific chemical signals in the termite *Schedorhinotermes* - production sites, chemistry, and behaviour, pp. 26–32, in F. G. Gribakin, K. Wiese, and A. V. Popov (eds.). Sensory Systems and Communication in Arthropods. Birkhäuser, Basel.

KULDOVÁ, J., HRDÝ, I., and SVATOŠ, A. 1999. Defense secretion of *Prorhinotermes simplex*: toxicity to insecticide susceptible and resistant house fly. *J. Chem. Ecol.* 25:657–662.

KRISTON, M. I., WATSON, J. A. L., and EISNER, T. 1977. Non-combative behaviour of large soldiers of *Nasutitermes exitiosus* (Hill): an analytical study. *Insect. Soc.* 24:103–111.

LADUGUIE, N., ROBERT, A., BONNARD, O., VIEAU, F., LE QUÉRÉ, J.-L., SEMON, E., and BORDEREAU, C. 1994. Isolation and identification of (3*Z*,6*Z*,8*E*)-3,6,8-dodecatrien-1-ol in *Reticulitermes santonensis* Feytaud (Isoptera, Rhinotermitidae): roles in worker trail-following and in alate sex-attraction behaviour. *J. Insect Physiol.* 40:781–787.

MASCHWITZ, U., and MÜHLENBERG, M. 1972. Chemische Gefahrenalarmierung bei einer Termite. *Naturwissenschaften* 59:516–517.

MATSUMURA, F., COPPEL, H. C., and TAI, A. 1968. Isolation and identification of termite trail following pheromone. *Nature* 219:963–964.

MCDOWELL, P. G., and OLOO, G. W. 1984. Isolation, identification, and biological activity of trail-following pheromone of termite *Trinervitermes bettonianus* (Sjöstedt) (Termitidae: Nasutitermitinae). *J. Chem. Ecol.* 10:835–851.

NOIROT, C. 1969. Glands and Secretions, pp. 89–123, in K. Krishna, and F. M. Weesner (eds.). Biology of Termites, Vol. 1. Academic, New York.

PASTEELS, J. M., and BORDEREAU, C. 1998. Releaser pheromones in termites, pp. 193–215, in R. K. Van der Meer, M. D. Breed, K. E. Espelie, and M. L. Winston (eds.). Pheromone Communication in Social Insects: Ants, Wasps, Bees and Termites. Westview, Boulder.

PEPPUY, A., ROBERT, A., SEMON, E., GINIES, C., LETTERE, M., BONNARD, O., and BORDEREAU, C. 2001. (*Z*)-dodec-3-en-1-ol, a novel termite trail pheromone identified after solid phase microextraction from *Macrotermes annandalei*. *J. Insect Physiol.* 47:445–453.

PEPPUY, A., ROBERT, A., and BORDEREAU, C. 2004. Species-specific sex pheromones secreted from new sexual glands in two sympatric fungus-growing termites from northern Vietnam, *Macrotermes annandalei* and *M. barneyi*. *Insect. Soc.* 51:91–98.

PISKORSKI, R., HANUS, R., VAŠÍČKOVÁ, S., CVAČKA, J., ŠOBOTNÍK, J., SVATOŠ, A., and VALTEROVÁ, I. 2007. Nitroalkenes and sesquiterpene hydrocarbons from the frontal gland secretion of three *Prorhinotermes* termite species. *J. Chem. Ecol.* 33:1787–1794.

QUENNEDEY, A., and DELIGNE, J. 1975. L´arme frontale des soldats de termites (Rhinotermitidae). *Insect. Soc.* 22:243–267.

QUINTANA, A., REINHARD, J., FAURE, R., UVA, P., BAGNÈRES, A.-G., MASSIOT, G., and CLÉMENT, J.-L. 2003. Interspecific variation in terpenoid composition of defensive secretions of European *Reticulitermes* termites. *J. Chem. Ecol.* 29:639–652.

REINHARD, J., and CLÉMENT, J. -L. 2002. Alarm reaction of European *Reticulitermes* termites to soldier head capsule volatiles (Isoptera, Rhinotermitidae). *J. Insect Behav.* 15:95–107.

REINHARD, J., LACEY, M. J., IBARRA, F., SCHROEDER, F. C., KAIB, M., and LENZ, M. 2002. Hydroquinone: a general phagostimulating pheromone in termites. *J. Chem. Ecol.* 28:1–14.

ROISIN, Y. 1988. Morphology, development and evolutionary significance of the working stages in the caste system of *Prorhinotermes* (Insecta, Isoptera). *Zoomorphology* 107:339–347.

ROISIN, Y., EVERAERTS, C., PASTEELS, J. M., and BONNARD, O. 1990. Caste-dependent reactions to soldier defensive secretion and chiral alarm/recruitment pheromone in *Nasutitermes princeps*. *J. Chem. Ecol.* 16:2865–2875.

ROISIN, Y., PEARCY, M., and RUPF, T. 2001. Foraging behaviour and nest moving in *Prorhinotermes inopinatus* (Rhinotermitidae), a termite without workers. *Proc. Eur. Congress IUSSI*, Berlin, p. 178.

RÖHRIG, A., KIRCHNER, W. H., and LEUTHOLD, R. H. 1999. Vibrational alarm communication in the African fungus-growing termite genus *Macrotermes* (Isoptera, Termitidae). *Insect. Soc.* 46:71–77.

SHELTON, T. G., and GRACE, J. K. 1996. Review of agonistic behaviors in the Isoptera. *Sociobiology* 28:155−176.

ŠOBOTNÍK, J., and HUBERT, J. 2003. The morphology of the exocrine glands of *Prorhinotermes simplex* (Isoptera: Rhinotermitidae)

and their ontogenetical aspects. *Acta Soc. Zool. Bohem.* 67:83–98.

ŠOBOTNÍK, J., WEYDA, F., and HANUS, R. 2003. Ultrastructure of epidermal glands in neotenic reproductives of the termite *Prorhinotermes simplex* (Isoptera: Rhinotermitidae). *Arthropod Struct. Devel.* 32:201–208.

THORNE, B. L., and HAVERTY, M. I. 1991. A review of intracolony, intraspecific, and interspecific agonism in termites. *Sociobiology* 19:115−145.

TRANIELLO, J. F. A., PRESTWICH, G. D., and THORNE, B. L. 1984. Chemical composition and efficacy of cephalic gland secretion of *Armitermes chagresi* (Isoptera: Termitidae). *J. Chem. Ecol.* 10:531–543.

VANDER MEER, R. K. 1983. Semiochemicals and the red imported fire ant (*Solenopsis invicta* Buren) (Hymenoptera: Formicidae). *Florida Entomol.* 66:139–161.

VRKOČ, J., and UBIK, K. 1974. 1-Nitro-*trans*-1-pentadecene as the defensive compound of termites. *Tetrahedron Lett.* 15:1463–1464.

VRKOČ, J., KŘEČEK, J., and HRDÝ, I. 1978. Monoterpenic alarm pheromones in two *Nasutitermes* species. *Acta Entom. Bohemoslov.* 75:1–8.

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Molecular Characterization and Expression Pattern of Two Pheromone-Binding Proteins from *Spodoptera litura* (Fabricius)

Wei-Ming Xiu · Yao-Zhen Zhou · Shuang-Lin Dong

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Abstract Pheromone perception is thought to be mediated by pheromone-binding proteins (PBPs) in the lymph surrounding the olfactory receptors. We cloned and characterized two PBP genes (*SlitPBP1* and *SlitPBP2*) from the common cutworm, *Spodoptera litura* (F.; Lepidoptera: Noctuidae), which encode PBPs belonging to two different PBP groups. Western blot analysis of the crude antennal extracts with SexigPBP1 antibody revealed a single immunoreactive band (much stronger in male than in female) of ~16 kDa, in agreement with the calculated values for SlitPBPs. From genomic DNA, two introns and a similar exon/intron structural pattern were identified in each PBP genes, but the introns differed in length within and between PBP genes. The expression patterns of two *SlitPBP* genes, with respect to tissue distribution and sex, were further investigated by reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time PCR. Although the two PBP genes were expressed only in the antennae of both sexes, reflecting the antennal specificity of PBPs, the transcription levels of PBP genes differed between the sexes and the genes. The transcription levels of *SlitPBP1* and *SlitPBP2* in females were only 2.1% and 7.0%, respectively, relative to those in males, and the levels of PBP2 compared with PBP1 were 31.4% and 95.3% in males and females, respectively. These differential expression levels might suggest different roles played by the two SlitPBPs in the perception of sex pheromone both in males and females.

Keywords Common cutworm · Expression pattern · Pheromone binding protein (PBP) · Real-time PCR

W.-M. Xiu · Y.-Z. Zhou · S.-L. Dong (✉)
Key Laboratory of Monitoring
and Management of Plant Diseases and Insects,
Ministry of Agriculture, Nanjing Agricultural University,
Nanjing 210095, China
e-mail: sldong@njau.edu.cn

W.-M. Xiu
Agro-Environmental Protection Institute, Ministry of Agriculture,
Tianjin 300191, China

Introduction

Pheromone communication in moths is extremely sensitive and selective to discriminate extremely similar compounds or isomers (Hansson 1995). Perception of pheromones in moths and other insects is mediated by specialized pheromone-sensing organs, the sensilla trichodea, located mainly on the antenna. An early step in perception involves pheromone-binding proteins (PBPs), which are localized in the lymph of the sensilla on the moth antennae (Vogt and Riddiford 1981; Steinbrecht et al. 1995; Vogt et al. 2002). The first PBP was identified by its ability to bind the sex pheromone in the silk moth, *Antheraea polyphemus* (Vogt and Riddiford 1981). Since then, PBPs have been identified from various moth species (Krieger et al. 1996; Robertson et al. 1999; Picimbon and Gadenne 2002; Abraham et al. 2005; de Santis et al. 2006; Forstner et al. 2006; Xiu and Dong 2007). These proteins display considerable diversity, sharing 32–92% amino acid identity (Abraham et al. 2005). PBPs are thought to transport the relatively hydrophobic sex pheromones through the lymph surrounding the sensory dendrites to specific receptor proteins on the membrane of olfactory neurons (Prestwich 1996). Several PBPs have been demonstrated to bind pheromonal compounds (Du and Prestwich 1995; Plettner et al. 2000; Bette et al. 2002; Klusák et al. 2003; Maida et al. 2003; Lautenschlager et al. 2007).

Recently, several occurrences of multiple PBPs from a single moth species have been reported, and in each case, the different PBPs were encoded by distinct loci (Maïbèche-Coisné et al. 1998; Maida et al. 2000; Bette et al. 2002; Picimbon and Gadenne 2002; Abraham et al. 2005; de Santis et al. 2006; Forstner et al. 2006; Xiu and Dong 2007). Considering the multicomponent nature of moth sex pheromone constitution, it has been suggested that PBPs in moths might be involved in discriminating among pheromone components. Ligand-binding experiments in some moths, especially in the gypsy moth, *Lymantria dispar* (L.), have indicated that the various PBPs showed specificity in binding to their respective pheromone components (Du and Prestwich 1995; Plettner et al. 2000; Bette et al. 2002; Maida et al. 2003). This suggests a potential strategy in pest control by interfering with the expression of a single PBP in a moth species.

The common cutworm, *Spodoptera litura* (F.; Lepidoptera: Noctuidae), and the beet armyworm, *Spodoptera exigua* (Hüber), are important congeneric pests of various agricultural crops. They are sympatric in many parts of China and other Asian countries. The sex pheromones for these species have been identified and used successfully in population forecasting and pest control by mating disruption and mass trapping (Nemoto et al. 1980; Wakamura and Takai 1995; Srinivas and Rao 1999). The sex pheromone of *S. litura* comprises *Z*9, *E*11-14:Ac, *Z*9, *E*12-14:Ac, *Z*9-14:Ac, and *E*11-14:Ac (Tamaki et al. 1973, 1976; Sun et al. 2003), whereas that of *S. exigua* is composed of *Z*9, *E*12-14:Ac, *Z*9-14:OH, *Z*9-14:Ac, and *Z*9,*E*12-14:OH (Wakamura et al. 1989; Mitchell and Tumlinson 1994; Dong and Du 2002). Although the two species employ chemically similar pheromone components and share two of them, they are reproductively isolated in field environments, and traps that are baited with two components of each species (with one component in common) selectively attracted only one species in field trials (our unpublished data). Therefore, it would be interesting to compare differences in pheromone perception between the two species.

Previously, we identified and described two PBPs from *S. exigua* (Xiu and Dong 2007). In the present study, we report the identification of two PBPs from *S. litura* (SlitPBPs); their tissue distribution pattern; and relative expression levels in male and female antennae. We also report the genomic sequences of both genes.

Methods and Materials

Insect Rearing and Tissue Collection *S. litura* were reared at 27°C (14:10 hr, L:D) in the laboratory on an artificial diet (Huang et al. 2002) and separated by sex as pupae. Antennae were collected from 3-d-old male and female moths. They were excised at the base and immediately transferred into Eppendorf tubes immersed in liquid nitrogen. The same procedure was used for collecting heads (without antennae), thoraces, abdomens, and legs. All tissues were stored at –75°C until used experimentally.

Nucleic Acid Manipulation Total RNA was extracted by homogenizing antennae or other tissues of male and female moths by using the SV total RNA isolation system kit (Promega, Madison, WI, USA) following the manufacturer's instructions. Total RNA was transcribed into single strand cDNA that served as templates for PCR amplification by extension of an oligo$(dT)_{18}$ primer with MMLV reverse transcriptase (Promega) at 42°C for 60 min. The reaction was stopped by heating at 95°C for 5 min.

Genomic DNA was prepared from an individual fourth instar larva. The larva was ground in liquid nitrogen and homogenized with 300 µl DNA extraction buffer [(100 mM Tris–HCl, pH 8.0; 50 mM ethylenediaminetetraacetic acid (EDTA); 200 mM NaCl; 1% sodium dodecyl sulfate (SDS)]. The homogenate was transferred to an Eppendorf tube, gently mixed with 20 µl Proteinase K (20 mg/ml), and incubated at 56°C for 3 hr. The tube was centrifuged for 10 min at 12,000×*g*. The supernatant was transferred to a new tube, and DNA was extracted from it with an equal volume of phenol:chloroform: isoamyl alcohol (25:24:1) followed by an equal volume of chloroform:isoamyl alcohol (24:1). Each time, the sample was centrifuged for 5 min at 12,000×*g* to collect the aqueous phases. Two-and-one-half volumes of chilled ethanol were added to the aqueous supernatant for DNA precipitation. The pellets were washed in 70% ethanol, briefly vacuum-dried, and the DNA was redissolved in 50 µl TE buffer (10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0; 100 µg/ml RNase A).

Molecular Cloning and Sequencing The cDNAs that encode PBPs of *S. litura* were amplified from antennal cDNA of males. A pair of degenerate primers (Table 1) was designed to match amino acid regions of FWK(R)EG(E)Y and HE(D)LN(K)WA from the alignment of PBP sequences from *Heliothis virescens* (Krieger et al. 1993, GenBank X96861), *Helicoverpa zea* (Callahan et al. 2000, GenBank AF090191), *Agrotis ipsilon* (Picimbon and Gadenne 2002, GenBank AY301986), *Agrotis segetum* (LaForest et al. 1999, GenBank AF134253), and *Mamestra brassicae* (Maïbèche-Coisné et al. 1998, GenBank AF051142 and AF051143). These were the same degenerate primers used to clone *S. exigua* PBPs from a previous study (Xiu and Dong 2007). Amplification was carried out in a PCR machine PTC-200 (MJ Research, Waltham, MA, USA) and included negative controls under the following conditions: 94°C for 3 min; 9 cycles at 94°C for 50 sec, 65°C for 1 min, and 72°C for 30 sec, with a decrease of the

Table 1 Oligonucleotide primers used for isolation and expression analysis of *S. litura* pheromone binding proteins (PBP)

Purpose/Primer Name	Sequence (5′—3′)
cDNA Isolation (RT-PCR)	
Sense	TTYTGGMRNGARGRNTA
Antisense	GCCCANTTNARNTCRTG
5′ and 3′ cDNA End Isolation (RACE)	
SlitPBP1	
5′GSP	GCACTTGGCTACCTCCAGCACCCTC
3′GSP	GAGGGTGCTGGAGGTAGCCAAGTGC
SlitPBP2	
5′GSP	GCCTCATCGGGAGTAGACTGTGCG
3′GSP	GGCTGTGCTATCCTCTGCCTCTCC
Genomic DNA Isolation	
SlitPBP1	
Sense	ATGGCGGTTTGTGTTTGTA
Antisense	TTATTTTCCATAGCGTTAGCAA
SlitPBP2	
Sense	CGTTCTGTCCATCGGTTAC
Antisense	ACACTTCAGCAAGCACCTC
Tissue Localization (RT-PCR)	
SlitPBP1	
Sense	ATGGCGGTTTGTGTTTGTA
Antisense	TTATTTTCCATAGCGTTAGCAA
SlitPBP2	
Sense	CGTTCTGTCCATCGGTTAC
Antisense	ACACTTCAGCAAGCACCTC
Actin	
Sense	ATCTGGCACACCTTCTACAACGA
Antisense	TCACGCACGATTTCCCTCTCA
Expression Analysis (Real-time PCR)	
SlitPBP1	
Sense	ATGGACGACCAGACGATG
Antisense	GGAAGCACTTGGCTACCTC
SlitPBP2	
Sense	TGTGCTATCCTCTGCCTCT
Antisense	TGTCCACTATCTGCTTCGC
Actin	
Sense	ATCCTCCGTCTGGACTTGG
Antisense	CGCACGATTTCCCTCTCA

annealing temperature by 1°C per cycle. This was followed by 25 cycles at 94°C for 50 sec, 55°C for 1 min, and 72°C for 30 sec, and final incubation for 10 min at 72°C. The reaction was performed in 50 μl with 3 μl single-stranded cDNA, 2.0 mM $MgCl_2$, 0.2 mM deoxyribonucleotide triphosphate, 1.5 μM of each primer, and 2.5 U Taq polymerase (Promega).

A rapid amplification of cDNA ends (RACE) procedure was employed to amplify the 5′ and 3′ end of the coding region using SMART RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) following the kit instructions. Gene-specific primers (GSP; Table 1) for 5′-RACE and 3′-RACE were derived from the sequence of PCR products. Amplification conditions were 94°C for 2 min; 28 cycles at 94°C for 30 sec, 65°C for 30 sec, and 72°C for 3 min.

Regions of the PBP gene were amplified from genomic DNA by PCR to ascertain the exon/intron structure of the genes. Specific primer pairs (Table 1) were chosen for the amplification. For *SlitPBP1*, touchdown PCR reactions were performed as follows: 94°C for 3 min; 6 cycles at 94°C for 50 sec, 58°C for 50 sec, and 72°C for 2 min, with a decrease of the annealing temperature by 1°C per cycle. Subsequently, 34 cycles at 94°C for 50 sec, 52°C for 50 sec, and 72°C for 2 min and followed by incubation for 10 min at 72°C. For *SlitPBP2*, the reactions were: 94°C for 3 min; 40 cycles at 94°C for 50 sec, 64°C for 50 sec, and 72°C for 2 min and followed by incubation for 10 min at 72°C. LA Taq polymerase (TaKaRa, Otsu, Shiga, Japan) was used following manufacturer's instructions.

PCR products were analyzed by 1.5% agarose-gel electrophoresis, and DNA purification was performed with a Wizard PCR Preps DNA purification kit (Promega). Purified products were subcloned in a T/A cloning plasmid by using the pGEM-T easy vector system (Promega) following manufacturer's instructions. Plasmid DNA was transformed into DH5α-competent cells. Positive clones (on the basis of restriction enzyme cleavage sites and PCR amplification) were sequenced by using dye-terminator chemistry and reactions on an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Sequence Analysis Sequences were identified by using the NCBI-BLAST network server (Altschul et al. 1990). The putative signal peptides and their cleavage sites were predicted with the SignalP V 3.0 program (Bendtsen et al. 2004; http://www.cbs.dtu.dk/services/SignalP/). The hydrophobicity profile was determined by the method of Kyte and Doolittle (1982). The sequence data were aligned and compared with Clustal X (1.81) (Thompson et al. 1997). The phylogenetic tree was constructed with Neighbor Joining (NJ) by the MEGA3.1 program (Kumar et al. 2004). Bootstrap analysis used 1,000 replications.

Protein Extraction and Immunological Detection of SlitPBPs To test the expression of the PBPs in *S. litura*, antennae of 3-d-old males and females were excised separately. Each sample of 200 antennae was homogenized in ice-cold homogenizers in 100 μl of 20 mM Tris–HCl (pH 8.0). The homogenized samples were centrifuged for 20 min at 12,000×*g* at 4°C, and the supernatants were centrifuged again under the same conditions. Total protein concentration was estimated according to the method of Bradford (Bradford 1976).

Total antennal proteins (40 μg) were separated by 15% SDS-polyacrylamide gel electrophoresis (PAGE) under reducing conditions (Laemmli 1970), and the proteins were

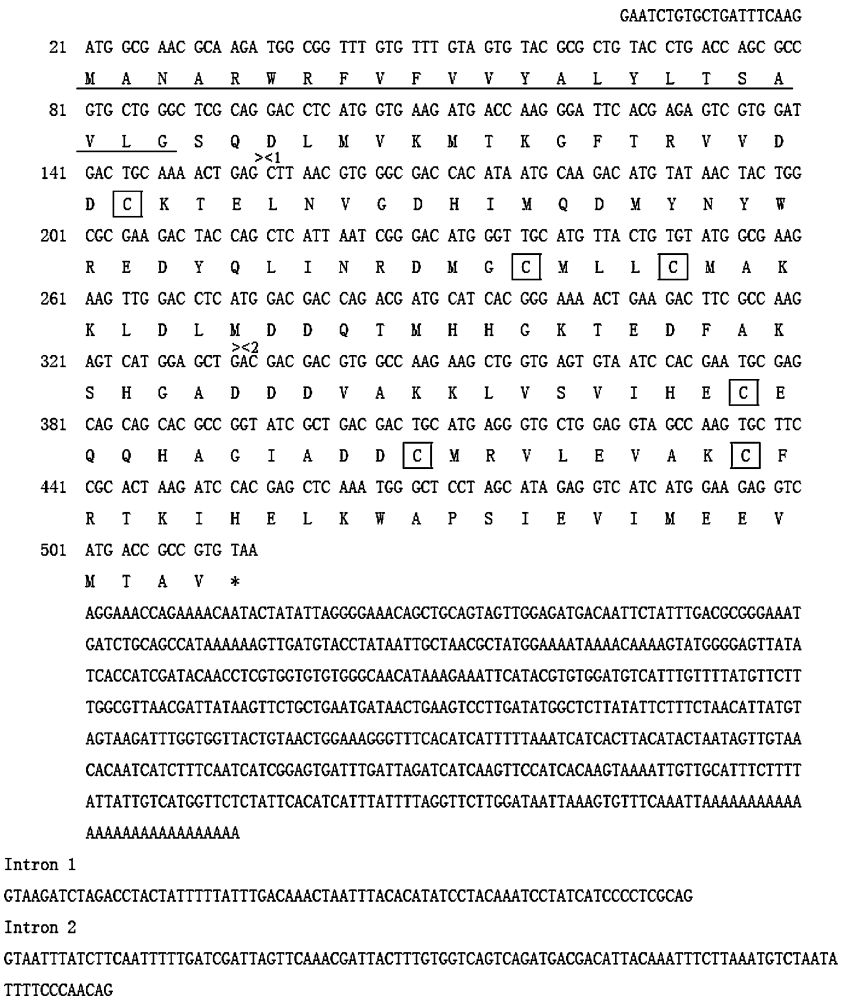

Fig. 1 cDNA sequence and predicted amino acid sequence of SlitPBP1. The stop codon is indicated with an *asterisk*. The signal peptide is *underlined*. The six conserved cysteines are *boxed*. Sites of intron one and two are marked with "><" above the sequence, and the intron sequences are given at the bottom of the figure

transferred to PVDF membranes (Millipore, Bedford, MA, USA). After transfer, the membranes were blocked with 5% dried nonfat milk powder in PBST buffer (PBS plus 0.05% Tween 20, pH 7.4), and incubated with mouse primary antiserum against SexigPBP1 (the antiserum was prepared by our laboratory) in PBST at an antibody dilution of 1:2,000 for 2 hr. After several washes with PBST, horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG as secondary antibody was applied for 2 hr, and 3,3′,5,5′-tetramethylbenzidine (TMB) was used as the staining substrate.

Distribution Pattern of SlitPBP Expression Tissue localization of expression of *SlitPBP*s was assessed by RT-PCR with the cDNA templates from antennae and other tissues of male and female moths, and genomic DNA. Specific primer pairs (Table 1) were derived from the cDNA sequences, spanning at least one predicted intron region, thus allowing us to distinguish genomic contaminants in the cDNA preparation. For testing the integrity of the cDNA templates, a control primer pair (Table 1) from the coding region of the *S. litura* β-actin gene (GenBank DQ494753) was used. PCR began at 94°C for 3 min, then 5 cycles at 95°C for 50 sec, 54°C for 50 sec, and 72°C for 2 min (with a decrease of the annealing temperature by 1°C per cycle); followed by 35 cycles at 95°C for 50 sec, 49°C for 50 sec, and 72°C for 2 min; with a final 10 min incubation at 72°C. PCR products were analyzed on 1.5% agarose gels.

Real-Time PCR Analysis of SlitPBP Expression Real-time PCR was performed in an ABI Prism 7000 Sequence Detection System (Applied Biosystems) with SYBR green dye bound to double strand DNA at the end of each elongation cycle (SYBR Green Realtime PCR Master Mix; TOYOBO, Osaka, Japan). Each pair of PCR primers was designed to span a cDNA exon–exon border to avoid amplification of potential traces of genomic DNA (Bohbot and Vogt 2005). *SlitPBP1* primers (Table 1) amplified a 169-bp product from cDNA. *SlitPBP2* primers (Table 1) amplified a 130 bp fragment from cDNA. As an endoge-

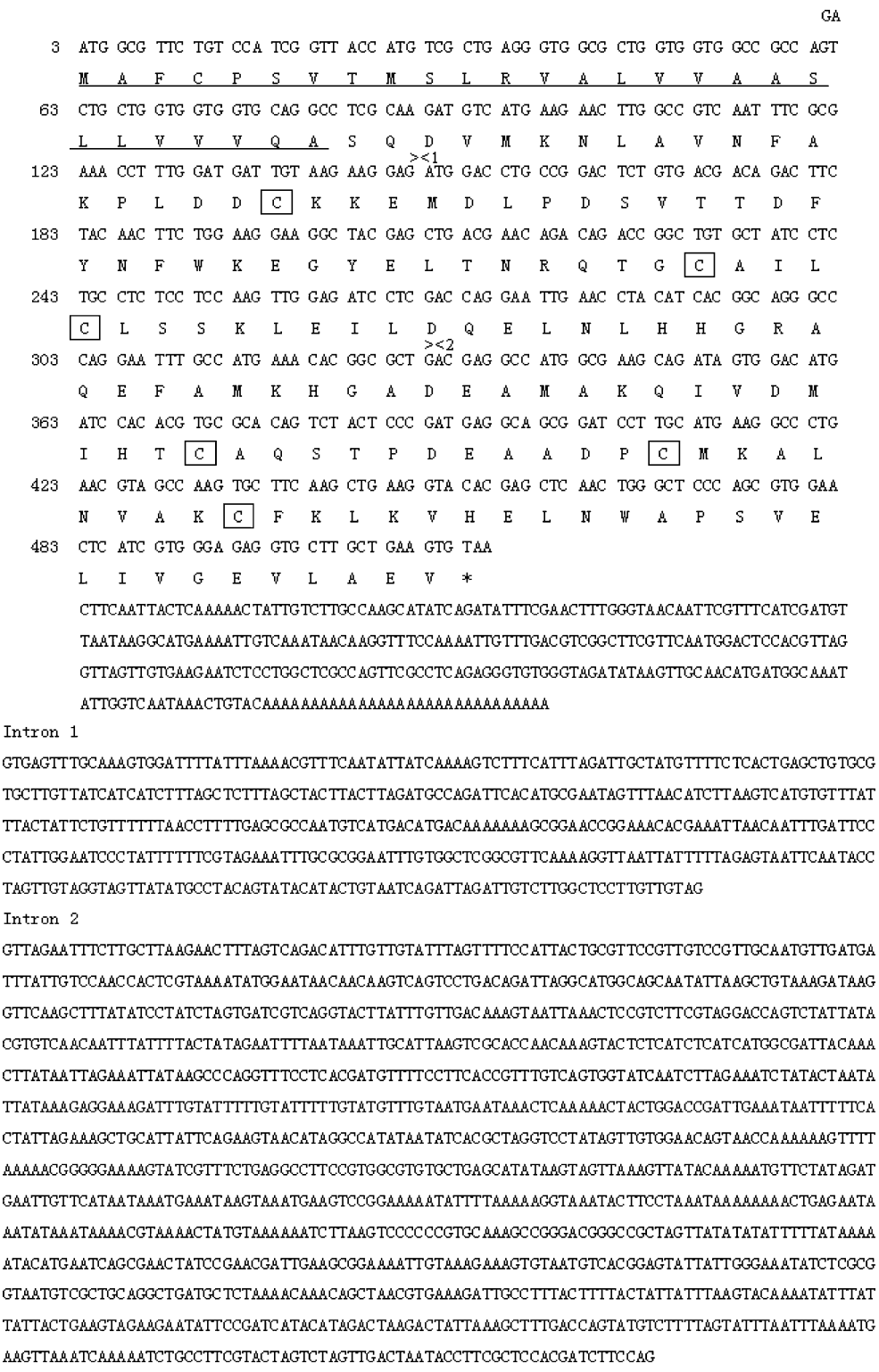

Fig. 2 cDNA sequence and predicted amino acid sequence of SlitPBP2. The stop codon is indicated with an *asterisk*. The signal peptide is *underlined*. The six conserved cysteines are *boxed*. Sites of intron one and two are marked with "><" above the sequence, and the intron sequences are given at the *bottom* of the figure

nous control to normalize the results of a variable target gene and to correct for sample-to-sample variation, the amplification size of β-actin gene primers (Table 1) was 220 bp.

Real-time PCR was conducted in 25 μl reactions that contained 12.5 μl of 2×SYBR Green PCR Master Mix, 0.5 μl of each primer (10 μM), 2.5 μl of sample cDNA, and 9 μl sterilized ultrapure H_2O (Millipore). To check reproducibility, test samples, endogenous control, and "no sample control" were done in triplicate. Cycling parameters included 94°C for 3 min, 40 cycles at 94°C for 30 sec, and

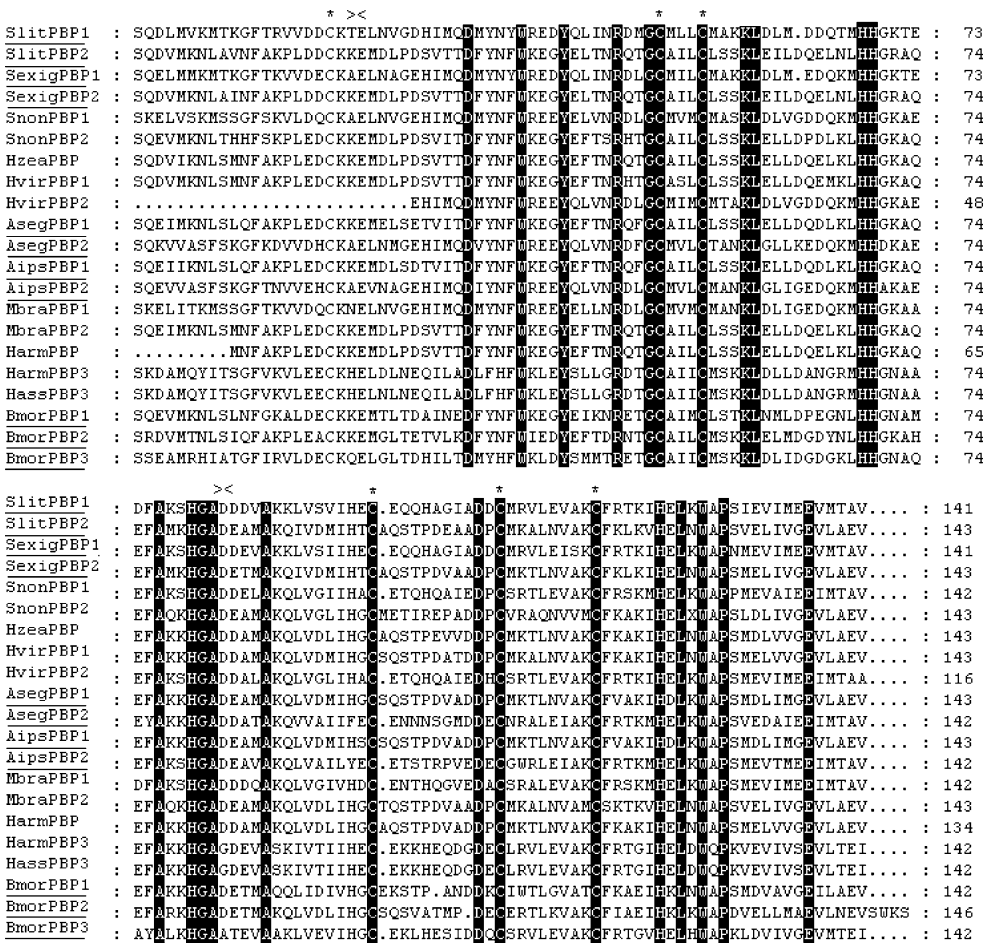

Fig. 3 Alignment of the mature SlitPBP1 and SlitPBP2 from *S. litura* with their orthologs from *Bombyx mori* and nine other species of Noctuidae: SlitPBP1 and SlitPBP2 are at the top, followed by SexigPBP1 (*S. exigua*, AY540316), SexigPBP2 (*S. exigua*, AY545636), SnonPBP1 (*Sesamia nonagrioides*, AY485219), SnonPBP2 (*S. nonagrioides*, AY485220), HzeaPBP (*Helicoverpa zea*, AF090191), HvirPBP1 (*Heliothis virescens*, X96861), HvirPBP2 (*H. virescens*, AY301988), AsegPBP1 (*A. segetum*, AF134253–AF134294), AsegPBP2 (*A. segetum*, AY301987), AipsPBP1 (*Agrotis ipsilon*, AY301985), AipsPBP2 (*A. ipsilon*, AY301986), MbraPBP1 (*Mamestra brassicae*, AF051143), MbraPBP2 (*M. brassicae*, AF051142), HarmPBP (*Helicoverpa armigera*, AJ278992), HarmPBP3 (*H. armigera*, AF527054), HassPBP3 (*Helicoverpa assulta*, DQ286414), BmorPBP1 (*Bombyx mori*, X94987), BmorPBP2 (*B. mori*, EMBL AM403100), and BmorPBP3 (*B. mori*, EMBL AM403101). Conserved amino acids in all PBPs are shown in a *black background*. Positions of six conserved cysteine residues are indicated by *asterisks*. Sites of the two introns are marked with "><", and the names of PBPs with intron data available are *underlined*

64.5°C for 45 sec. The products were analyzed by sequencing, agarose gel electrophoresis, and performing a melting curve, which indicated that each reaction did not produce nonspecific amplification.

Relative quantification was performed by using the comparative $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen 2001) to identify the amount of PBP mRNA in female antennal tissue relative to the amount in male tissue. All data were normalized to endogenous actin levels from the same individual sample. In the analysis of the relative fold-change, the male sample, which was one of the experimental samples, was taken as the calibrator (Mittapalli et al. 2006). Thus, the relative fold-change was assessed by comparing the expression level of *SexigPBP* in female moths to that in males. Calculations for relative quantification were performed, according to the instructions of the User Bulletin no. 2 from Applied Biosystems (Relative Quantification of Gene Expression; ABI Prism 7700 Sequence Detection System).

For a valid $\Delta\Delta C_T$ calculation with the comparative $2^{-\Delta\Delta C_T}$ method for relative quantification, the amplification efficiencies of the target and reference must be approximately equal. To confirm this, a pilot experiment was conducted to look at how ΔC_T ($C_{T,\text{Target}} - C_{T,\text{Actin}}$) varies with template dilution. Briefly, four serial tenfold dilutions of cDNA from each sample were amplified. For each dilution, amplifications were performed in triplicate with primers for PBP and actin. The average C_T was calculated for both PBP and actin and the ΔC_T was determined. A plot of the log cDNA dilution versus ΔC_T was made.

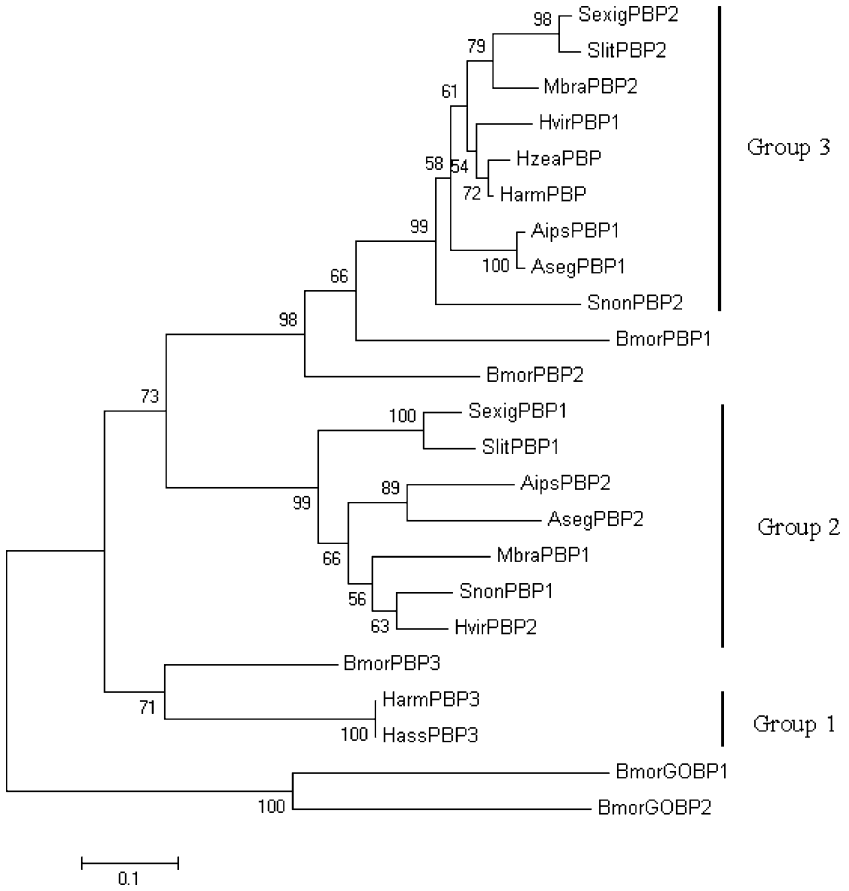

Fig. 4 Phylogenetic analysis based on SlitPBP1 and SlitPBP2 amino acid sequences. The tree was made with the neighbor-joining method with multiple alignments of amino acid sequences from Fig. 3 BmorGOBP1 and BmorGOBP2 from *Bombyx mori* were defined as the outgroups. Bootstrap analysis used 1,000 replications. The *bar* indicates phylogenetic distance value

Results

Identification of cDNA and Genomic DNA Sequences Encoding *S. litura* PBPs

The RT-PCR approach performed with a pair of degenerate primers identified two cDNA fragments of 275 and 281 bp, namely, *SlitPBP1* and *SlitPBP2*, respectively. A RACE procedure was further employed to obtain full-length sequences of the two genes from *S. litura*. The sequences of *SlitPBP1* and *SlitPBP2* (Figs. 1 and 2) were deposited in GenBank with the accession numbers DQ004497 and DQ114219, respectively.

The isolated cDNA clone encoding SlitPBP1 was 1,085 bp long and contained a 492-bp open reading frame for a polypeptide of 164 amino acids. The initial 23 amino acids are predicted as a signal peptide followed by the native protein of 141 amino acids with a molecular mass of 16,285 Da and an isoelectric point of 5.14. The isolated cDNA clone encoding SlitPBP2 (804 bp) comprised the complete PBP precursor consisting of a 27-amino acid signal peptide followed by the mature protein of 143 amino acids with a molecular mass of 15,998 Da and an isoelectric point of 4.92. The two PBPs display similar hydropathy profiles (not shown), and the presence of putative signal sequence suggest that these proteins are secreted into the extracellular fluid.

We aligned the mature amino acid sequences of SlitPBP1 and SlitPBP2 with PBPs from *Bombyx mori* (Lepidoptera: Bombycidae) and nine species of Noctuidae (Fig. 3). Both SlitPBP1 and SlitPBP2 have conserved motifs characteristic of lepidopteran PBPs, including the six cysteine residues that are believed to form three disulfide bridges, and the hydrophobic domains. The two SlitPBPs can be classified into different PBP groups (group 2 and group 3) by phylogenetic analysis and are closest phlylogenetically to PBPs from *S. exigua* (Fig. 4). Similarity analysis on the amino acid sequences of PBPs further confirmed the phylogenetic result. The two SlitPBPs share only 45% identity, but are much more identical to PBPs of the same group from other moth species. SlitPBP1 displays high identities with SexigPBP1 (87%), MbraPBP1 (70%), and SnonPBP1 (68%), whereas SlitPBP2 is more identical with SexigPBP2 (95%), MbraPBP2 (87%), and HvirPBP1 (84%; Table 2).

To understand the exon/intron structure of the genes, the two PBP genes were cloned and sequenced from the genomic DNA preparation. Both *SlitPBP1* and *SlitPBP2* contain two introns, and present a similar exon/intron structure. The two introns in *SlitPBP1* are located between Glu45 and Leu46 (intron 1, 76 bp) and inside the codon for Asp105 (intron 2,

Table 2 Percent identities of deduced amino acid sequence of full native proteins from pheromone binding proteins (PBPs) of the Noctuidae[a]

PBP	Harm PBP3	Hass PBP3	Slit PBP1	Sexig PBP1	Aips PBP2	Aseg PBP2	Mbra PBP1	Snon PBP1	Slit PBP2	Sexig PBP2	Aips PBP1	Aseg PBP1	Hvir PBP1	Hzea PBP	Mbra PBP2	Snon PBP2
HarmPBP3	142	**99%**	**51%**	**54%**	**44%**	**45%**	**50%**	**50%**	**46%**	**46%**	**43%**	**44%**	**46%**	**46%**	**45%**	**46%**
HassPBP3	141	142	**52%**	**54%**	**45%**	**46%**	**50%**	**51%**	**46%**	**46%**	**42%**	**44%**	**46%**	**46%**	**44%**	**45%**
SlitPBP1	73	74	141	**87%**	**63%**	**61%**	**70%**	**68%**	**45%**	**45%**	**45%**	**45%**	**46%**	**44%**	**44%**	**43%**
SexigPBP1	77	78	123	141	**69%**	**65%**	**71%**	**72%**	**42%**	**44%**	**46%**	**47%**	**44%**	**44%**	**44%**	**44%**
AipsPBP2	63	64	90	99	142	**76%**	**71%**	**70%**	**40%**	**40%**	**46%**	**46%**	**44%**	**44%**	**43%**	**43%**
AsegPBP2	65	66	88	93	109	142	**66%**	**67%**	**43%**	**41%**	**44%**	**45%**	**44%**	**46%**	**43%**	**42%**
MbraPBP1	71	72	100	102	102	95	142	**83%**	**42%**	**42%**	**47%**	**47%**	**46%**	**46%**	**44%**	**43%**
SnonPBP1	72	73	97	103	100	96	118	142	**43%**	**44%**	**48%**	**48%**	**48%**	**47%**	**44%**	**46%**
SlitPBP2	67	66	64	61	58	62	61	62	143	**95%**	**78%**	**77%**	**84%**	**83%**	**87%**	**73%**
SexigPBP2	67	66	65	63	58	59	61	64	137	143	**80%**	**79%**	**84%**	**86%**	**85%**	**72%**
AipsPBP1	62	61	65	66	66	64	68	69	112	115	143	**97%**	**83%**	**86%**	**83%**	**76%**
AsegPBP1	63	63	65	68	67	65	68	70	111	114	139	143	**83%**	**84%**	**83%**	**76%**
HvirPBP1	67	66	66	64	64	66	66	69	121	121	119	119	143	**92%**	**87%**	**84%**
HzeaPBP	67	66	64	63	64	64	66	68	120	123	123	121	132	143	**88%**	**86%**
MbraPBP2	65	64	63	63	62	62	63	64	125	122	119	119	125	126	143	**79%**
SnonPBP2	66	65	62	63	62	61	62	66	105	104	109	109	111	113	114	143

[a] Numbers in upper right (bold-faced font) are % identity of amino acid sequences and in lower left (normal font) show the number of residues that match exactly (identical residues) between the two sequences. The shaded table entries indicate the identities among PBPs of same group. PBP names are the same as in Fig. 3.

104 bp; Fig. 1). The introns in *SlitPBP2* are located between Glu49 and Met50 (intron 1, 437 bp) and inside the codon for Asp110 (intron 2, 2008 bp; Fig. 2). The ends of the introns have a typical GT–AG structure (Figs. 1 and 2).

SDS-PAGE and Western Blot Analysis of SlitPBPs To confirm the two SlitPBPs identified by the molecular means, SDS-PAGE and Western blot analysis with protein extracts from *S. litura* antenna were conducted (Fig. 5). An antenna-specific protein band was detected with the same molecular weight as that predicted from cloned SlitPBPs (~16 kD), and this protein reacted strongly with antibody against SexigPBP1. In addition, the targeted band intensity in male antennae was much higher than that in female antennae, in agreement with male-based detection of the female-produced *S. litura* sex pheromone.

Tissue Distribution of Expression RT-PCR experiments were performed by using specific primers (Table 1) to determine the tissue distribution of the PBPs from *S. litura* (Fig. 6). RT-PCR products of the predicted size were observed exclusively in reactions with antennal cDNA of both sexes. No specific product was observed with head (without antennae), thorax, abdomen, or leg cDNA. The integrity of the cDNA templates prepared from different tissues was verified by primers specific for the *actin* gene. In all cDNA preparations, an *Actin* amplification product of the correct size was amplified. Larger bands that might have been generated from amplification of genomic DNA were not found. Therefore, *SlitPBP1* and *SlitPBP2* are only expressed in antennae.

PBP Gene Expression Between the Sexes Real-time PCR was performed to compare the transcription levels of *SlitPBP1* and *SlitPBP2* from antennae of male with female moths. For each sex, cDNA was synthesized from mRNA isolated from pools of 200 antennae. To validate the $\Delta\Delta C_T$ calculation, a pilot experiment was conducted to look at how ΔC_T ($C_{T,Target} - C_{T,Actin}$) varies with a tenfold serial dilution of template cDNA. The results indicated that the absolute value of the slope of the entire dilution curve is close to zero (data not shown). Therefore, the efficiencies of the target and reference genes are similar, and the $\Delta\Delta C_T$ calculation method can be used for the relative quantification.

The levels of transcription of PBP genes in antennae were higher in male than in female moths. The amounts of *SlitPBP1* and *SlitPBP2* mRNA in female antennae were about 2.1% and 7.0%, respectively, relative to those in male antennae. The transcription level of *SlitPBP2* was 31.4% of that of *SlitPBP1* in male moths, but *SlitPBP1* and *SlitPBP2* had similar amounts (100:95.3) in female tissues (Table 3).

Discussion

Two novel PBPs were identified from the antennae of *S. litura.* They bear all the hallmarks of the PBP family including a signal peptide, a major hydrophobic domain, and particularly the six conserved cysteine residues among Lepidopteran OBPs. These six cysteine residues are connected by three interlocking disulfide bridges in the native OPPs (reviewed by Pelosi et al. 2006). Western blot analysis with antiserum against SexigPBP1 indicates a single protein band of the correct size in both male and female antennae, further supporting the identification of the two SlitPBPs. The identification of these two SlitPBPs adds new members to the moth PBP family, and enables future studies on structure–function relationship in PBPs, partic-

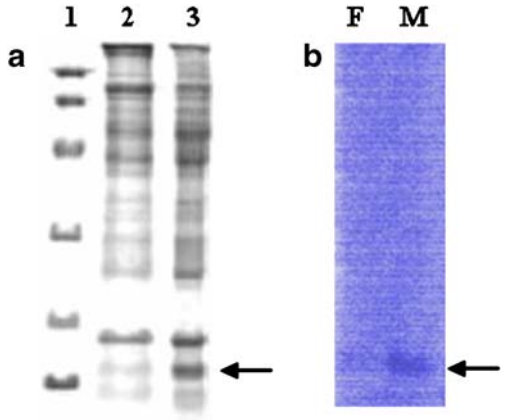

Fig. 5 SDS-PAGE and Western blots of crude extracts from female and male *S. litura* antenna probed with mouse primary antiserum against SexigPBP1 (i.e., an antibody from the congener). **a** SDS-PAGE of crude extracts from *S. litura* female and male antenna. *Lane 1*: low molecular mass markers (from the bottom to the top: 14.3 kD, 20.1 kD, 29.0 kD, 44.3 kD, 66.4 kD, 97.2 kD); *Lane 2*: Crude extracts from female antenna; and *Lane 3*: Crude extracts from male antenna. **b** Western blots of crude extracts from female and male *S. litura* antenna. *F* Female, *M* male. Arrows indicate the expected PBP proteins

ularly with respect to their discriminating ability among different pheromone components.

PBPs had been long regarded as specific to male antennae since their first identification in *A. polyphemus* (Vogt and Riddiford 1981; Laue and Steinbrecht 1997). However, PBP expression in female antennae now has been detected in many moth species (Krieger et al. 1996; Nagnan-Le Meillour et al. 1996; Callahan et al. 2000; Picimbon and Gadenne 2002; Maida et al. 2005; de Santis et al. 2006; Xiu and Dong 2007). In our present study with *S. litura*, we also found that both PBPs are also expressed in female antenna (Fig. 6). In fact, PBPs have been detected in antennae of all female moths examined so far when RT-PCR is used, a method far more sensitive than Western blot analysis. Therefore, it seems all female moths might express PBP in the antenna, although the expression levels might be lower than in male antennae and could vary according to species. Several studies have shown female autodetection of sex pheromone by electroantennography assay (Ljungberg et al. 1993; Ochieng et al. 1995; Schneider et al. 1998; Fan et al. 2003). The possible capacity for females to have a feedback control of their pheromone release has been proposed by Vogt et al. (2002). Nevertheless, the function and behavioral significance of PBPs in female moths remains elusive.

It is commonly recognized that the discrimination of pheromone components is performed by highly specific receptor proteins on the dendrite membrane of the olfactory neurons (Krieger et al. 2005; Nakagawa et al. 2005). In addition, the finding of multiple PBPs in a single insect species suggests that PBPs may also play a role in the differentiation of pheromone components by different binding capacity with pheromones. Quantification of the relative expression levels of each PBP could provide some clue for elucidating the functions of different PBPs. Unfortunately, the traditional immunoblot method with a PBP antibody does not differentiate different PBPs. It lacks selectivity among PBPs, even from different moth species. RT-PCR with specific primers can differentiate PBPs, but it is not a quantitative method. In the present study, we quantified the relative expression levels of the two SlitPBPs by using real-time PCR, and found that the expression levels of PBP1 is three times higher than that of PBP2 in male antennae, and this relative expression between PBP1 and PBP2 agrees with the quantitative ratio (100:27) of two

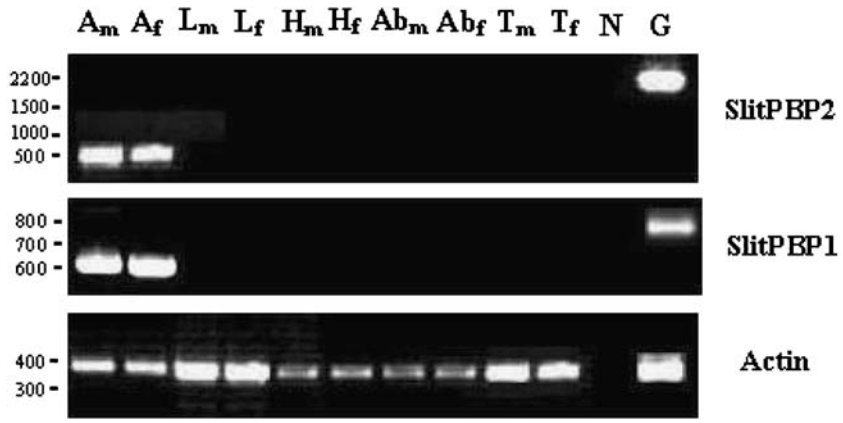

Fig. 6 Tissue-specific expression of *SlitPBP1* and *SlitPBP2*. RT-PCRs were performed by using RNAs isolated from the different tissues. Amplification products were analyzed on agarose gels and visualized by UV illumination after staining with ethidium bromide. Based on the primer design, the sizes of the expected PCR-products were 617 bp for *SlitPBP1*, 507 bp for *SlitPBP2*, and 377 bp for *Actin* (control). A_m Male antenna, A_f female antenna, H_m male head (without antenna), H_f female head (without antenna), T_m male thorax, T_f female thorax, Ab_m male abdomen, Ab_f female abdomen, L_m male leg, L_f female leg, *N* no-template (negative control) ensures the specificity of the amplifications, *G* genomic DNA template reaction indicates the relative position of PCR product derived from genomic DNA contamination in experimental samples. The position of molecular weight markers (bp) is indicated on the *left side*

Table 3 Relative quantification gene expression of *S. litura* pheromone-binding proteins (PBP) and β-actin by real-time PCR

Gene	PBP C_T[a]	Actin C_T[a]	ΔC_T[b]	$\Delta\Delta C_T$[c]	$2^{-\Delta\Delta C_T}$ (Range)[d]	$\Delta\Delta C_T$[e]	$2^{-\Delta\Delta C_T}$ (Range)[f]
Male PBP1	20.84±0.40	31.43±0.18	−10.60±0.43	0.00±0.43	1.000 (0.742–1.347)	0.00±0.43	1.000 (0.742–1.347)
Male PBP2	22.49±0.32	31.43±0.18	−8.93±0.14	1.67±0.14	0.314 (0.285–0.346)	0.00±0.14	1.000 (0.908–1.102)
Female PBP1	15.38±0.15	20.40±0.25	−5.02±0.32	0.00±0.32	1.000 (0.801–1.248)	5.58±0.32	0.021 (0.018–0.026)
Female PBP2	15.31±0.19	20.40±0.25	−5.09±0.10	0.07±0.10	0.953 (0.889–1.02)	3.84±0.10	0.070 (0.065–0.075)

[a] Mean±SE
[b] Mean PBP C_T−mean actin C_T±SE
[c] Mean ΔC_T−mean $\Delta C_{T,\ PBP1}$±SE
[d] Normalized amount of PBP relative to PBP1
[e] Mean ΔC_T−mean $\Delta C_{T,\ male}$±SE
[f] Normalized amount of PBP relative to male antennae

main active sex pheromone components (*Z*9,*E*11-14:Ac to *Z*9,*E*12-14:Ac) in the female sex pheromone gland in *S. litura* (Sun et al. 2002). However, it is unlikely that the two PBPs selectively bind to two different pheromone components, respectively, because in *S. exigua*, *Z*9,*E*12-14:Ac is the most abundant pheromone component, and the expression level of *SexiPBP1* is also much higher than that of *SexiPBP2* (Xiu and Dong 2007). Binding assays between PBPs and pheromone components would provide a clearer picture of whether and to what extent binding selectivity exists in *S. litura* and *S. exigua*.

Insects appeared in the geological record about 400 million years ago (Mya) and include more than 800,000 identified species with upper estimates ranging from 1.5–30 million species (Erwin 1982; Kristensen 1991; Vogt et al. 2002). The Noctuidae are thought to have evolved as a taxon as early as 100 Mya (Pashley and Ke 1992). The two *SlitPBPs* and other moth PBP genes share the same exon/intron number and conserved exon/intron boundaries and positions (Fig. 3), which suggests a common PBP ancestor of these genes. A previous phylogenetic analysis of deduced amino acid sequences revealed three distinct groups of Noctuid PBPs (Xiu and Dong 2007). We elected to name these group-1 through group-3, with group-1 being the most ancestral. The clustering suggests that two duplication events might have occurred during noctuid PBP gene evolution, but we hypothesized that these duplications occurred before the differentiation of the Noctuidae (Xiu and Dong 2007). The two duplication events lead to the three distinct PBP groups, and group-2 and group-3 PBP have been found commonly in the Noctuidae, whereas Group-1 PBP have been identified rarely in these species (Fig. 4).

In contrast to the similarities among the amino acid sequences of PBPs of each group, the introns of PBPs display greater variation, particularly in their lengths (Table 4). First, both introns of group-2 PBP genes in *S. litura* and *S. exigua* are only about 100 bp, much shorter than introns from group-2 PBP genes of other species. Second, when compared with the sequences of two *Agrotis* moths, introns in group-3 PBP genes are much longer than those in group-2 genes. These differences indicate that the introns are under weaker selective pressure than exons in the evolution of moth PBPs. More generalized conclusions on introns and their implications in PBP evolution can be proposed when additional intron data become available from additional noctuid species.

Table 4 Intron lengths of known PBPs of the Noctuidae and Bombycidae

Family	Species	PBP Group in Noctuidae	PBP Name	GenBank Accession Number	First Intron (bp)	Second Intron (bp)	Second Intron/First Intron in Length
Bombycidae	*B. mori*		BmorPBP1	Gene Scaffold010822	701	427	
			BmorPBP2		782	630	
			BmorPBP3		229	701	
Noctuidae	*A. ipsilon*	Group-2	AipsPBP2	AY973627	458	564	1.23
		Group-3	AipsPBP1	AY973626	312	1,056	3.38
	A. segetum	Group-2	AsegPBP2	AY973628	362	512	1.41
		Group-3	AsegPBP1	AF134294	318	993	3.12
	S. litura	Group-2	SlitPBP1	EF052847	76	104	1.41
		Group-3	SlitPBP2	EF052848	437	1,251	2.86
	S. exigua	Group-2	SexigPBP1	EF052846	87	142	1.63
		Group-3	SexigPBP2	EF052849	295	644	2.18

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References

ABRAHAM, D., LÖFSTEDT, C., and PICIMBON, J. F. 2005. Molecular characterization and evolution of pheromone binding protein genes in *Agrotis* moths. *Insect Biochem. Mol. Biol.* 35:1100–1111.

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W., and LIPMAN, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

BENDTSEN, J. D., NIELSEN, H., VONHEIJNE, G., and BRUNAK, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340:783–795.

BETTE, S., BREER, H., and KRIEGER, J. 2002. Probing a pheromone binding protein of the silkmoth *Antheraea polyphemus* by endogenous tryptophan fluorescence. *Insect Biochem. Mol. Biol.* 32:241–246.

BOHBOT, J., and VOGT, R. G. 2005. Antennal expressed genes of the yellow fever mosquito (*Aedes aegypti* L.); characterization of odorant-binding protein 10 and takeout. *Insect Biochem. Mol. Biol.* 35:961–979.

BRADFORD, M. A. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

CALLAHAN, F. E., VOGT, R. G., TUCKER, M. L., DICKENS, J. C., and MATTOO, A. K. 2000. High level expression of "male specific" pheromone binding proteins (PBPs) in the antennae of female noctuid moths. *Insect Biochem. Mol. Biol.* 30:507–514.

DE SANTIS, F., FRANÇOIS, M. C., MERLIN, C., PELLETIER, J., MAÏBÈCHE-COISNÉ, M., CONTI, E., and JACQUIN-JOLY, E. 2006. Molecular cloning and in situ expression patterns of two new pheromone-binding proteins from the corn stemborer, *Sesamia nonagrioides*. *J. Chem. Ecol.* 32:1703–1717.

DONG, S. L., and DU, J. W. 2002. Chemical identification and field tests of sex pheromone of beet armyworm *Spodoptera exigua*. *Acta Phytophylacica Sin.* 29:19–24.

DU, G., and PRESTWICH, G. D. 1995. Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry* 34:8726–8732.

ERWIN, T. L. 1982. Tropical forests: their richness in Coleoptera and other arthropod species. *Coleop. Bull.* 36:74–75.

FAN, W. M., SHENG, C. F., and SU, J. W. 2003. Electrophysiological and behavioral responses of both sexes of the cotton bollworm, *Helicoverpa armigera* Hübner to sex pheromones. *Acta Entomologica Sinica* 46:138–143.

FORSTNER, M., GOHL, T., BREER, H., and KRIEGER, J. 2006. Candidate pheromone binding proteins of the silkmoth *Bombyx mori*. *Invert. Neurosci.* 8:177–187.

HANSSON, B. S. 1995. Olfaction in Lepidoptera. *Experientia* 51:1003–1027.

HUANG, C. X., ZHU, L. M., NI, J. P., and CHAO, X. Y. 2002. A method of rearing the beet armyworm *Spodoptera exigua*. *Entomological Knowledge* 39:229–231.

KLUSÁK, V., HAVLAS, Z., ULISEK, L., VONDRÁSEK, J., and SVATOS, A. 2003. Sexual attraction in the silkworm moth: Nature of binding of bombykol in pheromone binding protein-an *ab initio* study. *Chem. Biol.* 10:331–340.

KRIEGER, J., GANSSLE, H., RAMING, K., and BREER, H. 1993. Odorant binding proteins of *Heliothis virescens*. *Insect Biochem. Mol. Biol.* 23:449–456.

KRIEGER, J., VONNICKISCH-OSENEGK, E., MAMELI, M., PELOSI, P., and BREER, H. 1996. Binding proteins from the antennae of *Bombyx mori*. *Insect Biochem. Mol. Biol.* 26:297–307.

KRIEGER, J., GROSSE-WILDE, E., GOHL, T., and BREER, H. 2005. Candidate pheromone receptors of the silkmoth *Bombyx mori*. *Eur. J. Neurosci.* 21:2167–2176.

KRISTENSEN, N. P. 1991 Phylogeny of extant hexapodspp.125–140, in CSIRO (ed.). The Insects of Australia: A Textbook for Students and Research Workers. Melbourne University Press, Melbourne, Australia.

KUMAR S., TAMURA K., and NEI M. 2004. MEGA3: Integrated software for molecular evolutionary genetics analysis and sequence alignment. *Brief. Bioinform.* 5:150–163.

KYTE, J., and DOOLITTLE, R. F. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* 157:105–132.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LAFOREST, S. M., PRESTWICH, G. D., and LÖFSTEDT, C. 1999. Intraspecific nucleotide variation at the pheromone binding protein locus in the turnip moth, *Agrotis segetum*. *Insect Mol. Biol.* 8:481–490.

LAUE, M., and STEINBRECHT, R. A. 1997. Topochemistry of moth olfactory sensillae. *Int. J. Insect Morphol. Embryol.* 26:217–228.

LAUTENSCHLAGER, C., LEAL, W. S., and CLARDY, J. 2007. *Bombyx mori* pheromone-binding protein binding nonpheromone ligands: Implications for pheromone recognition. *Structure* 15:1148–1154.

LIVAK, K. J., and SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method. *Methods* 25:402–408.

LJUNGBERG, H., ANDERSON, P., and HANSSON, B. S. 1993. Physiology and morphology of pheromone specific sensilla on the antennae of male and female *Spodoptera littoralis* (Lepidoptera: Noctuidae). *J. Insect Physiol.* 39:253–260.

MAÏBÈCHE-COISNÉ, M., JACQUIN-JOLY, E., FRANCOIS, M. C., and NAGNAN-LEMEILLOURM, P. 1998. Molecular cloning of two pheromone binding proteins in the cabbage armyworm *Mamestra brassicae*. *Insect Biochem. Mol. Biol.* 28:815–818.

MAIDA, R., KRIEGER, J., GEBAUER, T., LANGE, U., and ZIEGELBERGER, G. 2000. Three pheromone binding proteins in olfactory sensilla of the two silkmoth species *Antheraea polyphemus* and *Antheraea pernyi*. *Eur. J. Biochem.* 267:2899–2908.

MAIDA, R., ZIEGELBERGER, G., and KAISSLING, K. E. 2003. Ligand binding to six recombinant pheromone binding proteins of *Antheraea polyphemus* and *Antheraea pernyi*. *J. Comp. Physiol. B* 173:565–573.

MAIDA, R., MAMELI, M., MÜLLER, B., KRIEGER, J., and STEINBRECHT, R. A. 2005. The expression pattern of four odorant-binding proteins in male and female silk moths, *Bombyx mori*. *J. Neurocytology* 34:149–163.

MITCHELL, E. R., and TUMLINSON J. H. 1994. Response of *Spodoptera exigua* and *S. eridania* (Lepidoptera: Noctuidae) males to synthetic pheromone and *S. exigua* females. *Florida Entomol.* 77:237–247.

MITTAPALLI, O., WISE, I. L., and SHUKLE, R. H. 2006. Characterization of a serine carboxypeptidase in the salivary glands and fat body of the orange wheat blossom midge, *Sitodiplosis mosellana* (Diptera: Cecidomyiidae). *Insect Biochem. Mol. Biol.* 36:154–160.

NAGNAN-LE MEILLOUR, P., HUET, J. C., MAÏBECHE, M., PERNOLLET, J. C., and DESCOINS, C. 1996. Purification and characterization of multiple forms of Odorant/Pheromone binding proteins in the antennae of *Mamestra brassicae* (Noctuidae). *Insect Biochem. Mol. Biol.* 26:59–67.

NAKAGAWA, T., SAKURAI, T., NISHIOKA, T., and TOUHARA, K. 2005. Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307:1638–1642.

NEMOTO, H., TAKAHASHI, K., and KUBOTA, A. 1980. Reduction of the population density of *Spodoptera litura* (F.) (Lepidoptera: Noctuidae) using a synthetic sex pheromone. I. Experiment in taro field. *Jap. J. Appl. Entomol. Zool.* 24:211–216.

OCHIENG, S. A., ANDERSON, P., and HANSSON, B. S. 1995. Antennal lobe projection patterns of olfactory receptor neurons involved in sex pheromone detection in *Spodoptera littoralis* (Lepidoptera: Noctuidae). *Tissue Cell* 27:221–232.

PASHLEY, D. P., and KE, L. D. 1992. Sequence evolution in mitochondrial ribosomal and ND-1 genes in Lepidoptera: implications for phylogenetic analyses. *Mol. Biol. Evol.* 9:1061–1075.

PELOSI, P., ZHOU, J. J., BAN, L. P., and CALVELLO, M. 2006. Soluble proteins in insect chemical communication. *Cell. Mol. Life Sci.* 63:1658–1676.

PICIMBON, J. -F., and GADENNE, C. 2002. Evolution of noctuid pheromone binding proteins: identification of PBP in the black cutworm moth, *Agrotis ipsilon*. *Insect Biochem. Mol. Biol.* 32:839–846.

PLETTNER, E., LAZAR, J., PRESTWICH, E. G., and PRESTWICH, G. D. 2000. Discrimination of pheromone enantiomers by two pheromone binding proteins from the gypsy moth *Lymantria dispar*. *Biochemistry* 39:8953–8962.

PRESTWICH, G. D. 1996. Proteins that smell: Pheromone recognition and signal transduction. *Bioorgan. Med. Chem.* 4:505–513.

ROBERTSON, H. M., MARTOS, R., SEARS, C. R., TODRES, E. Z., WALDEN, K. K. O., and NARDI, J. B. 1999. Diversity of odorant binding proteins revealed by an expressed sequence tag project on male *Manduca sexta* moth antennae. *Insect Mol. Biol.* 8: 501–518.

SCHNEIDER, D., SCHULZ, S., PRIESNER, E., ZIESMANN, J., and FRANCKE, W. 1998. Autodetection and chemistry of female and male pheromone in both sexes of the tiger moth *Panaxia quadripunctaria*. *J. Comp. Physiol. A* 182:153–161.

SRINIVAS, K., and RAO, P. A. 1999. Management of *Spodoptera litura* (F.) infesting groundnut by mating disruption technique with synthetic sex pheromone. *J. Entomol. Res.* 23:115–119.

STEINBRECHT, R. A., LAUE, M., and ZIEGELBERGER, G. 1995. Immunolocalization of pheromone-binding protein and general odorant binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. *Cell Tissue Res.* 282:203–217.

SUN, F., DU, J. W., and CHEN, T. H. 2003. The behavioral responses of *Spodoptera litura* (F.) males to the female sex pheromone in wind tunnel and field trapping tests. *Acta Entomologica Sinica* 46:126–130.

SUN, F., HU, Y. Y., and DU, J. W. 2002. The sex pheromone communication system of *Spodoptera litura* (Fabricius). *Acta Entomological Sinica* 45:404–407.

TAMAKI, Y., NOGUCHI, H., and YUSHIMA, T. 1973. Sex pheromone of *Spodoptera litura* (F.) (Lepidoptera: Noctuidae): isolation, identification and synthesis. *Appl. Ent. Zool.* 8:200–203.

TAMAKI, Y., OSAWA, T., YUSHIMA, T., and NOGUCHI H. 1976. Sex pheromone and related compounds secreted by the virgin female of *Spodoptera litura* (F.). *Jap. J. Appl. Ent. Zool.* 20: 81–86.

THOMPSON, J. D., GIBSON, T. J., PLEWNIAK, F., JEANMOUGIN, F., and HIGGIN, D. G. 1997. The Clustal X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acid Res.* 25:4876–4882.

VOGT, R. G., and RIDDIFORD, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163.

VOGT, R. G., ROGERS, M. E., FRANCO, M. D., and SUN, M. 2002. A comparative study of odorant binding protein genes: differential expression of the PBP1-GOBP2 gene cluster in *Manduca sexta* (Lepidoptera) and the organization of OBP genes in *Drosophila melanogaster* (Diptera). *J. Exp. Biol.* 205:719–744.

WAKAMURA, S., and TAKAI, M. 1995. Communication disruption for control of the beet armyworm, *Spodoptera exigua* (Hübner), with synthetic sex pheromone. *Jap. Agric. Res.* 29:125–130.

WAKAMURA, S., TAKAI, M., KOZAI, S., INOUE, H., YAMASHITA, I., KAWAHARA, S., and KAWAMURA, M. 1989. Control of the beet armyworm *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) using synthetic sex pheromone. I. Effect of communication disruption in Welsh onion fields. *Appl. Entomol. Zool.* 24: 387–397.

XIU, W. M., and DONG, S. L. 2007. Molecular characterization of two pheromone binding proteins and quantitative analysis of their expression in the beet armyworm, *Spodoptera exigua* Hübner. *J. Chem. Ecol.* 33:947–961.

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Bidens pilosa L. Exhibits High Sensitivity to Coumarin in Comparison with Three Other Weed Species

Érica Marusa Pergo · Denise Abrahim · Patrícia Cristina Soares da Silva · Kátia Aparecida Kern · Lucas Jonatas Da Silva · Elemar Voll · Emy Luiza Ishii-Iwamoto

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Abstract Nine natural plant compounds were screened for phytotoxicity to *Bidens pilosa* L. a troublesome weed in field and plantation crops. The sensitivity of three other weed species to coumarin, the most active identified compound, was also evaluated. Coumarin, at a concentration of 500 μM, had little effect on germination and growth of *Senna obtusifolia* L., *Euphorbia heterophylla* L., and *Ipomoea grandifolia* L. when compared with its effects on *B. pilosa* L. In a concentration range of 10–100 μM, coumarin caused a dose-dependent inhibition of germination and growth of *B. pilosa* L. The measurements of some parameters of energy metabolism revealed that coumarin-treated root tissues exhibited characteristics of seedlings in an earlier stage of growth, including higher respiratory activity and higher activities of alcohol dehydrogenase and lipoxygenase. These results suggest that coumarin inhibition of germination and growth of *B. pilosa* L. was not a consequence of an impairment of energy metabolism. Rather, it seems to act as a cytostatic agent, retarding germination. At concentrations above 50 μM, coumarin increased lipoxygenase activity and the level of conjugated dienes of root extracts, suggesting that it may induce oxidative stress in seedling roots.

É. M. Pergo · D. Abrahim · P. C. Soares da Silva · K. A. Kern · L. J. Da Silva · E. L. Ishii-Iwamoto (✉)
Laboratory of Biological Oxidations,
Department of Biochemistry, University of Maringá,
87020900 Maringá, Brazil
e-mail: eliiwamoto@uem.br

E. Voll
Brazilian Agricultural Research Corporation-EMBRAPA,
Londrina, Brazil

Keywords *Bidens pilosa* L. · *Asteraceae* · Seed germination · Seedling growth · Respiratory energy metabolism · Alcohol dehydrogenase · Lipoxygenase · Coumarin

Abbreviations

AOX	alternative oxidase
COX	cytochrome oxidase
DTT	dithiothreitol
EDTA	ethylene diamide tetracetic acid
KCN	potassium cyanide
MDA	malondialdehyde
TBA	2-thiobarbituric acid
TCA	trichloroacetic acid

Introduction

Bidens pilosa L., an annual native of tropical America, is a dicot weed of the *Asteraceae* family. The species is a troublesome weed in field and plantation crops in more than 40 countries (Holm et al. 1977). In Brazil, it is responsible for yield losses of several crops, particularly soybean. It was demonstrated that *B. pilosa* L. first evolved resistance to herbicides known as acetolactase synthase inhibitors in 1993, and there were estimates that the resistant biotype continues to increase its distribution and prevalence (Christoffoleti and Foloni 1999). Because of increasing incidence of weeds evolving resistance to many commercial herbicides, there is growing interest in the development of alternative methods for weed control based on natural products. Plants produce thousands of secondary products that represent a large reservoir of novel chemical structures with biological activity. The use of plant species

with weed-suppressing ability thus has been considered for biological weed management in crop production (Putnam and Duke 1974; Macías 1995; Wu et al. 1999; Duke et al. 2000; Vyvyan 2002; Khanh et al. 2006). Numerous secondary plant products of the phenolic, flavonoid, and terpenoid classes have been implicated as compounds responsible for plant growth suppression (Reigosa et al. 1999; Vaughn and Spencer 1993; Dudai et al. 1999; Duke et al. 2000; Chon and Kim 2004; Kohli et al. 2006). However, there are fewer studies that are concerned with modes of action. Mitochondrial respiratory metabolism is essential to produce energy and precursors for biosynthesis of new cellular structures. An effect on respiratory metabolism could be a mode of action of natural compounds in suppressing the germination and growth of weeds.

In view of this, the aim of the present work was to identify which among nine compounds exerts high and selective phytotoxicity on *B. pilosa* L. We chose representative compounds of the phenolic and terpenoid classes, namely, caffeic, *p*-coumaric, ferulic, protocatechuic, and vanillic acids, coumarin, flavone, camphor, and eucalyptol. The effects of coumarin, the most active identified compound, on biochemical processes, critical for seed germination and seedling growth, were also evaluated. For the latter purpose, respiratory activity and the activity of alcohol dehydrogenase (EC 1.1.1.1) in seedling roots during postgerminative growth were measured. Some parameters of oxidative stress were also evaluated, including lipoxygenase activity (EC 1.13.11.12) and the content of malondialdehyde and conjugated dienes.

Methods and Materials

Reagents Camphor, caffeic acid, eucalyptol, *p*-coumaric acid, coumarin, ferulic acid, flavone, protocatechuic acid, vanillic acid, NAD^+, linolenic acid, and 2-thiobarbituric acid (TBA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Reagents were of the purest grade available.

Seed Germination and Growth Seeds of *B. pilosa* L., *Euphorbia heterophylla* L., *Senna obtusifolia* L., and *Ipomoea grandifolia* L. were purchased from a commercial supplier (Cosmos Agrícola Produtos e Serviços Rurais Ltda, Brazil). Seeds were surface-sterilized in a 1.0% sodium hypochlorite solution. After washing in distilled water, seeds were placed on a double sheet of germination paper in plastic germination boxes (gerbox; 110×110 mm), moistened with 5–12 ml of distilled water or plant natural compound solutions at a concentration of 500 μM. Ferulic, caffeic, vanillic, and protocatechuic acids were dissolved in distilled water. Camphor, eucalyptol, flavone, and coumarin were dissolved in a 0.1% dimethylsulfoxide solution. Flavone was assayed at a concentration of 250 μM because of its low solubility in the 0.1% dimethylsulfoxide solution. Coumarin was assayed in a concentration range of 10 to 100 μM. Controls were performed to exclude the interference of dimethylsulfoxide, but no significant changes in seedling growth were found. Each treatment was applied to three plates (replicates), and each replicate consisted of 50 seeds distributed over gerbox. Experiments were repeated four to six times. Boxes were placed in a growth chamber programmed for the following regime for *B. pilosa* L.: 8/16 hr L/D, 30°/20°C. *E. heterophylla* L. seeds were allowed to germinate and grow at 25°C and on a 12/12 hr L/D photoperiod. The regimes for *S. obtusifolia* L. and *I. grandifolia* L. were 30°C and 12/12 hr L/D photoperiod. The photon flux density of the growth chamber was approximately 230 μmol m^{-2} s^{-1} photon flux. A seed was considered germinated when the radicle was 2.0 mm or longer. Seeds that had germinated at 2, 4, or 6 d were selected for growth tests. Seedlings were removed, dried on filter paper, and the primary roots were excised for measurements of their length and fresh weight. Data were expressed as centimeters or milligrams per root. The mean germination time was calculated according to Eq. 1 (Labouriau and Osborn 1984):

$$\bar{t} = \sum n_i t_i \Big/ \sum n_i \qquad (1)$$

$\bar{t}$ Mean germination time
n Number of germinated seeds between the times t_{i-1} and t_i

Respiration of Excised Primary Roots Oxygen consumption of primary roots from *B. pilosa* L. seedlings was measured polarographically at 25°C with a Clark-type electrode positioned in a closed plexi-glass chamber. Primary roots were removed from seedlings and rinsed in distilled water. For each measurement, samples of six roots were cut into segments 5–10 mm long as measured from the growth apex, weighed, and placed immediately in the oxygen electrode vessel that contained 2 ml of nutrient solution (pH 5.8) containing 2 mM $Ca(NO_3)_2$, 2 mM KNO_3, 0.43 mM NH_4Cl, 0.75 mM $MgSO_4$, and 20 μM NaH_2PO_4 (Larkin 1987). For estimating the contribution of the mitochondrial cytochrome oxidase (COX; KCN-sensitive respiration) and mitochondrial alternative oxidase (AOX) plus extramitochondrial oxidases (KCN-insensitive respiration) to the overall O_2 uptake, 270 μM potassium cyanide (KCN) were added to the reaction medium. Oxygen uptake was monitored for 12–15 min. Uptake rates were calculated from the polarographic records considering an initial concentration of dissolved oxygen of 240 μM at

25°C (Estabrook 1967) and referred to the fresh weight of the roots.

Alcohol Dehydrogenase Activity Alcohol dehydrogenase activity was assayed in root extracts from seedlings grown in the absence of coumarin for 2, 3, or 4 d or in the presence of coumarin (10–50 μM) for 4 d. Primary roots (approximately 0.2 g fresh weight) were excised from the seedlings, weighed, and transferred to a mortar, thoroughly mixed with 3.0 ml of a medium that contained 50 mM Tris–HCl (pH 7.4), 1.0 mM ethylene diamide tetracetic acid, and 2.0 mM dithiothreitol. Extracts were centrifuged for 20 min at 20,000×*g* and 5°C. The supernatant was decanted and used as the enzyme source. Alcohol dehydrogenase activity was measured according to Lee (1982). The reaction medium contained 50 mM Tris–HCl (pH 7.4), 1.0 mM NAD^+, and 200 μl of enzyme extract. The reaction was initiated by the addition of 120 μM *n*-propanol. Enzyme activity was evaluated as the initial rate of NAD^+ reduction, which was calculated from the increase in absorbance at 340 nm. Enzyme activity was expressed as μmol min^{-1} (g fresh weight)$^{-1}$.

Lipoxygenase Activity Lipoxygenase activity was assayed in the root extracts from seedlings grown in the absence of coumarin for 2, 3, or 4 d or in the presence of coumarin (10–50 μM) for 4 d. Primary roots (approximately 0.2 g fresh weight) were weighed and transferred to a mortar and thoroughly mixed with 1.5 ml of a cold 50 mM K-phosphate (pH 7.0) solution containing 0.1% Triton X-100 (*v*/*v*). Extracts were centrifuged for 10 min at 12,000×*g* and 5°C. The supernatant was decanted and used as the enzyme source. Lipoxygenase was measured polarographically with a Clark-type oxygen electrode according to Siedow and Girvin (1980). The reaction medium contained 200 mM K-phosphate (pH 7.0) and 200 μl of enzyme extract. The reaction was initiated by the addition of linolenic acid (3.0 mM final concentration), dissolved in Tween 20. Oxygen uptake was monitored for 12–15 min, and the enzyme activity was expressed as μmol O_2 min^{-1} (g root fresh weight)$^{-1}$. Controls were run to exclude solvent effects.

Lipid Peroxidation Products The level of lipid peroxidation in primary root extracts was measured in terms of malondialdehyde (MDA) and conjugated diene contents. Approximately 200 mg of excised roots were homogenized in 4.0 ml of 96% (*v*/*v*) ethanol. The content of malondialdehyde (MDA) was assayed in 3.0 ml of the homogenate (Heath and Packer 1968). An equal volume of 10% trichloroacetic acid that contained 0.5% TBA was added to the homogenate. The mixture was heated to 95°C for 30 min and cooled quickly in an ice bath. After centrifuging at 10,000×*g* for 10 min, absorbance of the supernatant at 532 nm was read. The value for nonspecific absorbance at 600 nm was subtracted. The concentration of MDA was calculated by using its extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol (g root fresh weight)$^{-1}$.

For the conjugated diene measurement, a 1.0-ml aliquot of homogenate was mixed into an equal volume of 96% ethanol and centrifuged at 12,000×*g* for 10 min (Boveris et al. 1980). Absorbance of the supernatant was read at 234 nm, and the nonspecific absorbance at 500 nm was subtracted. Concentration of the conjugated dienes was calculated by using the extinction coefficient of 2.65×10^4 M^{-1} cm^{-1} and expressed as μmol (g root fresh weight)$^{-1}$.

Statistical Analysis The data shown in the graphs and tables were expressed as means±standard errors (SEM) of independent preparations. Data were analyzed with Student's *t* test or analysis of variance (ANOVA), significant differences between means being identified by Duncan's test. The comparisons are given in the text as probability values (*P*). $P\leq0.05$ being adopted as the minimum criterion of significance. The ID_{50} was computed by numerical interpolation by means of a cubic spline function. Statistical analyses were performed by using the Statistica™ software package.

Results

Effects on Germination and Growth The present work revealed different effects and potencies for each tested compound on *B. pilosa* (Table 1). Germination and seedling growth inhibition among the cinnamic acid derivatives varied greatly. At 500 μM, ferulic acid reduced germination to 42% of the control at the second day, but when germination occurred, the lengths and the fresh weights of the seedling roots were not modified. After 2 days, no significant modifications were observed. Vanillic acid, in contrast, did not affect seed germination, but reduced the growth of seedling roots at the fourth day. Caffeic, *p*-coumaric, and protocatechuic acids were inactive.

The actions of monoterpenes were also variable. Whereas eucalyptol was inactive, camphor inhibited both germination and growth of *B. pilosa*. At 500 μM, it reduced germination by 81% and 40% on the second and fourth days, respectively. The lengths and the fresh weights of the seedling roots were 39% and 51% reduced, respectively, on the second day. On the fourth day, the corresponding values were 19% and 11%.

Flavone, at 250 μM, had weak activities on *B. pilosa*. Germination was 13% reduced at day 4, but the lengths and

Table 1 Germination percentage, length, and fresh weight of primary roots of *B. pilosa* L. incubated for 2 or 4 d in water (control), in 1% (*v*/*v*) DMSO, in 250 μM flavone or in 500 μM caffeic, *p*-coumaric, ferulic, protocatechuic and vanillic acids, camphor, eucalyptol, and coumarin

Compound (500 μM)	Germination (%)		Root Length (cm)		Root Fresh Weight (mg per root)	
	Growth Period (d)					
	2	4	2	4	2	4
Control (*N*=19)	25.7±3.10	77.43±1.56	0.66±0.02	1.12±0.04	1.27±0.07	2.4±0.10
DMSO 1% (*N*=8)	19.2±2.33	72.80±2.80	0.64±0.04	1.07±0.05	1.18±0.09	2.24±0.10
Caffeic acid (*N*=6)	28.0±3.10	82.0±4.60	0.74±0.04	1.14±0.10	1.28±0.05	2.44±0.16
Coumaric Acid (*N*=4)	23.0±5.70	80.0±7.50	0.61±0.05	0.93±0.07	1.15±0.11	2.36±0.18
Ferulic acid (*N*=6)	10.67±2.29*	73.0±4.75	0.62±0.07	0.95±0.06	1.10±0.17	1.64±0.07*
Protocatechuic acid (*N*=4)	26.0±3.16	84.50±5.50	0.60±0.02	1.15±0.09	1.13±0.04	2.34±0.23
Vanillic acid (*N*=4)	15.5±4.79	64.0±12.36	0.59±0.03	0.69±0.09*	1.09±0.03	1.72±0.32*
Camphor (*N*=5)	3.60±0.98*	44.0±3.52*	0.39±0.11*	0.87±0.06*	0.58±0.26*	1.99±0.08
Eucalyptol (*N*=5)	21.2±1.63	70.80±3.93	0.73±0.01	1.16±0.06	1.19±0.10	2.27±0.17
Flavone (*N*=5)	20.8±2.87	63.2±3.01*	0.66±0.05	1.05±0.05	1.38±0.17	2.28±0.19
Coumarin (*N*=5)	0.0*	0.0*	–	–	–	–

Values are expressed as mean ± SE. Significant differences between treated seeds and the respective controls are indicated.
*$P \leq 0.05$, ANOVA with Duncan's multiple range test.

the fresh weights of the seedling roots of germinated seeds were not modified.

Coumarin was the most phytotoxic among all assayed compounds. At the 500 μM concentration, it completely suppressed germination of *B. pilosa*.

Effects of Coumarin on Germination and Growth of E. heterophylla, I. grandifolia, and S. obtusifolia To evaluate whether the phytotoxicity of coumarin is species specific, the effects of 500 μM coumarin on germination and growth of *I. grandifolia*, *S. obtusifolia*, and *E. heterophylla* were examined (Table 2). Germination of *S. obtusifolia* was completely inhibited by the second day, but by the fourth day, the number of germinated seeds was not different from that of the control. No significant modification was found in the germination of *E. heterophylla* and *I. grandifolia*, although coumarin affected seedling growth. The lengths of primary roots of *E. heterophylla* and *S. obtusifolia* were reduced without significant reduction in fresh weights, whereas in *I. grandifolia*, a reduction in both growth parameters was observed by the second day. Comparison among species reveals that coumarin was more phytotoxic

Table 2 Germination percentage, length, and fresh weight of primary roots of *E. heterophylla* L., *I. grandifolia* L., and *S. obtusifolia* L. incubated for 2 or 4 d in 1% (*v*/*v*) DMSO or in 500 μM coumarin

Plant species	Condition	Germination (%)		Root Length (cm)		Root Fresh Weight (mg per root)	
		Growth Period (d)					
		2	4	2	4	2	4
Euphorbia heterophylla	Control (*N*=4)	45.60±3.43	54.67±3.92	1.06±0.10	3.80±0.21	5.90±0.53	24.19±2.43
	Coumarin 500 μM (*N*=4)	34.67±4.37	51.50±3.78	0.28±0.01* (−74%)	1.47±0.06* (−61%)	4.52±1.04	22.74±0.20
Ipomoea grandifolia	Control (*N*=4)	13.4±1.54	22.2±0.49	0.51±0.05	1.98±0.17	4.00±0.33	10.34±0.8
	Coumarin 500 μM (*N*=4)	10.4±0.98	17.4±1.25	0.29±0.02* (–43%)	0.75±0.04* (–63%)	2.92±0.16* (−27%)	10.01±0.36
Senna obtusifolia	Control (*N*=5)	6.20±0.58	18.4±2.1	0.35±0.04	0.69±0.024	4.48±0.32	10.53±0.39
	Coumarin 500 μM (*N*=5)	0±0* (−100%)	12.6±0.81	0±0* (−100%)	0.41±0.03* (−41%)	0±0* (−100*)	10.4±0.76

Values are expressed as mean±SE. Significant differences between treated seeds and the respective controls are indicated.
*$P \leq 0.05$, ANOVA with Duncan's multiple range test.

to *I. grandifolia* than to *E. heterophylla* and *S. obtusifolia*. However, the phytotoxicity of coumarin on *B. pilosa* was significantly higher (at the same concentration [500 μM] ,it completely suppressed germination of this weed [Table 1]). This finding led us to perform a more extensive investigation of coumarin effects on *B. pilosa*.

Effects of Coumarin on Germination and Growth of B. pilosa When *B. pilosa* seeds were incubated in the presence of coumarin in the concentration range of 10–100 μM for 6 d, both seed germination and seedling root growth were significantly inhibited (Fig. 1). At d 2, complete suppression of seed germination was observed with coumarin at the 50 μM concentration or higher (Fig. 1a). The calculated ID_{50} were 10.6±2.1 μM, 23.8±4.5 μM, and 21.3±4.4 μM for germination, root length, and root fresh weight, respectively. At subsequent periods (d 4 and 6), germination and root growth were also reduced, though to lesser degrees. The ID_{50} for germination was increased to 46.5± 2.7 and 89.9±2.1 μM on the fourth and sixth day of incubation, respectively. Mean germination time increased from 88.4±4.5 hr in the control to 94.0±2.7, 105.8±2.6, 122.8±1.1, and 129.1±2.8 hr with 10, 25, 50, and 100 μM coumarin, respectively. Root fresh weight was reduced to a lesser extent compared to reduction of root length. At d 6, for example, coumarin up to 50 μM had not affected root fresh weight, while an inhibitory action on root length was observed with 10 μM coumarin.

After 4 d of incubation, the first leaves were visible, indicating that photosynthesis had started to contribute to seedling energy metabolism. Thus, the subsequent experiments were performed with seedlings grown for a maximum of 4 d, assuring that the contribution of mitochondrial respiration to seedling energy metabolism was predominant.

Effects of Coumarin on Respiratory Activity of Excised Primary Roots and on Activities of Alcohol Dehydrogenase and Lipoxygenase in Primary Root Extracts Respiratory activity of primary roots and activity of alcohol dehydrogenase and lipoxygenase in the control series (absence of coumarin) were measured in seedlings grown for 2, 3, and 4 d. As shown (Fig. 2), all these parameters were higher shortly after the emergence of primary roots (d 2) and decreased progressively during the subsequent growth period. From the second to the fourth day, the overall O_2 consumption rates were reduced by 40% on a fresh-mass basis. The relative contribution of the KCN-sensitive respiration to the overall respiration decreased from 73.9% at d 2 to 68.9% at d 4. The decline in the activities of alcohol dehydrogenase and lipoxygenase during the growth period was more accentuated (Fig. 2). At d 3 and 4, alcohol dehydrogenase activity was, respectively, 33.2% and 8.6% of that one found on d 2. Lipoxygenase activity decreased

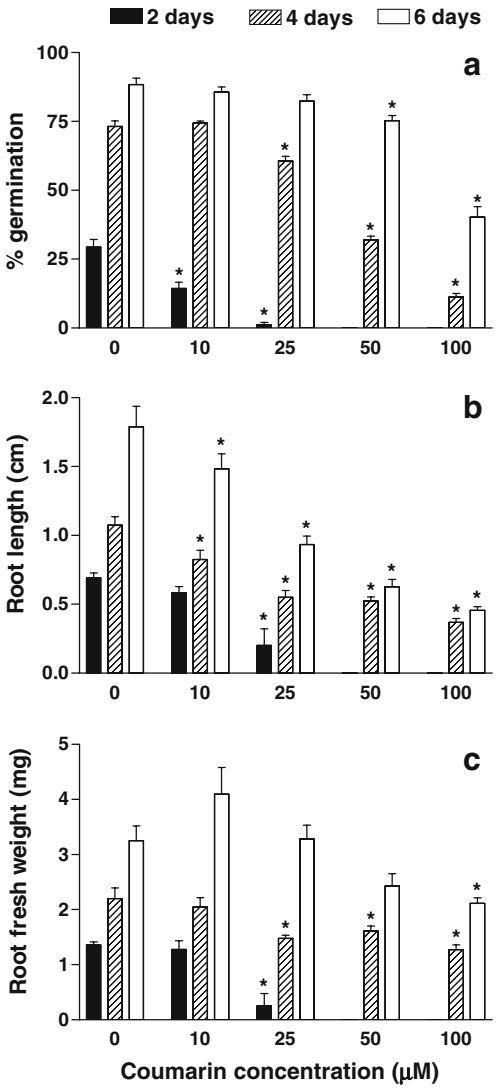

Fig. 1 The effects of coumarin on germination (**a**), root length (**b**), and root fresh weight (**c**) of *Bidens pilosa* L. Seeds were germinated and grown on the following regime: 8 hr light, at 30°C, 16 hr dark, at 20°C, photon flux density of approximately 230 μmol m^{-2} s^{-1}. Coumarin (10–100 μM) was added to the nutrient solution, and at each experimental interval (2, 4, or 6 d), roots were excised and their lengths and fresh weights were measured. All values are means of three to five independent experiments. *Error bars* are SEM. Significant differences between coumarin-treated and untreated seedlings were identified by ANOVA with Duncan's testing (*$P<0.05$)

28.4% on d 3 and 14.3% on d 4 relative to the value found on the second day.

The effects of coumarin at a concentration range of 10–100 μM were evaluated only in seedlings grown for 4 d (Fig. 3). At shorter time intervals, insufficient material was available for measurements because of the strong inhibition in seedling growth.

In contrast to what happened with germination and seedling growth (Fig. 1), overall respiration rates of root apices from seedlings grown for 4 d were stimulated in a dose-dependent manner by coumarin up to 50 μM

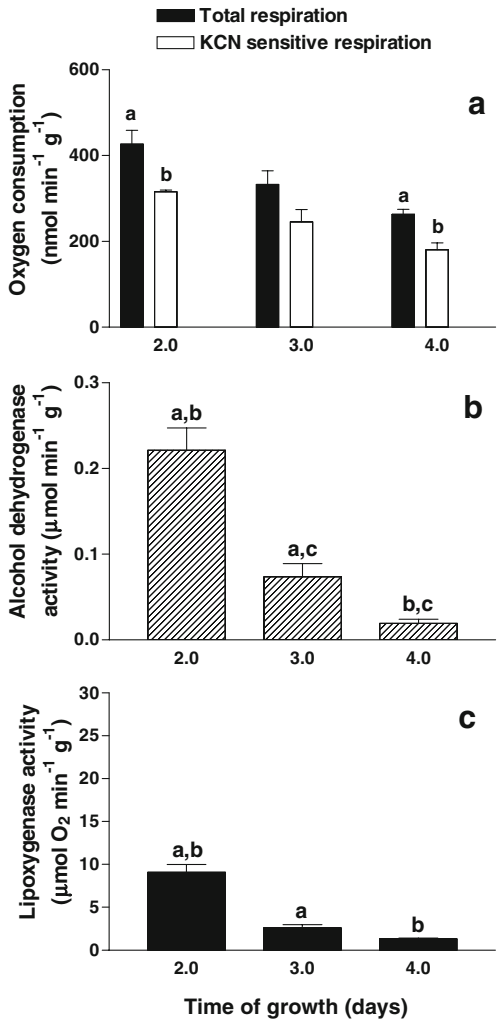

Fig. 2 Time course of respiration rates (**a**), alcohol dehydrogenase (**b**), and lipoxygenase (**c**) activities in roots of *B. pilosa* L. seedlings. In **a**, primary root tip samples were removed from seedlings and added without delay to the oxygen electrode vessel, containing 2.0 ml of nutrient medium in the absence or presence of 270 µM KCN. Oxygen consumption was followed polarographically over approximately 12–15 min. Total respiration: rate of oxygen consumption in the absence of inhibitors; KCN-sensitive respiration: difference between the rates of oxygen consumption measured in the absence and presence of KCN; Alcohol dehydrogenase activity (**b**) was measured in reaction medium containing 1.0 mM NAD^+ and 120 µM *n*-propanol. Lipoxygenase activity (**c**) was measured polarographically in the presence of 3.0 mM linolenic acid. Each data point is the mean value of four (**a**), three (**b**), or three (**c**) independent experiments. *Error bars* are SEM. *Pairs of letters* indicate statistical significance as determined by ANOVA with Duncan's testing ($P<0.05$)

(Fig. 3a). KCN-sensitive respiration was similarly stimulated, so that its relative contribution to overall respiration was not modified. It constituted 68.9% of overall respiration in the control condition and 67.8, 68.3, 70.8, and 66.4% in the presence of 10, 25, 50, and 100 µM coumarin, respectively.

Activities of alcohol dehydrogenase and lipoxygenase in root extracts from seedlings grown for 4 d were also stimulated in a dose-dependent manner by coumarin. The activity of alcohol dehydrogenase increased 4.6- and 9.3-fold, in the presence of 25 and 50 µM coumarin, respectively (Fig. 3b). Under the same conditions, the lipoxygenase activity increased 5.7- and 14.7-fold, compared to untreated seedlings (Fig. 3c).

To examine the possibility that lipoxygenase stimulation was a response to a cellular oxidative stress condition, we measured the content of malondialdehyde (MDA) and conjugated dienes in roots of seedlings grown in the presence of 25 and 50 µM coumarin (Table 3). An increment of nearly fourfold in conjugated dienes content

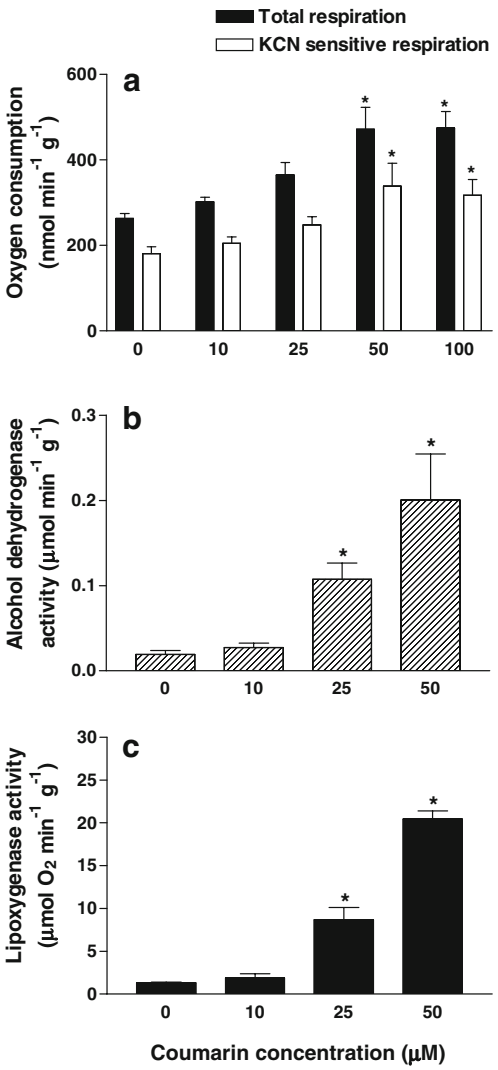

Fig. 3 Effects of coumarin on respiration rates (**a**), alcohol dehydrogenase (**b**), and lipoxygenase (**c**) activities in roots of *B. pilosa* L. seedlings. Seedlings were grown for 4 d in the absence or presence of coumarin (concentration range 10–100 µM). Respiration rates and the activities of lipoxygenase and alcohol dehydrogenase were measured as described in legend of Fig. 2. All values are the means of 4 (**a**), 3–4 (**b**), or 3 (**c**) independent experiments. *Error bars* are SEM. Significant differences between coumarin-treated and untreated seedlings were identified by ANOVA with Duncan's testing (*$P<0.05$)

Table 3 Effects of coumarin (25 and 50 μM) on the content of malondialdehyde (MDA) and conjugated dienes in primary roots from *Bidens pilosa* seedlings grown for 4 days

Coumarin Concentration	MDA (N=3)	Conjugated Dienes (N=6)
μM	nmol (g root fresh weight)$^{-1}$	μmol (g root fresh weight)$^{-1}$
0	14.3±2.4	3.18±0.21[a]
25	8.85±0.8	3.78±0.49[b]
50	12.3±1.4	15.68±2.06[a,b]

Values are expressed as mean±SE. Pairs of letters in each column indicate statistical significance as determined by ANOVA with Duncan's testing (P<0.05)

was found in 50 μM-treated seedlings, with no significant changes in MDA content.

Discussion

The study revealed different effects and potencies on *B. pilosa* for each compound tested. Coumarin was the most phytotoxic. Among the cinnamic derivatives, only ferulic and vanillic acids were active. Cinnamic acid derivatives possess in their structures a phenyl group with different substituents. Ferulic and vanillic acids possess in common the methoxyl substituent, which is absent in the other cinnamic acid derivatives. This group seems to be a critical factor in the inhibition of germination and growth of *B. pilosa*. This characteristic seems uncommon in other plant species such as *Arabidopsis thaliana* (Reigosa and Pazos-Malvido 2007) and *Amaranthus retroflexus* (Reigosa et al. 1999). In those studies, radicle growth of both species was reduced by ferulic and vanillic acids and also by *p*-coumaric and protocatechuic acids, which do not possess a methoxy group substituent.

Activities of the monoterpenes eucalyptol and camphor were also different. Both terpenes possess in common a hydrocarbonated cyclical structure and an oxygenated substituent, an ether function in eucalyptol and a ketone function in camphor. Camphor's higher solubility (Vaughn and Spencer 1993; Fischer et al. 1994) could have exerted some influence on its *B. pilosa* activity. Solubility did not seem to be a differential factor for coumarin and flavone activity. Both compounds are practically insoluble in water, but despite the fact that they are structurally related, flavone had weak activities on *B. pilosa*. The benzopyranone group of coumarin is an integral part of the structure of flavone, which possesses a phenyl substituent in position 2 of the benzopyranone group. This substituent apparently suppresses biological activity of the benzopyranone group in *B. pilosa*. This interpretation is consistent with the work of Richard et al. (1950), which demonstrated that the introduction of substituents in positions 3 and 4 of the benzopyranone group reduces the inhibitory potential of derivatives on the growth of *Avena* roots.

The phytotoxicity of coumarin seems to be species specific, as *E. heterophylla*, *S. obtusifolia*, and *I. grandifolia* were less sensitive. Coumarin and its derivatives are produced by plants of almost all families and are found on the surfaces of leaves, seeds, and fruits (Zobel and Brown 1995; Chon et al. 2003; Chon and Kim 2004; Khanh et al. 2006). There are a number of reports concerning the effects of coumarin on crop species, the responses also being species specific and concentration dependent (Murray et al. 1982). For example, at 680 μM, coumarin completely inhibits root growth of *Cucumis sativus* and *Zea mays* seedlings, but causes only slight inhibition of *Pisum sativum* root growth (Kupidlowska et al. 1994). Inhibition of *Triticum turgidum* ssp. *durum* seed germination was reported to occur at concentrations above 200 μM (Abenavoli et al. 2004, 2006). From this study, it is clear that *B. pilosa* has high sensitivity to coumarin in comparison with most assayed weed or crop species.

Several explanations for coumarin inhibitory action on germination have been proposed, including inhibition of cellulose synthesis (Hara et al. 1973), auxin-like activity (Jansson and Svensson 1980), inhibition of photosynthesis (Moreland and Novitzky 1987), uncoupling of mitochondrial oxidative phosphorylation (Knypl 1964; Yakushkina and Starikova 1978), blocking of the cell cycle (Zobel and Brown 1995), antimitotic action (Podbiekowska et al. 1994), inhibition of cell division and cell elongation (Svensson 1972), and inhibition of amino acid transport and protein synthesis (Van Sumere et al. 1972). The simplest explanation for the observed reduction in root seedling growth associated with increased KCN-sensitive respiration is that in *B. pilosa*, coumarin is acting as an uncoupler of mitochondrial oxidative phosphorylation. In this context, the observed increase in alcohol dehydrogenase activity would be interpreted as a compensatory increase of anaerobic ATP synthesis. However, comparisons between the metabolic parameters measured in seedlings grown in the presence of coumarin for 4 days and those of the control series (absence of coumarin) measured at days 2, 3, and 4 point to an alternative mechanism. Respiratory activity and the activities of alcohol dehydrogenase and lipoxygenase in the control series were higher shortly after the emergence of primary roots (d 2) and decreased progressively during the growth period. These results suggest that ATP production was provided by alcohol dehydrogenase activity only in the early stage of root growth with subsequent predominance of the mitochondrial ATP-generation pathway. The decline in KCN-sensitive respiration observed during the growth period

may represent a reduction in ATP demand due to progressive reduction in the root relative growth.

From these observations, it is plausible to suggest that the observed higher root respiration and alcohol dehydrogenase and lipoxygenase activities of seedlings grown for 4 days in the presence of coumarin are not consequences of an impairment on energy metabolism, but represent the metabolic status of seedlings at a different physiological age, i.e., in an earlier stage of growth. Coumarin probably acted by inducing a delay in seed germination and seedling growth. This conclusion is corroborated by the observation that the mean values of all parameters measured in seedlings grown in the presence of 50 μM coumarin for 4 days were statistically equal to those of seedlings grown for 2 days in the absence of coumarin. The only exception was the lipoxygenase activity, which was substantially higher in the presence of 50 μM coumarin.

Although the exact mechanism of germination and growth inhibition remains to be elucidated, our results corroborate the hypothesis that coumarin acts as a cytostatic agent, as suggested by its reported effects on mitosis and cell division (Svensson 1972; Podbiekowska et al. 1994; Zobel and Brown 1995).

Coumarin at higher concentrations also exerts an additional phytotoxic action in *B. pilosa* as indicated by the higher activity of lipoxygenase and the high content of conjugated dienes in root extracts. Activation of lipoxygenase is believed to be one of the immediate responses to changes in cell membrane structure induced by different agents including oxygen reactive species that can be generated in response to a variety of stress conditions (Siedow 1991; Porta and Rocha-Sosa 2002; Blokhina et al. 2003). The MDA content was presumably not increased because the products of lipid peroxidation were further oxidized or metabolized (Beuge and Aust 1978; Muscari et al. 1990). The hypothesis that coumarin induces a condition of oxidative stress was also suggested by Abenavoli et al. (2003, 2006) based on changes in antioxidant enzyme activities in durum wheat (*Triticum turgidum*) seedlings. In this plant species, however, the effects occurred at a concentration of 1,000 μM.

From the present study, it can be concluded that coumarin has a strong and selective ability to suppress the germination and growth of *B. pilosa* and might be effectively exploited as a natural herbicide. For example, the use of plants with high coumarin content (Macías et al. 1993; Chon et al. 2003; Chon and Kim 2004; Khanh et al. 2006) could be used in intercropping systems to reduce *B. pilosa* germination and growth.

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References

ABENAVOLI, M. R., SORGONÃ, A., SIDARI, M., BADIANI, M., and FUGGI, A 2003. Coumarin inhibits the growth of carrot (*Daucus carota* L. cv. Saint Valery) cells in suspension culture. *J. Plant Physiol.* 160:227–237.

ABENAVOLI, M. R., SORGONÀ, A., SIDARI, M., ALBANO, A., and CACCO, G. 2004. Coumarin differentially affects the morphology of different root types of maize seedlings. *J. Chem. Ecol.* 30:1871–1883.

ABENAVOLI, M. R., CACCO, G., SORGONÀ, A., MARABOTTINI, R., PAOLACCI, A. R., CIAFFI, M., and BADIANI, M. 2006. The inhibitory effect of coumarin on the germination of durum wheat (*Triticum turgidum* ssp. *durum*, CV. SIMETO) seeds. *J. Chem. Ecol.* 32:489–506.

BEUGE, J. A., and AUST, S. D. 1978. Microsomal lipid peroxidation. *Method. Enzymol.* 52:302–310.

BLOKHINA, O., VIROLAINEN, E., and FAGERSTED, V. K. 2003. Antioxidants, oxidative damage and oxygen deprivation stress: a review. *Ann. Bot.* 91:179–194.

BOVERIS, A., CADENAS, E., and CHANCE, B. 1980. Low level chemiluminescence of the lipoxygenase reaction. *Photobiochem. Photobiophys.* 1:175–182.

CHON, S. U., and KIM, Y. M. 2004. Herbicidal potential and quantification of suspected allelochemicals from four grass crop extracts. *J. Agron. Crop Sci.* 190:145–150.

CHON, S. U., KIM, Y. M., and LEE, J. C. 2003. Herbicidal potencial and quantification of causative allelochemicals from several *Compositae* weeds. *Weed Res.* 43:444–450.

CHRISTOFFOLETI, P. J., and FOLONI, L. 1999. Dose-response curves of resistant and susceptible *Bidens pilosa* to ALS inhibitor herbicide, pp. 159–162, in Proceedings from the Brighton Crop Protection Conference: weeds. November, 1999, London, UK.

DUDAI, N., POLJAKOFF-MAYBER, A., MAYER, A. M., PUTIEVSKY, E., and LERNER, H. R. 1999. Essential oils as allelochemical and their potential use as bioherbicides. *J. Chem. Ecol.* 25:079–1089.

DUKE, S. O., DAYAN, F. E., OMAGNI, J. G., and IMANDO, A. M. 2000. Natural products as sources of herbicides: current status and future trends. *Weed Res.* 40:99–111.

ESTABROOK, R. W. 1967. Mitochondrial respiratory control and polarographic measurements of ADP/O ratio. *Method. Enzymol.* 10:41–47.

FISCHER, N. H., WILLIAMSON, G. B., WEIDENHAMER, J. D., and RICHARDSON 1994. In search of allelopathy in the Florida scrub: the role of terpenoids. *J. Chem. Ecol.* 20:1355–1379.

HARA, M., UMETSU, N., MIYAMOTO, C., and TAMARI, K. 1973. Inhibition of the biosynthesis of plant cell wall materials, especially cellulose biosynthesis by coumarin. *Plant Cell Physiol.* 14:11–28.

HEATH, R. L., and PACKER, L. 1968. Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acids peroxidation. *Arch. Biochem. Biophys.* 125:189–198.

HOLM, L. G., PLUCKNETT, D. L., PANCHO, J. V., and HERBERGER, J. P. 1977. The World's Worst Weeds: Distribution and Biology. The University Press of Hawaii, Honolulu.

JANSSON, E., and SVENSSON, S. B. 1980. Coumarin effects on *Glycine max* hypocotyls explants. *Physiol. Plantarum* 48:486–490.

KHANH, T. D., CHUNG, I. M., TAWATA, S., and XUAN, T. D. 2006. Weed suppression by *Passiflora edulis* and its potential allelochemicals. *Weed Res.* 46:296–303.

KNYPL, J. S. 1964. Coumarin-induced respiration of sunflower. *Physiol. Plantarum* 17:771–778.

KOHLI, R. K., BATISH, D. R., and SINGH, H. P. 2006. Allelopathic interactions in agroecosystems, pp. 465–493, in M. V. Reigosa, N. Pedrol, and L. González (eds.). Allelopathy: A Physiological Process with Ecological Implications Springer, Netherlands.

KUPIDLOWSKA, E., KOWALEC, M., SULKOWSKI, G., and ZOBEL, A. M. 1994. The effects of coumarin on root elongation and ultrastructure of meristematic cell protoplast. *Ann. Bot.* 73:525–530.

LABOURIAU, L. G., and OSBORN, J. H. 1984. Temperature dependence of the germination of tomato seeds. *J. Therm. Biol.* 9:285–294.

LARKIN, P. J. 1987. Calmodulin levels are not responsible for aluminum tolerance in wheat. *Aust. J. Plant Physiol.* 14:377–387.

LEE, C. Y. 1982. Alcohol dehydrogenase from *Drosophila melanogaster*. *Method. Enzymol.* 89:445–450.

MACÍAS, F. 1995. Allelopathy in the search for natural herbicide models, pp. 310–329, in Inderjit, M. M. Dakshini, and F. A. Einhellig (eds.). Allelopathy: Organisms, Processes, and Applications American Chemical Society, Washington DC.

MACÍAS, F., GALINDO, J. C. G., MASSANET, G. M., RODRIGUEZ-LUÍS, F., and ZUBÍAS, E. 1993. Allelochemicals from *Pilocarpus goudotianus* leaves. *J. Chem. Ecol.* 19:1371–1379.

MORELAND, E. D., and NOVITZKY, W. P. 1987. Effects of coumarins and flavonoids on isolated chloroplast and mitochondria, pp. 247–261, in G. R. Waller (ed.). Allelochemicals: Role in Agriculture and ForestryAmerican Society Series. Americal Chemical Society, Washington, DC.

MURRAY, R. D. H., MÉNDEZ, J., and BROWN, S. A. 1982. The Natural Coumarins: Occurrence, Chemistry and Biochemistry. J. Wiley and Sons, Chichester.

MUSCARI, C. L., FRASCARO, M., GUARNIERI, C., and CALDARERA, C. I. M. 1990. Mitochondrial function and superoxide generation from submitochondrial particles of aged rat hearts. *Biochim. Biophys. Acta* 1015:200–204.

PODBIEKOWSKA, M., KUPIDLOWSKA, E., WALEZA, M., DOBRZYNSKA, K., LOUIS, S. A., KEIGHTLEY, A., and ZOBEL, A. M. 1994. Coumarin as antimitotics. *Int. J. Pharm.* 32:262–273.

PORTA, H., and ROCHA-SOSA, M. 2002. Plant lipoxygenases. Physiological and Molecular Features. *Plant Physiol.* 130:15–21.

PUTNAM, A. R., and DUKE, W. B. 1974. Biological suppression of weeds: evidence for allelopathy in accessions of cucumber. *Science* 185:370–372.

REIGOSA, M. J., and PAZOS-MALVIDO, E. 2007. Phytotoxic effects of 21 plant secondary metabolites on *Arabidopsis thaliana* germination and root growth. *J. Chem Ecol.* 33:1456–1466.

REIGOSA, M. J., SOUTO, X. C., and GONZÁLEZ, L. 1999. Effect of phenolic compounds on the germination of six weeds species. *Plant Growth Reg.* 28:83–88.

RICHARD, H., GOODWIN, A. E., and TAVES, C. 1950. The effect of coumarin derivatives on the growth of *Avena* roots. *Amer. J. Bot.* 37:224–231.

SIEDOW, J. N. 1991. Plant lipoxygenase: structure and function. *Annu. Rev. Plant Physiol.* 42:145–188.

SIEDOW, J. N., and GIRVIN, M. E. 1980. Alternative respiratory pathway. *Plant Physiol.* 65:669–674.

SVENSSON, S. B. 1972. The effect of coumarin on growth, production of dry matter, protein and nucleic acids in roots of maize and wheat and the interactions of coumarin with metabolic inhibitors. *Physiol. Plantarum* 27:13–24.

VAN SUMERE, C. F., COTTENIE, J., DE GREEF, J., and KINT, J. 1972. Biochemical studies in relation to the possible germination regulatory role of naturally occurring coumarin and phenolic. *Recent Adv. Phytochemistry* 4:165–221.

VAUGHN, S. F., and SPENCER, G. F. 1993. Volatile monoterpenes as potential parent structures for new herbicides. *Weed Sci.* 41:114–119.

VYVYAN, J. R. 2002. Allelochemicals as leads for new herbicides and agrochemicals. *Tetrahedron* 58:1631–1646.

YAKUSHKINA, N. I., and STARIKOVA, V. T. 1978. Effects of coumarin and gibberellin on certain aspects of the energy metabolism of corn seedlings. *Fiziol. Rast.* 24:1211–1216.

WU, H., PRATLEY, J., LEMERLE, D., and HAIG, T. 1999. Crop cultivars with allelopathic capability. *Weed Res.* 39:171–180.

ZOBEL, A. M., and BROWN, S. A. 1995. Coumarins in the interactions between the plant and its environment. *Allelopathy J.* 2:9–20.

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Increased Terpenoid Accumulation in Cotton (*Gossypium hirsutum*) Foliage is a General Wound Response

Stefan Opitz · Grit Kunert · Jonathan Gershenzon

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Abstract The subepidermal pigment glands of cotton accumulate a variety of terpenoid products, including monoterpenes, sesquiterpenes, and terpenoid aldehydes that can act as feeding deterrents against a number of insect herbivore species. We compared the effect of herbivory by *Spodoptera littoralis* caterpillars, mechanical damage by a fabric pattern wheel, and the application of jasmonic acid on levels of the major representatives of the three structural classes of terpenoids in the leaf foliage of 4-week-old *Gossypium hirsutum* plants. Terpenoid levels increased successively from control to mechanical damage, herbivory, and jasmonic acid treatments, with *E*-β-ocimene and heliocide H_1 and H_4 showing the highest increases, up to 15-fold. Herbivory or mechanical damage to older leaves led to terpenoid increases in younger leaves. Leaf-by-leaf analysis of terpenes and gland density revealed that higher levels of terpenoids were achieved by two mechanisms: (1) increased filling of existing glands with terpenoids and (2) the production of additional glands, which were found to be dependent on damage intensity. As the relative response of individual terpenoids did not differ substantially among herbivore, mechanical damage, and jasmonic acid treatments, the induction of terpenoids in cotton foliage appears to represent a non-specific wound response mediated by jasmonic acid.

Keywords *Gossypium hirsutum* · Cotton · *Spodoptera littoralis* · Terpenoid aldehydes · Monoterpenes · Sesquiterpenes · Pigment glands · Constitutive plant defense · Jasmonic acid · Mechanical damage · Induction

Introduction

Plants produce a broad range of defense chemicals that act as deterrents or toxins against herbivores and pathogens. Several different strategies have evolved concerning the deployment of such compounds. When the synthesis of defense chemicals occurs solely after initial attack, this reduces metabolic costs (Gershenzon 1994), as defenses are produced only when needed. Such induced defenses have ecological advantages (Agrawal and Karban 1999) and might be a useful strategy for plants that are sporadically attacked. However, plants that risk frequent and heavy damage may be better protected by investing in constitutive defense compounds (Wittstock and Gershenzon 2002). The accumulation of constitutive defenses within plants usually conforms to expectations of the 'optimal defense theory' (ODT), which predicts that the highest protection level will be found in parts with the highest fitness value such as young tissues and reproductive organs (McKey 1979; Rhoades 1979). This has been shown for a variety of plant secondary metabolites from different classes such as alkaloids (Hartmann and Zimmer 1986; Baldwin 2001), glucosinolates (Porter et al. 1991. Brown et al. 2003), furanocoumarins (Berenbaum and Zangerl 1999), phenylpropanoids (Harborne 1991; Opitz and Schneider 2002), and terpenes (Gershenzon and Croteau 1991). Under abiotic or biotic stress, plants often exhibit induced responses by enhancing the accumulation of constitutive compounds in certain tissues that increases their deterrence or toxicity to

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S. Opitz (✉) · G. Kunert · J. Gershenzon
Department of Biochemistry,
Max Planck Institute for Chemical Ecology,
Hans-Knöll-Strasse 8,
07745 Jena, Germany
e-mail: opitz@ice.mpg.de

enemies (Baldwin 1988; Wittstock and Gershenzon 2002; Vazquez-Flota et al. 2004). These changes also may be rationalized as a way for plants to reduce the costs of defense by producing high concentrations of constitutive defenses only when and where these are needed. However, more information is required to understand how the levels of constitutive defenses change under different stresses in plants on an organ-by-organ basis.

Among the vast number of defensive compounds in plants, terpenoids form the largest group with over 25,000 known structures (Buckingham 1994). Some of the best-studied examples for constitutive plant defenses are terpenoid volatiles such as monoterpenes (C_{10}), which are considered defensive against herbivores and pathogens (Gershenzon and Dudareva 2007). Monoterpenes are frequently constituents of oils and resins that are accumulated in complex secretory structures such as glandular trichomes, secretory cavities, or resin ducts (Fahn 1979). Among the most comprehensively studied systems are monoterpenes in the glandular trichomes of peppermint (Gershenzon et al. 2000; McConkey et al. 2000). However, less is known about the accumulation of other classes of terpenes.

Cotton (*Gossypium hirsutum*) is particularly suitable for the study of terpenoid accumulation because a large variety of terpenes are produced constitutively in all photosynthetically active parts of the plant and stored in subepidermal glands. Beside monoterpenes, *G. hirsutum* produces sesquiterpenes (C_{15}; Elzen et al. 1985) and terpenoid aldehydes, such as hemigossypolone (C_{15}) and the heliocides H_1 to H_4 (C_{25}; Fig. 1; Hedin et al. 1992). All three classes of terpenoids in cotton are biosynthetically related (Stipanovic et al. 1977, 1978a; Davis and Essenberg 1995). The precursor of sesquiterpenes, farnesyl diphosphate, is formed by addition of a C_5 isopentenyl diphosphate unit to geranyl diphosphate, the precursor of all monoterpenes. The C_{25} heliocides are formed from Diels–Alder-type reactions of the C_{15} hemigossypolone (derived from δ-cadinene) to the monoterpene myrcene for the formation of heliocides H_2 and H_3 or the monoterpene *E*-β-ocimene for the formation of heliocides H_1 and H_4 (Fig. 2).

The accumulation of terpenoid aldehydes in cotton leaves was shown to be increased after herbivory (Bezemer et al. 2004), and these compounds have been considered to function as feeding deterrents on generalist herbivores such as *Spodoptera exigua* (McAuslane et al. 1997). The heliocides specifically have been correlated with anti-feedant effects and are locally and systematically induced in response to herbivore attack (Karban and Carey 1984; Croxford et al. 1989; Alborn et al. 1996). However, besides the terpenoid aldehydes, little information is available on the accumulation of other terpenes found in subepidermal glands. In studying the control of terpenoid accumulation in cotton, it is of interest to determine if higher concentrations arise from increases in gland number or from increases in the amount of terpenoids per gland (McAuslane et al. 1997). Increasing numbers of subepidermal glands in cotton leaves were observed after attacks of spider mites and caterpillars (McAuslane et al. 1997; Agrawal and Karban 2000).

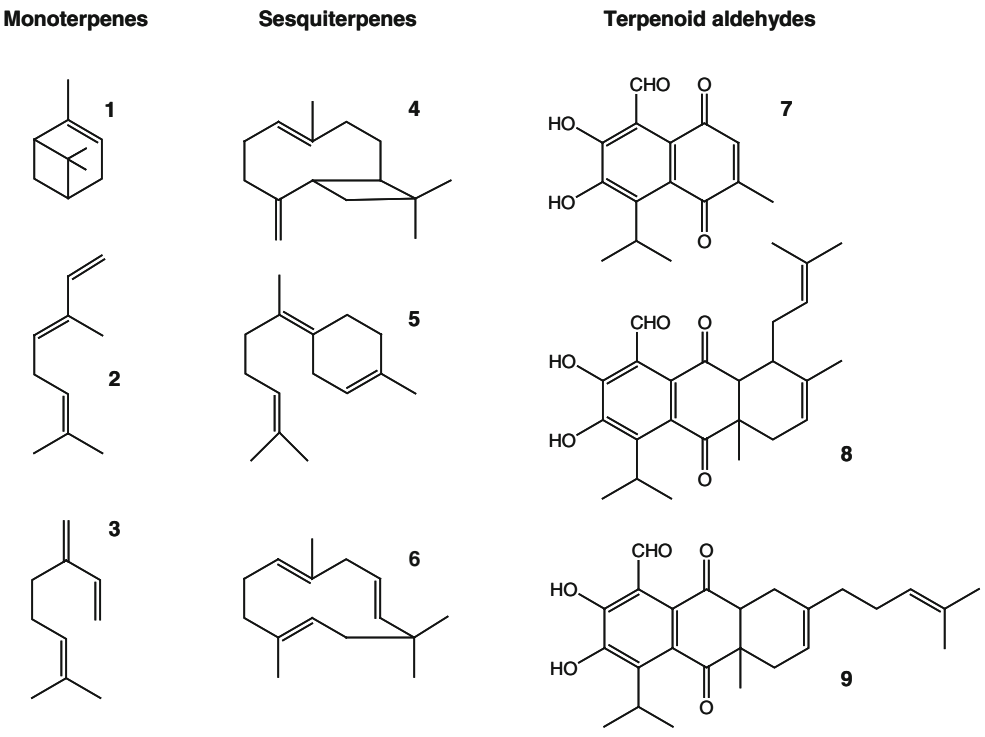


Fig. 1 Terpenoid products of three structural classes, which are accumulated in glands of the foliage of *Gossypium hirsutum*. *1* α-pinene, *2* (*E*)-β-ocimene, *3* myrcene, *4* (*E*)-β-caryophyllene, *5* γ-bisabolene, *6* α-humulene, *7* hemigossypolone, *8* heliocide H_1, *9* heliocide H_2

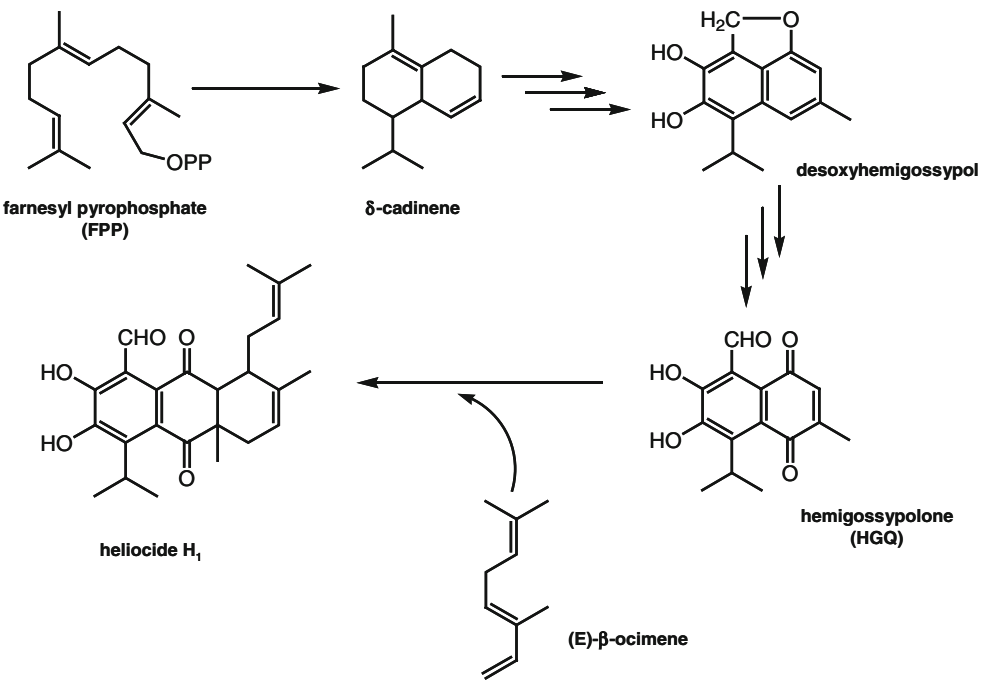


Fig. 2 Proposed scheme for the biosynthesis of heliocide H_1 in cotton

To learn more about the control of terpenoid accumulation in cotton, we compared the response of plants to herbivore damage, mechanical damage, and the application of jasmonic acid (JA), a ubiquitous mediator of defense responses in the plant kingdom (Browse 2005). Differences in the reaction of plants to these treatments were investigated with respect to plant growth, gland production, and accumulation of major compounds from three classes of terpenoids: terpenoid aldehydes, monoterpenes, and sesquiterpenes at the level of the individual leaf. We also examined how the accumulation was influenced by the number of glands in the total foliage or the relative filling of glands with terpenoids.

Methods and Material

Plants G. hirsutum L. of a single variety, 'Deltapine acala 90' (Delta and Pine Company—Hollandala, MS, USA), were reared in a growth chamber under high light intensity [1 mmol $(m^2)^{-1}$ s^{-1} of photosynthetically active radiation], a 15-h light period, 28°C/23°C (day/night), and 65% relative humidity. Plants were established in pots containing 'Seramis'-clay granulate (Masterfood GmbH, Verden/Germany), which were watered daily and fertilized weekly with a complete fertilizer [1 g/l Flory 3 (N/P/K, 15:10:15), Euflor/Germany]. Under these conditions, optimal growth and production of the various terpenoids was obtained.

For all experiments, leaves were numbered sequentially such that number 1 refers to the first true leaf initiated right after the cotyledons (Fig. 3). In this study, leaves 1–7 were investigated and collectively referred to as total foliage.

Herbivores Eggs of the generalist herbivore, *Spodoptera littoralis* (Boisd.; Lepidoptera, Noctuidae), Egyptian cotton worm, were obtained from Syngenta (Basel, Switzerland). After hatching, larvae were reared in 2-l plastic boxes

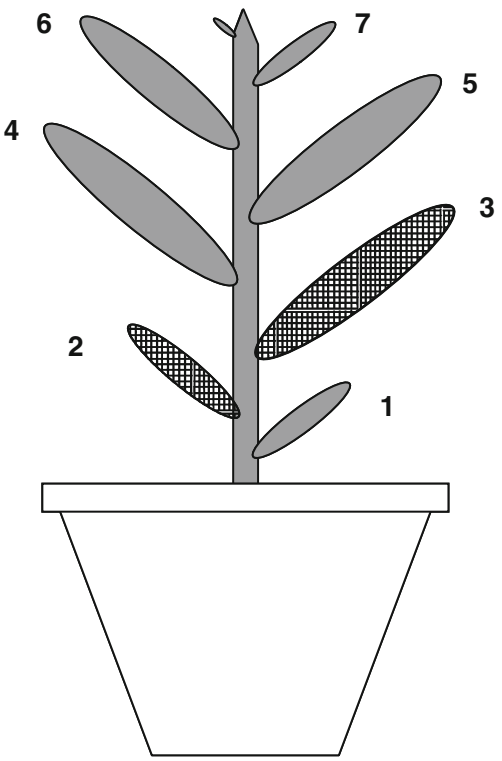


Fig. 3 Sketch of a *Gossypium hirsutum* plant at the end of experiment 1, approximately 1-month-old. Leaves are numbered according to the order of development. *Grid pattern* indicate leaves, which were damaged either mechanically or by caterpillars of *Spodoptera littoralis*

containing artificial diet, which consisted of 500 g of bean flour, 500 ml water, 9 g ascorbic acid, 5 g ethyl-4-benzoic acid, 0.6 ml α-tocopherol, 9 ml Mazola germ oil, 4 ml 3.7% formaldehyde, and 650 ml 7.5% agar solution. Fresh diet was applied every third day. Larvae were reared at constant 20°C, 50–60% relative humidity, and ambient light conditions. Two weeks after hatching, larvae reached the third instar and were used for the study.

Experiment 1

Treatment of Cotton Plants Three weeks after germination, when plants had developed four to five true leaves, the length of the midrib of leaf 4 was determined as a good measure for growth stage. Next, 24 plants at comparable growth stage were transferred into a second growth chamber (Vötsch, Germany) with abiotic conditions identical to the chamber where plants were reared. Here, plants were maintained separately in 250-ml plastic pots and watered twice a day to ensure adequate water supply. After 24 h, plants were divided into four treatment groups of six individuals each. Plants of one group were damaged mechanically three times on leaf 2 on the first day and three times on leaf 3 on the fourth day by using a fabric pattern wheel. In every case, leaves were scratched four times in parallel to the midrib (two times on each side). For a second group, three caterpillars of *Spodoptera littoralis* were caged on each plant on leaf 2 during the first day and on leaf 3 during the fourth day until larvae consumed at least one third of the available leaf material. Plants of the third group were watered with a 100-μM JA solution in water (Sigma) during the first day. JA concentrations in this range are commonly used to induce the biosynthesis of secondary products in plants (Koch et al. 1999; van Poecke and Dicke 2002). Afterward, this solution was replaced by pure tap water. A control group of plants was not treated at all. One week after the start of treatments, all true leaves were harvested separately, frozen in liquid nitrogen, and stored at −20°C until further analyses.

Foliar Glands Just after removal, all leaves were scanned to calculate leaf areas by using the program ImageJ (Wayne Rasband, National Institutes of Health, USA). In this procedure, herbivore-damaged leaves were used as templates to reconstruct the areas of undamaged leaves. In addition, surface view pictures were taken of two circular 24-mm^2 sections from the center of each leaf half with a stereomicroscope connected to a digital camera. In doing so, leaves were illuminated from below with a lamp to visualize filled subepidermal glands as pigmented spots. After the number of filled glands was counted, gland densities for 24-mm^2 sections were calculated. Based on these sections, gland numbers and gland densities per leaf and per total foliage were extrapolated. These values were slightly underestimated because of higher gland densities at leaf edges (personal observations).

Analysis of Monoterpenes and Sesquiterpenes True leaves were separately analyzed for their content of constitutive terpenes. Depending on available material, 30–100 mg of ground frozen leaf were extracted with 300–500 μl pentane/*n*-hexane 1:1. Additionally, 2 μg of nonylacetate were added as internal standard before extracts were shaken for 3 h at room temperature. After extracts were cooled to 4°C, they were filtered through cotton and Na_2SO_4 to remove remaining water. A subset of extracts was analyzed qualitatively by gas chromatography–mass spectrometry (GC–MS) on a Hewlett-Packard 6890 gas chromatograph (injector temperature 220°C; 1 μl splitless injection) coupled to a Hewlett-Packard 5973 quadrupole mass selective detector. Separations were performed on a DB-5MS column (30 m×0.25 mm×0.25 μm film; Agilent Technology) with 2 ml min^{-1} helium as the carrier gas. The following temperature gradient was used: 40°C for 3 min, increased to 90°C at 5°C/min, further increased to 140°C at 40°C/min, followed by an increase to 160°C at 4°C/min, maintained at 160°C for 3 min, and a final heating to 300°C for 3 min. Mass spectrometry was performed with an ionization potential of 70 electron volts and a scan range of *m*/*z* from 50 to 300. Compound identification was based on comparisons with mass spectra in the Wiley and National Institute of Standards and Technology (NIST) libraries or on direct comparison of mass spectra and retention times with available standards. For quantification, all samples were analyzed on a Hewlet-Packard 6890 gas chromatograph and a flame ionization detector (temperature 250°C). The separation procedure was identical to the method for GC–MS analysis. Terpenes were quantified by using nonylacetate and corrected with response factors according to Scanlon and Willis (1985). Concentrations and levels of terpenes per subepidermal gland were calculated for single leaves and for total foliage. Eight individual monoterpenes and sesquiterpenes were analyzed, which represent more than 90% of the total terpenes in *G. hirsutum*.

Analysis of Terpenoid Aldehydes True leaves were separately analyzed for their content of terpenoid aldehydes according to Stipanovic et al. (1988). Depending on available material, 30–100 mg of ground frozen material were extracted ×3 with 3 ml of ethyl acetate/*n*-hexane (1:3) and 50 μl of 10% HCL. Extracts were shaken at 5°C for 3 h. Afterwards, extracts were removed from the leaf material, evaporated with nitrogen, and redissolved in 40% H_2O and 60% solvent B (see below) from the following high-performance liquid chromatography (HPLC) proce-

dure. Reversed phase HPLC analysis was performed on a Chromolith LC-18 column (Merck; 5 μm; 100×4 mm) with single wavelength detection at 272 nm. The column was eluted with H_2O + 0.05% trifluoracetic acid (solvent A) and EtOH/MeOH/MeCN/EtOAc/iPrOH, 26.6:7.4:40.5:6.1:19.4 (solvent B), with the following gradient: 50% B (0 min)–50% B (5 min)–61% B (5.1 min)–61% B (20 min). Flow rate was held constant at 2 ml min^{-1}, and temperature was kept at 25°C. Five compounds, the hemigossypolone (C_{15}) and the heliocides 1–4 (C_{25}), were identified by their retention times according to the literature (Stipanovic et al. 1988) and additionally by liquid chromatography–mass spectrometry (LC–MS) measurements. Quantification was done by using standard curves of purified compounds. Concentrations and levels per subepidermal gland were calculated for single leaves and for the total foliage. Hemigossypolone and the four heliocides represent approximately 90% of the total terpenoid aldehyde content. As a sesquiterpene aldehyde, hemigossypolone was included in our analysis with other terpenoid aldehydes rather than with the sesquiterpenes. In comparison to other cotton varieties (Bezemer et al. 2004), the terpenoid aldehyde gossypol (C_{30}) was found only in traces by LC–MS and was not quantified.

Experiment 2

Eighteen 2-wk-old plants at the growth stage of two true leaves were chosen and prepared for treatments in a way comparable to the first experiment. Plants were divided into three groups. Plants of one group were damaged mechanically nine times on leaves 1 and 2 during six consecutive days by using a mechanical fabric pattern wheel (procedure see experiment 1). For a second group, two caterpillars of *Spodoptera littoralis* were caged on each plant on one cotyledon during day 1, on leaf 1 during day 3, and on leaf 2 during day 5 until larvae consumed at least one third of the available leaf material. A control group was not treated at all. One week after the start of treatments, leaf 4 from each plant was harvested. Leaf areas and gland number per leaf were determined according to experiment 1. Afterwards, leaves were frozen in liquid nitrogen and stored at −20°C until further analyses of mono- and sesquiterpenes. Levels of terpene classes were calculated based on the levels of eight individual compounds that represent more than 90% of the accumulated monoterpenes and sesquiterpenes in *G. hirsutum.*

Statistics—Experiment 1

Total Foliage The effects of mechanical damage, herbivory, and JA application on foliage area, levels of total monoterpenes, sesquiterpenes, and terpenoid aldehydes, as well as on levels of individual major terpenoids, were analyzed with analyses of variance. Data were tested for normality and equal variances. For normally distributed data, one-way analyses of variance (ANOVAs) were used. Differences between treatments and controls were tested for significance by using Dunn's post hoc tests. Data not normally distributed were analyzed by using Kruskall–Wallis one-way ANOVAs on ranks and Dunn's post hoc tests. Analyses were performed with Sigma Stat 2.03. The effect of foliage area and treatment on the total number of glands was tested by analysis of codeviance. Generalized linear models (GLM) with the log link as link function were performed. To deal with overdispersion, a quasi-Poisson distribution was used in the models instead of Poisson distribution. The models were simplified by removing non-significant terms and by factor-level reduction (Crawley 2002). These analyses were performed in *R*, version 2.4.1. All data are presented as mean ± SE except when it is mentioned otherwise.

Single Leaves The effects of leaf position, treatment, and their interactions on levels of terpenoids per gland were tested by using nested two-way ANOVA. For this purpose, terpenoid data were root- or log-transformed to normalize them. For single leaf positions, the effects of the treatments on the leaf area were tested with one-way ANOVAs. The effect of leaf area and treatment on the total number of glands was tested by analysis of codeviance (GLM see above). To analyze the effect of leaf area and treatment on gland density, levels of terpenoids per gland and terpenoid concentrations per milligram fresh leaf material ANCOVAs were used. These analyses were performed in *R*, version 2.4.1. Changes in per gland concentrations of individual terpenoids at specific leaf positions after treatments were tested with Sigma Stat 2.03. For normally distributed data, one-way ANOVAs were used. Differences between treatments and controls were tested for significance by using Dunn's post hoc tests. Data not normally distributed were analyzed using Kruskall–Wallis one-way ANOVAs on ranks and Dunn's post hoc tests.

Statistics—Experiment 2

Single Leaves The effect of the treatments on the areas of leaf 4 was tested with one-way ANOVA. The effect of leaf area and treatment on the total number of glands was tested by analysis of codeviance (GLM see above). To analyze the influence of leaf area and treatments on the accumulation of mono- and sesquiterpenes an ANCOVA was achieved. All analyses were performed in *R*, version 2.4.1.

Results

Experiment 1

Terpenoid Accumulation in Total Cotton Foliage after Induction The accumulation of terpenoid classes among the four different treatments was first compared on the basis of total foliage (Fig. 4). For monoterpenes, sesquiterpenes, and terpenoid aldehydes, there was a gradual increase in the following order: control, mechanical damage, herbivory, and JA treatment (ANOVA on ranks: monoterpenes, $H=9.420$, $P=0.024$; sesquiterpenes, $H=14.140$, $P=0.003$; terpenoid aldehydes, $H=19.760$, $P<0.001$). Levels of all three classes were elevated in herbivore-damaged (Dunn's tests: monoterpenes, $Q=2.572$; sesquiterpenes, $Q=2.490$; aldehydes, $Q=2.939$; all $P<0.05$) and JA-treated plants (Dunn's tests: monoterpenes, $Q=2.735$; sesquiterpenes, $Q=3.674$; aldehydes, $Q=4.164$; all $P<0.05$) in comparison to the controls.

All individual compounds showed the same pattern that was seen for total terpenoid accumulation: a gradual increase from control to mechanical damage, herbivory, and JA treatment. In 11 out of 13 compounds, this increase was significant (Table 1) for herbivore and JA-treated plants compared to the control plants, while mechanically damaged plants showed tendencies for increased accumulation. Among monoterpenes, considerable differences in accumulation after treatment were shown. (*E*)-β-ocimene increased up to eightfold after both herbivory and JA treatment in comparison to controls, whereas myrcene levels slightly but significantly increased after herbivory and JA treatment, and the amounts of α- and β-pinene did not show significant differences between treatments and control. Little variation in the induction pattern occurred when comparing individual sesquiterpenes. Levels of all major sesquiterpenes, including β-caryophyllene, α-humulene, γ-bisabolene, and β-bisabolol doubled after herbivory and were 2.0–2.6-fold higher after JA treatment in comparison to control plants. Among terpenoid aldehydes, considerable differences appeared. Whereas the amounts of hemigossypolone were doubled, levels of heliocides, especially H_1 and H_4, increased much more after herbivory and JA treatment compared to the controls. Heliocide H_4, for example, showed up to a ninefold increase after herbivory and up to a 15-fold increase after JA treatment in comparison to control plants. For H_1, these increases were approximately five- and tenfold, respectively. Meanwhile, the highest levels of H_2 and H_3 were found in the JA-treated plants, reaching up to 3.3-fold that of control levels.

Growth and Subepidermal Gland Production of Cotton Foliage ANOVA showed an effect of the treatments on the total leaf area ($F=10.44$, $P<0.001$). Whereas controls, mechanically and herbivore-damaged plants were highly comparable in size (mean, 496–513 cm^2), JA-treated plants (mean=306 cm^2) showed a reduction in the total area of foliage down to 60% that of the controls (Bonferoni post hoc test. $P<0.05$).

Because of significantly smaller leaves in the JA-treated plants, the effect of treatment on the production of subepidermal glands was tested by using a GLM with leaf area as a covariable. For all treatments, this covariable showed an impact on the number of glands ($t=8.113$, $P<0.001$; Fig. 5). In comparison to the controls, an increase in the number of glands was found for herbivore-damaged plants ($t=4.147$, $P<0.001$) and for plants that were treated with JA ($t=5.585$, $P<0.001$). Because there were no differences between mechanically damaged and non-treated plants, the data of both groups were combined in this analysis.

Single Leaf Analysis To assess more precisely plant response to the treatments, analyses were carried out at the level of individual leaves. For all treatments, the distribution of leaf areas followed a consistent pattern within plants with the biggest leaves at intermediate positions (Fig. 6a). Whereas no differences in leaf area

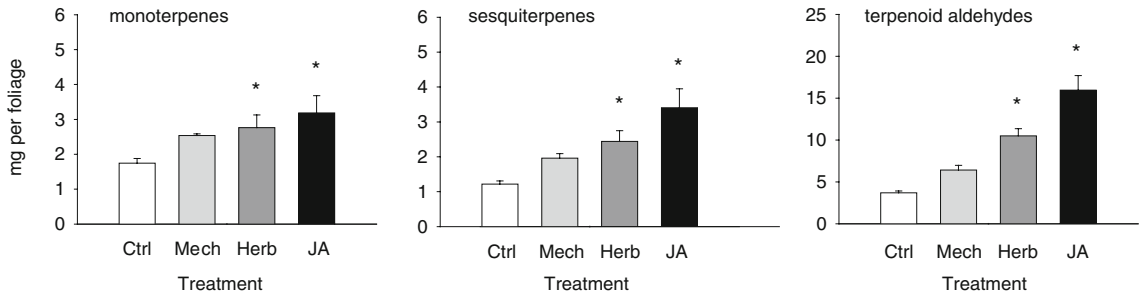


Fig. 4 Mean (±SE, $N=6$) levels of terpenoids in the total foliage of 4-week-old cotton plants, which were either damaged at leaves 2 and 3 mechanically (*Mech*), fed upon by larvae of *Spodoptera littoralis* (Herb), treated with jasmonic acid (*JA*), or left as untreated controls (*Ctrl*) 7 days previously. *Asterisks* indicate significant differences between treatment and control plants based on Kruskall–Wallis one-way ANOVAs on ranks and Dunn's post hoc tests. *$P<0.05$

Table 1 Mean levels (±SD, $N=6$) of terpenoid products in total foliage of 1-month-old *G. hirsutum* plants that were either mechanically damaged with a fabric pattern wheel, damaged by larvae of *Spodoptera littoralis* (herbivory), treated with jasmonic acid (JA), or left as untreated controls

Compound	ANOVA ($df=3$)		Levels of terpenoids (mg)			
	F/H	P Value	Control	Mechanical damage	Herbivory	JA
Monoterpenes						
α-Pinene	7.213	0.065	1.11±0.08	1.26±0.11	1.31±0.25	1.45±0.44
β-Pinene	7.647	0.054	0.20±0.02	0.23±0.02	0.25±0.05	0.26±0.08
Myrcene	10.167	0.017	0.49±0.06	0.68±0.04	0.78**±0.15	0.80**±0.33
(E)-β-Ocimene	17.487	<0.001	0.06±0.02	0.25±0.06	0.47**±0.11	0.49**±0.26
Sesquiterpenes						
(E)-β-Caryophyllene	16.340	<0.001	0.50±0.07	0.76±0.12	0.95**±0.21	1.32**±0.50
α-Humulene	9.509	<0.001	0.14±0.02	0.22±0.03	0.27 *±0.06	0.36 *±0.13
γ-Bisabolene	18.007	<0.001	0.23±0.04	0.34±0.06	0.45**±0.10	0.60**±0.19
β-Bisabolol	14.340	0.002	0.46±0.05	0.59±0.09	0.82**±0.18	0.91**±0.26
Terpenoid aldehydes						
Hemigossypolone	15.287	0.002	1.59±0.30	2.16±0.44	3.14**±0.40	3.10**±0.83
Heliocide 1	20.487	<0.001	0.59±0.14	1.65±0.37	3.35**±1.11	5.95**±1.93
Heliocide 2	21.031	<0.001	0.97±0.21	1.45±0.42	1.83 *±0.37	3.06 *±0.74
Heliocide 3	23.938	<0.001	0.37±0.07	0.56±0.15	0.70 *±0.15	1.23 *±0.30
Heliocide 4	20.247	<0.001	0.17±0.08	0.59±0.25	1.47**±0.39	2.60**±0.78

The effect of treatment on terpenoid levels were tested using one-way ANOVAs. For normally distributed data, one-way ANOVAs were performed (F values). For not normally distributed data, Kruskall–Wallis one-way ANOVAs on ranks were performed (H values).
*$P<0.05$, significant differences from the control according to Bonferoni post hoc tests
**$P<0.05$, significant differences from the control according to Dunn's post hoc tests

were evident among mechanically damaged, herbivore-damaged, and control plants, plants treated with JA exhibited significant smaller areas for leaves 3–6 in comparison to corresponding control leaves (ESM Tables 3 and 4). Because of the negative impact of JA treatment on the leaf area, the following analyses of the effect of treatments on gland and terpenoid production were performed by using leaf area as covariable (statistical results see ESM Tables 3 and 4).

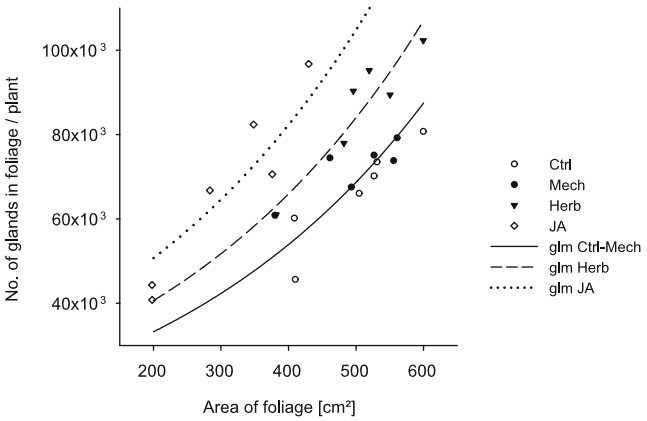


Fig. 5 Effect of leaf area and treatment on the number of glands in the total foliage of 4-week-old cotton plants, which were either damaged at leaves 2 and 3 mechanically (*Mech*), fed upon by larvae of *Spodoptera littoralis* (*Herb*), treated with jasmonic acid (*JA*), or left as untreated controls (*Ctrl*) 7 days previously. Regressions were drawn based on transformed data from a generalized linear model (*GLM*) with a quasi-Poisson error distribution. Data for control and mechanically damaged plants were combined due to factor level reduction

Gland number per leaf revealed a similar distribution pattern among leaves for all treatments: The oldest leaf 1 contained the lowest gland number, which increased gradually toward leaf 5 and again decreased in the expanding leaves 6 and 7 (Fig. 6b). Young leaves (5–7) of herbivore-treated plants showed significantly elevated numbers of glands in comparison to corresponding leaves of control plants. Lower but still significant increases in gland numbers were found for leaves 2, 3, and 7 (youngest leaf) of plants that were treated with JA compared to control leaves. Mechanical damage showed nearly no effect on gland number.

Changes in leaf area and gland numbers after treatment should have an impact on gland densities. Independent of treatment, gland densities increased gradually from the oldest leaf 1 toward the youngest leaf 7 (Fig. 6c). This pattern was enhanced in herbivore-damaged and JA-treated plants because of significantly higher gland densities in young leaves (5, 6, 7) compared to those leaves from the control plants. Mechanical damage did not cause an increased gland density in any leaf.

To analyze how terpenoid production was altered by treatment, total terpenoid levels per gland were calculated. Treatments had a considerable effect on the pattern of terpenoids per gland among leaves (Fig. 6d). Control plants showed highest levels in the oldest leaf and lowest levels in the youngest one. In contrast, mechanically and herbivore-

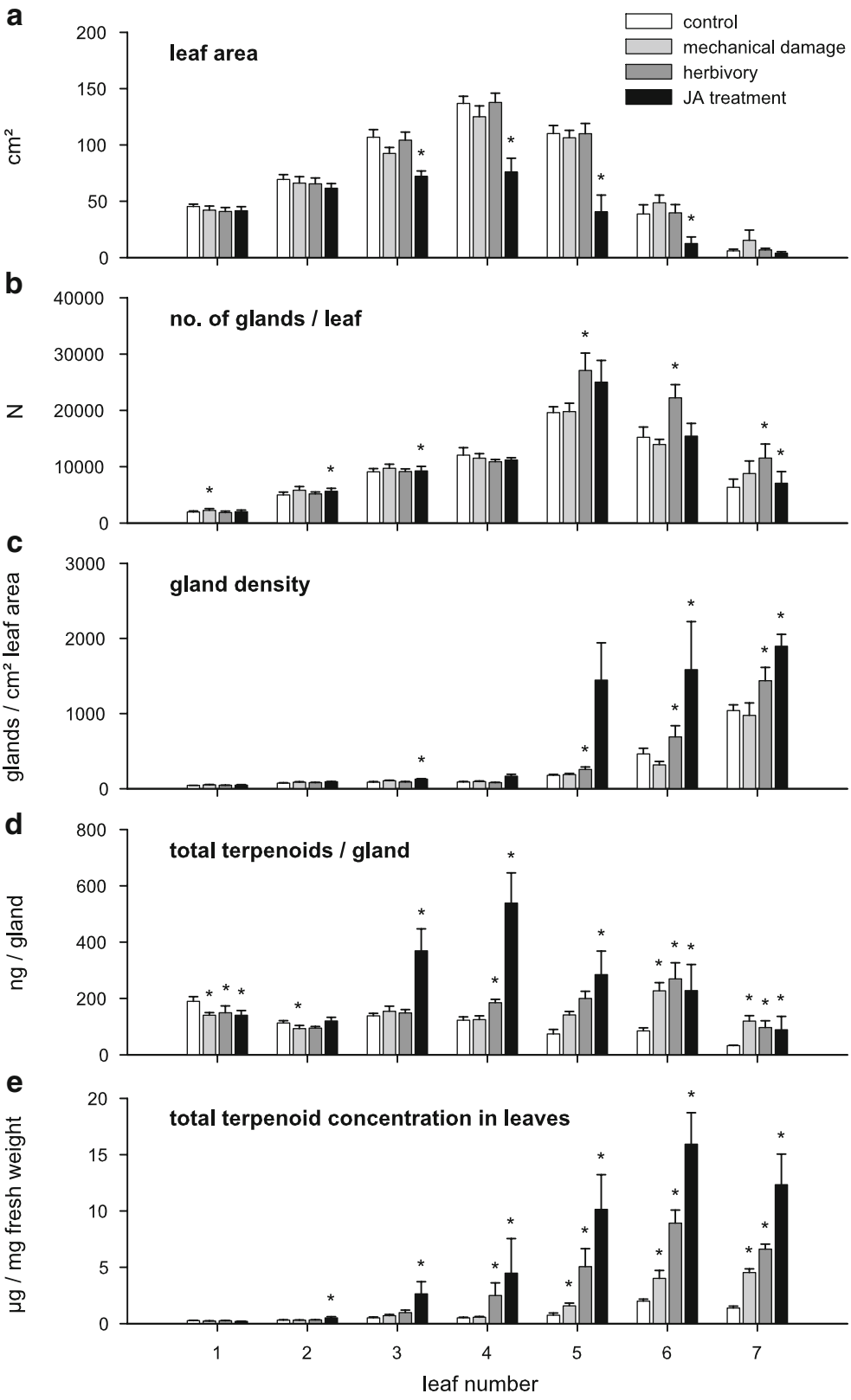


Fig. 6 Mean (±SE, N=3–6) leaf area, number of glands, gland density, terpenoid level per gland, and terpenoid concentration of 4-week-old cotton plants, which were either damaged at leaves 2 and 3 mechanically (Mech), fed upon by larvae of *Spodoptera littoralis* (Herb), or treated with jasmonic acid (JA) 7 days previously. Areas of herbivore-damaged leaves 2 and 3 were extrapolated from remaining leaf material. For each leaf position, *asterisks* indicate significant differences from corresponding control leaves based on one-way ANOVAs (**a**), on analyses of codeviance with leaf area as covariable (**b**), and ANCOVAs with leaf area as covariable (**c**–**e**). *P<0.05

damaged plants exhibited highest values in younger leaves, having significantly elevated total terpenoid levels in comparison to those of control leaves. Plants that were treated with JA showed significantly elevated levels of total terpenoids per gland in young and intermediate leaves in comparison to the controls.

The increase in total terpenoid and gland production caused by the treatments as well as altered leaf areas had a considerable impact on the pattern of the total terpenoid concentrations in leaves. Total terpenoid concentrations showed a consistent distribution pattern among leaves for all treatments (Fig. 6e). There was a gradual increase starting from the oldest leaf 1 toward the youngest leaves 6 and 7. All treatments enhanced this pattern by causing significantly higher concentrations in intermediate and young leaves in comparison to the leaves of corresponding control plants. The lowest effect was found for mechanically damaged plants, whereas plants treated with JA reached the highest concentration with mean levels of approximately 16 μg terpenoids per milligram fresh weight.

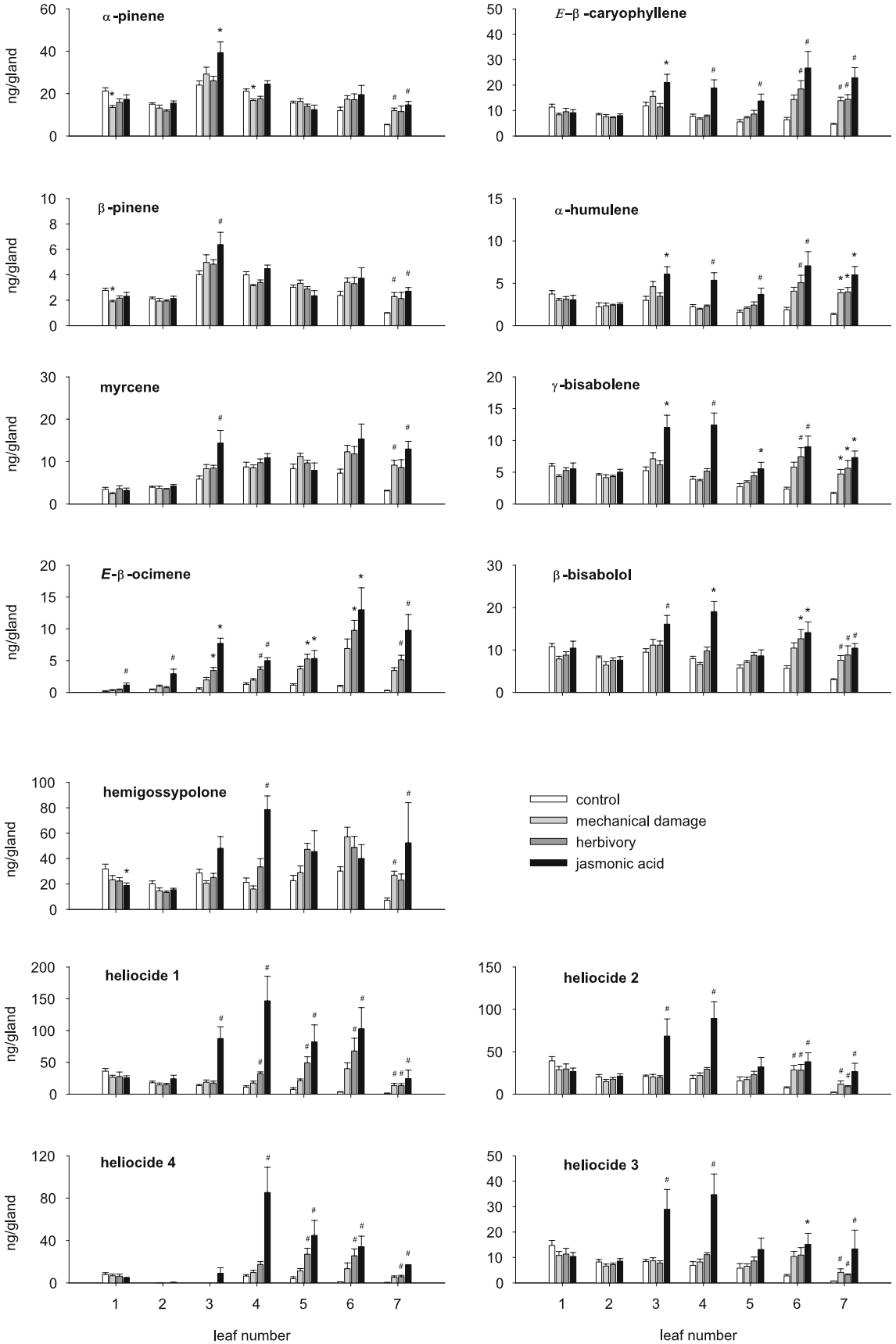
The analyses of individual terpenoids revealed a significant impact of treatment, leaf position, and interactions between these two factors on levels of all compounds per gland (Table 2). The lowest effects of treatment were found for the monoterpenes α- and β-pinene, whereas the biggest changes appeared for (*E*)-β-ocimene and heliocides 1 and 4. Differences of single terpenoid levels per gland among leaves appeared to be biggest for (*E*)-β-ocimene and heliocides 4, but in case of the latter, this was due to its unusual absence in leaves 2 and 3 (Fig. 7). Significant interactions between treatment and leaf position revealed that changes in levels of total and single terpenoids of induced plants were not consistent in all leaves, which is illustrated in Fig. 7. As with total terpenoid levels per gland (Fig. 6d), mechanical damage and herbivory caused significantly elevated accumulation of individual compounds per gland in the youngest leaves in comparison to control plants. In contrast, JA treatment induced elevated levels in young but also in intermediate leaves compared to control leaves. JA treatment led to the highest increases of most compounds, especially for heliocides, in comparison to those in the corresponding leaves of control plants.

Experiment 2

Morphological and Chemical Changes in Young Leaves after Induction In addition to herbivore-treated plants and untreated controls, this supplementary experiment also included mechanically damaged plants that were injured more frequently and over a longer period than in experiment 1. ANOVA showed an effect of the treatments on areas of young leaves (leaf 4; $F=5.97$, $P=0.012$; Fig. 8a, ESM Table 5). Herbivory reduced the areas of these leaves in comparison to those of the corresponding leaves of the control plants ($t=-3.445$, $P=0.004$). Therefore, the effect of mechanical damage and herbivory on the production of glands were tested by using a GLM with leaf area as a covariable. For both treatments, leaf area showed an impact on the number of glands ($t=2.256$, $P=0.041$). In comparison to the control plants, a significant increase in the number of glands was found for mechanically damaged plants ($t=2.599$, $P<0.021$) and an even more pronounced

Table 2 Results of nested two-way ANOVA for the effects of treatment (mechanical damage, herbivory, jasmonic acid application), leaf position, and interactions on levels of accumulated terpenoids per gland in plants of *G. hirsutum*

Compound	Treatment (*df*=3)		Leaf Number (*df*=6)		Treatment × Leaf Number (*df*=18)	
	F	*P*	*F*	*P*	*F*	*P*
Total terpenoids	37.81	<0.001	17.45	<0.001	6.78	<0.001
Monoterpenes						
α-Pinene	6.70	<0.001	39.98	<0.001	4.02	<0.001
β-Pinene	4.34	0.006	42.48	<0.001	3.67	<0.001
Myrcene	10.25	<0.001	51.60	<0.001	4.06	<0.001
(E)-β-Ocimene	106.72	<0.001	79.24	<0.001	6.50	<0.001
Sesquiterpenes						
(E)-β-Caryophyllene	24.13	<0.001	13.30	<0.001	6.59	<0.001
α-Humulene	20.56	<0.001	9.66	<0.001	3.21	<0.001
γ-Bisabolene	37.08	<0.001	11.33	<0.001	5.74	<0.001
β-Bisabolol	23.15	<0.001	12.48	<0.001	4.99	<0.001
Terpenoid aldehydes						
Hemigossypolone	10.16	<0.001	15.48	<0.001	6.47	<0.001
Heliocide 1	64.63	<0.001	13.78	<0.001	8.88	<0.001
Heliocide 2	27.13	<0.001	17.65	<0.001	4.94	<0.001
Heliocide 3	30.60	<0.001	17.66	<0.001	4.83	<0.001
Heliocide 4	55.70	<0.001	104.05	<0.001	8.59	<0.001


α-pinene
E–β -caryophyllene
β-pinene
α-humulene
myrcene
γ-bisabolene
E-β -ocimene
β-bisabolol
hemigossypolone
heliocide 1
heliocide 2
heliocide 4
heliocide 3
ng/gland
leaf number
control
mechanical damage
herbivory
jasmonic acid

◀ **Fig. 7** Mean (±SE, N=3–6) concentrations of terpenoids per gland among true leaves of 4-week-old *Gossypium hirsutum* plants, which were either damaged at leaves 2 and 3 mechanically (*Mech*), fed upon by larvae of *Spodoptera littoralis* (*Herb*), treated with jasmonic acid (*JA*), or left as untreated controls (*Ctrl*) 7 days previously. *Asterisks* indicate significant differences (P<0.05) from corresponding control leaves based on one-way ANOVA's and Bonferoni post hoc tests. *Pound signs* indicate significant differences (P<0.05) from corresponding control leaves based on Kruskall–Wallis one-way ANOVAs on ranks and Dunn's post hoc tests

increase for herbivore-treated plants (t=3.938, P=0.002; Fig. 8b).

The accumulation of mono- and sesquiterpenes in leaf 4 after treatment followed a comparable pattern: a gradual increase from control to mechanical damage and herbivory (ANOVA: monoterpenes, F=8.13, P=0.004; sesquiterpenes, F=27.43, P<0.001; Fig. 8c,d). Levels of monoterpenes were elevated after herbivory in comparison to the controls (t=4.029, P=0.001). Levels of sesquiterpenes were elevated after mechanical damage (t=5.115, P<0.001) and herbivory (t=7.197, P<0.001) in comparison to the controls.

Discussion

Cotton (*G. hirsutum*) accumulates a large variety of terpenes constitutively, including monoterpenes (C_{10}), sesquiterpenes (C_{15}), and terpenoid aldehydes (C_{15}, C_{25}, and C_{30}). This study is the first to describe the accumulation of all three of these classes after actual or simulated herbivory. Increased levels of terpenoids were observed in the total foliage 1 week after mechanical damage, feeding by *Spodoptera littoralis* caterpillars, or treatment with JA, in comparison to the untreated controls. A closer look revealed that, after damage to older leaves (leaves 2 and 3 numbered from the base), the increase was restricted mainly to young leaves (leaves 5–7). Similarly, studies on cotton that investigate the effects of herbivory by larvae of *S. exigua* and the wireworm *Agriotes lineatus* also showed elevated concentrations of terpenoid aldehydes in young cotton leaves (McAuslane et al. 1997; McAuslane and Alborn 1998; Bezemer et al. 2004). Such an induction pattern is in agreement with ODT, which predicts that an increase in the accumulation of defense chemicals occurs preferentially in plant parts with the highest fitness value such as young tissues or reproductive organs (McKey 1979; Frischknecht et al. 1987; Ohnmeiss and Baldwin 2000).

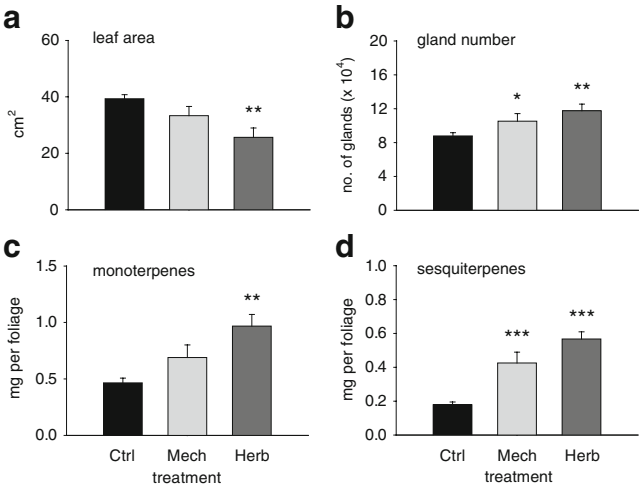


Fig. 8 Mean (±SE, N=6) leaf area, number of glands, and monoterpene- and sesquiterpene levels of leaf 4 from 3-week-old *Gossypium hirsutum* plants, which were either damaged mechanically at leaves 1 and 2 (*Mech*), fed upon by larvae of *Spodoptera littoralis* (*Herb*) on one cotyledon, leaves 1 and 2, or left as untreated controls (*Ctrl*). *Asterisks* indicate significant differences from corresponding control leaves based on one-way ANOVAs (**a**), on analyses of codeviance with leaf area as covariable (**b**), and ANCOVAs with leaf area as covariable (**c**, **d**). *P<0.05; **P<0.01; ***P<0.001

All three classes of cotton terpenoids are stored in subepidermal pigment glands found in leaves and other organs. After treatment, *G. hirsutum* displayed two ways to achieve elevated terpenoid accumulation: (1) production of additional glands and (2) increased filling of existing glands (Fig. 6). We showed that production of additional glands was restricted to leaves that were still under development or newly formed after treatment. This trend also has been observed after attack by spider mites or larvae of *S. exigua* on cotton (McAuslane et al. 1997; Agrawal and Karban 2000). In other plants, such as birch and tomato, an elevated number of defense structures like glandular trichomes is also produced in young leaves after induction by herbivores (Boughton et al. 2005; Valkama et al. 2005). Besides the fact that, according to ODT, plants have been selected to increase their resistance to herbivores especially in young tissue, the formation of subepidermal pigment glands in cotton might be necessarily restricted to newly developing leaves because of developmental constraints.

In counting the number of cotton leaf glands under a stereomicroscope, we may have overestimated the production of new glands after treatment if herbivory triggered the filling of pre-existing glands that were unpigmented (H. T. Alborn, personal communication). However, by using several microscopic techniques, we did not find any evidence for unfilled glands in the leaves analyzed. Therefore, we assume that actual or simulated herbivory does indeed induce increased numbers of glands in cotton.

The increased filling of existing glands with terpenoids was shown in the youngest leaves of induced plants for all major terpenoid classes. However, considerable differences in the magnitude of this additional accumulation were observed among individual terpenoid compounds. Among terpenoid aldehydes, levels of heliocides H_1 and H_4, showed the highest increase after treatments. Other studies on cotton species also have demonstrated that these two

heliocides increase more than H_2, H_3, and hemigossypolone (HGQ) after herbivory by *Spodoptera* species (McAuslane et al. 1997; McAuslane and Alborn 1998; Agrell et al. 2004; Bezemer et al. 2004). Here, we showed that levels of (*E*)-β-ocimene increased in a magnitude similar to H_1 and H_4, and that this compound increased most among monoterpenes and sesquiterpenes. Interestingly, (*E*)-β-ocimene is a direct precursor in the formation of H_1 and H_4, combining with hemigossypolone in a Diels–Alder-type reaction (Stipanovic et al. 1978a; Fig. 2). Therefore, in non-treated plants, the biosynthesis of this monoterpene could represent a limiting step in the production of the appropriate heliocides. This proposition is supported by the fact that, in cotyledons of *G. hirsutum*, which do not contain any heliocides, hemigossypolone is present (S. Opitz, unpublished), but (*E*)-β-ocimene is absent. A comparable regulation mechanism that involves myrcene could determine the levels of H_2 and H_3 (Stipanovic et al. 1977, 1978b).

Our study compared the terpenoid content of total cotton foliage after three different treatments and showed a gradual increase in terpenoid levels in the following order: mechanical damage with a fabric pattern wheel, herbivory by *S. littoralis* caterpillars, and application of JA. However, a single incidence of mechanical injury is hard to compare with continuous feeding damage by an insect. Studies on lima bean have demonstrated that both the intensity and frequency of mechanical damage can alter the reaction of a plant (Mithöfer et al. 2005). This is consistent with the results of experiment 2 where more frequent mechanical damage caused significantly higher numbers of glands (Fig. 8), a response not detectable after the less frequent mechanical damage in experiment 1 (Fig. 5 and 6). However, in both experiments, herbivory induced stronger reactions of plants than mechanical damage. Elicitors found in the regurgitate of herbivores are known to trigger the biosynthesis of defense metabolites (Alborn et al. 1997). If such elicitors play a role in cotton defense reactions, mechanical injury may never result in terpenoid accumulation comparable to that caused by herbivory.

The strongest increase in both terpenoid and gland production appeared in plants that were treated with JA, a ubiquitous plant hormone known to mediate defense responses to biotic and abiotic stresses (Browse 2005). A previous study with cotton showed that the application of the methylated derivative, methyl jasmonate, induced the synthesis and emission of volatile terpenes, a response also observed for herbivore-damaged plants (Rodriguez-Saona et al. 2001). Obviously, JA plays an important role in mediating damage-induced signaling in *G. hirsutum*. Given the effects of exogenous application, herbivory can be assumed to trigger elevated internal levels of JA as in other species (Baldwin et al. 1997; Creelman and Mullet 1997). For example, in tobacco, lima bean, or maize, internal JA levels were determined in ranges of single nanograms per gram fresh weight, showing 20- to 40-fold increases within the first hours after induction (Baldwin et al. 1997; Koch et al. 1999; Schmelz et al. 2003). However, internal JA levels of cotton still need to be investigated. If the irrigation of plants with 100 μM JA in our study led to a non-physiological high internal concentration of JA, this could explain why JA treatment induced terpenoid accumulation to a greater extent than herbivory. We observed additionally that JA inhibited leaf development, a response also seen in other plants (Sembdner and Parthier 1993).

Studies on the induction of chemical defense in plants after herbivory always raise questions about the specificity of such reactions to particular enemies. Certain plants have been shown to induce different spectra of defenses in response to different herbivores (Turlings et al. 1998; Traw and Dawson 2002; Delphia et al. 2007). However, in this study, we did not find substantial differences in the pattern of induction among treatments for the major terpenoids measured (see Table 1). The amounts of single compounds varied in magnitude, leading to changes in the terpenoid profile, but these profile changes tended to be comparable among treatments. The induction of elevated terpenoid accumulation in leaves of *G. hirsutum* may thus be a non-specific reaction of plants to damage. Similar non-specific reactions to damage are known for terpenes in other species (Banchio et al. 2005), as well as for alkaloids (Frischknecht et al. 1987; Baldwin et al. 1997), phenolics (Cipollini 1997), and glucosinolates (Bodnaryk 1992).

The fact that cotton plants react to damage with elevated levels of terpenoids accumulated in the subepidermal pigment glands of their foliage suggests that these compounds function in plant defense. Caterpillar species such as *S. exigua* or *Heliothis virescens* prefer feeding on glandless instead of glanded cultivars of *G. hirsutum*, thus supporting the argument that the gland terpenes play a key role in defense of cotton against herbivores (Montandon et al. 1986; McAuslane and Alborn 2000). Leaf material from plants of glanded cultivar lines that had been induced by herbivory showed increased deterrent or toxic effects on *Spodoptera* species compared to material from uninduced plants, a finding thought to be due to their elevated contents of terpenoid aldehydes (Alborn et al. 1996; McAuslane et al. 1997; Anderson et al. 2001). Indeed, terpenoid aldehydes such as gossypol, hemigossypolone, and the heliocides H_1 and H_2 exhibit strong toxicity to caterpillars of *H. virescens* and *Pectinophora gossypiella* after addition to artificial diet (Elliger et al., 1978). While the feeding deterrent effects of induced cotton foliage are usually attributed to elevated terpenoid aldehydes contents (Hedin et al. 1992; McAuslane et al. 1997; McAuslane and Alborn 2000), we showed that cotton plants also increase their

levels of monoterpenes and sesquiterpenes. Thus, these compounds may also participate in defense against herbivores. A sesquiterpene, caryophyllene oxide has been demonstrated to synergize the negative effect of gossypol, the dominant terpenoid aldehyde in roots and seeds of cotton, on larval development of *H. virescens* (Gunasena et al. 1988). Additionally, caryophyllene retarded the growth and delayed the time of development of these larvae. Other studies have shown that single sesquiterpenes such as β-bisabolene impair the development of insect herbivores and deter them from feeding (Bowers et al. 1976; Gonzalez-Coloma et al. 1995; Zipfel 2007).

Beside their function against herbivores, terpenoids are also considered to play a defensive role against fungi or pathogens. Because plants are exposed especially to infestation at wound sites, the elevated terpenoid levels in damaged leaves might have a critical role in helping plants to cope with pathogens. For cotton, studies have shown that certain terpenoid aldehydes possess antifungal and anti-pathogen activities (Zhang et al. 1993; Abraham et al. 1999). A good example for the antibiotic activity of terpenoids in other species comes from conifers where the growth and germination of bark beetle associated pathogens is inhibited (Keeling and Bohlmann 2006). In addition, analyses of terpene-rich essential oils from a variety of plant species have shown antibacterial and antifungal activities, indicating the potential role of these substances in plant defense (Oyedeji and Afolayan 2005; Ozer et al. 2007).

In summary, our results demonstrate that elevated levels of terpenoids in cotton leaves after real and simulated herbivory represent a general wound response that is mediated by JA. The increase in terpenoids is due to the production of additional glands, in which terpenoids are stored, as well as the increased filling of existing glands. As all three classes of terpenoids (monoterpenes, sesquiterpenes, and terpenoid aldehydes) were elevated after damage, these substances may act synergistically in defense against herbivores or pathogens (Stipanovic et al. 1988).

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References

ABRAHAM, K. J., PIERCE, M. L., and ESSENBERG, M. 1999. The phytoalexins desoxyhemigossypol and hemigossypol are elicited by Xanthomonas in Gossypium cotyledons. *Phytochemistry* 52:829–836.

AGRAWAL, A. A., and KARBAN, R. 1999. Why induced defenses may be favored over constitutive strategies in plants, pp. 45–61, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible DefensesPrinceton University Press, Princeton.

AGRAWAL, A. A., and KARBAN, R. 2000. Specificity of constitutive and induced resistance: pigment glands influence mites and caterpillars on cotton plants. *Entomol. Exp. Appl.* 96:39–49.

AGRELL, J., ANDERSON, P., OLESZEK, W., STOCHMAL, A., and AGRELL, C. 2004. Combined effects of elevated CO2 and herbivore damage on alfalfa and cotton. *J. Chem. Ecol.* 30: 2309–2324.

ALBORN, H. T., RÖSE, U. S. R., and MCAUSLANE, H. J. 1996. Systemic induction of feeding deterrents in cotton plants by feeding of Spodoptera spp. larvae. *J. Chem. Ecol.* 22:919–932.

ALBORN, T., TURLINGS, T. C. J., JONES, T. H., STENHAGEN, G., LOUGHRIN, J. H., and TUMLINSON, J. H. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276:945–949.

ANDERSON, P., JONSSON, M., and MORTE, U. 2001. Variation in damage to cotton affecting larval feeding preference of Spodoptera littoralis. *Entomol. Exp. Appl.* 101:191–198.

BALDWIN, I. T. 1988. The alkaloidal responses of wild tobacco to real and simulated herbivory. *Oecologia* 77:378–381.

BALDWIN, I. T. 2001. An ecologically motivated analysis of plant–herbivore interactions in native tobacco. *Plant Physiol.* 127:1449–1458.

BALDWIN, I. T., ZHANG, Z. P., DIAB, N., OHNMEISS, T. E., MCCLOUD, E. S., LYNDS, G. Y., and SCHMELZ, E. A. 1997. Quantification, correlations and manipulations of wound-induced changes in jasmonic acid and nicotine in *Nicotiana sylvestris*. *Planta* 201: 397–404.

BANCHIO, E., ZYGADLO, J., and VALLADARES, G. R. 2005. Quantitative variations in the essential oil of *Minthostachys mollis* (Kunth.) Griseb. in response to insects with different feeding habits. *J. Agric. Food Chem.* 53:6903–6906.

BERENBAUM, M. R., and ZANGERL, A. R. 1999. Coping with life as a menu option: inducible defenses of the wild parsnip, pp. 10–32, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible DefensesPrinceton University Press, Princeton.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WACKERS, F. L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30:53–67.

BODNARYK, R. P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31:2671–2677.

BOUGHTON, A. J., HOOVER, K., and FELTON, G. W. 2005. Methyl jasmonate application induces increased densities of glandular trichomes on tomato, *Lycopersicon esculentum*. *J. Chem. Ecol.* 31:2211–2216.

BOWERS, W. S., OHTA, T., CLEERE, J. S., and MARSELLA, P. A. 1976. Discovery of insect anti-juvenile hormones in plants. *Science* 193:542–547.

BROWN, P. D., TOKUHISA, J. G., EICHELT, M., and GERSHENZON, J. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of Arabidopsis thaliana. *Phytochemistry* 62:471–481.

BROWSE, J. 2005. Jasmonate: an oxylipin signal with many roles in plants. *Plant Hormones* 72:431–456.

BUCKINGHAM, J. (eds.). 1994. Dictionary of Natural Products. Chapman & Hall, London.

CIPOLLINI, D. F. 1997. Wind-induced mechanical stimulation increases pest resistance in common bean. *Oecologia* 111:84–90.

CRAWLEY, M. J. 2002. Statistical Computing. Wiley, West Sussex.

CREELMAN, R. A., and MULLET, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355–381.

CROXFORD, A. C., EDWARDS, P. J., and WRATTEN, S. D. 1989. Temporal and spatial variation in palatability of soybean and cotton leaves following wounding. *Oecologia* 79:520–525.

DAVIS, G. D., and ESSENBERG, M. 1995. (+)-Delta-cadinene is a product of sesquiterpene cyclase activity in cotton. *Phytochemistry* 39:553–567.

DELPHIA, C. M., MESCHER, M. C., and DE MORAES, C. M. 2007. Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defenses on host–plant selection by thrips. *J. Chem. Ecol.* 33:997–1012.

ELLIGER, C. A., CHAN, B. G., and WAISS, A. C. 1978. Relative toxicity of minor cotton terpenoids compared to gossypol. *J. Econ. Entomol.* 71:161–164.

ELZEN, G. W., WILLIAMS, H. J., BELL, A. A., STIPANOVIC, R. D., and VINSON, S. B. 1985. Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* cultivars and lines by gas chromatography. *J. Agric. Food Chem.* 33:1079–1082.

FAHN, A. 1979. Secretory Tissues in Plants. Academic, London.

FRISCHKNECHT, P. M., BATTIG, M., and BAUMANN, T. W. 1987. Effect of drought and wounding stress on indole alkaloid formation in *Catharanthus roseus*. *Phytochemistry* 26:707–710.

GERSHENZON, J. 1994. Metabolic costs of terpenoid accumulation in higher plants. *J. Chem. Ecol.* 20:1281–1328.

GERSHENZON, J., and CROTEAU, R. 1991. Terpenoids, pp. 165–219, in G. A. Rosenthal, and M. R. Berenbaum (eds.). Herbivores, Vol. 1: Their Interaction with Secondary Plant MetabolitesAcademic, San Diego.

GERSHENZON, J., and DUDAREVA, N. 2007. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3:408–414.

GERSHENZON, J., MCCONKEY, M. E., and CROTEAU, R. B. 2000. Regulation of monoterpene accumulation in leaves of peppermint. *Plant Physiol.* 122:205–213.

GONZALEZ-COLOMA, A., REINA, M., CABRERA, R., CASTANERA, P., and GUTIERREZ, C. 1995. Antifeedant and toxic effects of sesquiterpenes from *Senecio palmensis* to Colorado potato beetle. *J. Chem. Ecol.* 21:1255–1270.

GUNASENA, G. H., VINSON, S. B., WILLIAMS, H. J., and STIPANOVIC, R. D. 1988. Effects of caryophyllene, caryophyllene oxide, and their interaction with gossypol on the growth and development of *Heliothis virescens* (F) (Lepidoptera, Noctuidae). *J. Econ. Entomol.* 81:93–97.

HARBORNE, J. B. 1991. Flavonoid pigments, pp. 389–426, in G. A. Rosenthal, and M. R. Berenbaum (eds.). Herbivores, Vol. 1: Their Interaction with Secondary Plant Metabolites Academic, San Diego.

HARTMANN, T., and ZIMMER, M. 1986. Organ-specific distribution and accumulation of pyrrolizidine alkaloids during the life history of two annual Senecio species. *J. Plant Physiol.* 122:67–80.

HEDIN, P. A., PARROTT, W. L., and JENKINS, J. N. 1992. Relationships of glands, cotton square terpenoid aldehydes, and other allelochemicals to larval growth of *Heliothis virescens* (Lepidoptera, Noctuidae). *J. Econ. Entomol.* 85:359–364.

KARBAN, R., and CAREY, J. R. 1984. Induced resistance of cotton seedlings to mites. *Science* 225:53–54.

KEELING, C. I., and BOHLMANN, J. 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol.* 170:657–675.

KOCH, T., KRUMM, T., JUNG, V., ENGELBERTH, J., and BOLAND, W. 1999. Differential induction of plant volatile biosynthesis in the lima bean by early and late intermediates of the octadecanoid-signaling pathway. *Plant Physiol.* 121:153–162.

MCAUSLANE, H. J., and ALBORN, H. T. 1998. Systemic induction of allelochemicals in glanded and glandless isogenic cotton by Spodoptera exigua feeding. *J. Chem. Ecol.* 24:399–416.

MCAUSLANE, H. J., and ALBORN, H. T. 2000. Influence of previous herbivory on behavior and development of Spodoptera exigua larvae on glanded and glandless cotton. *Entomol. Exp. Appl.* 97:283–291.

MCAUSLANE, H. J., ALBORN, H. T., and TOTH, J. P. 1997. Systemic induction of terpenoid aldehydes in cotton pigment glands by feeding of larval Spodoptera exigua. *J. Chem. Ecol.* 23:2861–2879.

MCCONKEY, M. E., GERSHENZON, J., and CROTEAU, R. B. 2000. Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. *Plant Physiol.* 122:215–223.

MCKEY, D. 1979. Distribution of secondary compounds in plants, pp. 45–133, in G. A. Rosenthal, and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary MetabolitesAcademic, New York.

MITHÖFER, A., WANNER, G., and BOLAND, W. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137: 1160–1168.

MONTANDON, R., WILLIAMS, H. J., STERLING, W. L., STIPANOVIC, R. D., and VINSON, S. B. 1986. Comparison of the development of *Alabama argillacea* (Hubner) and *Heliothis virescens* (F) (Lepidoptera, Noctuidae) fed glanded and glandless cotton leaves. *Environ. Entomol.* 15:128–131.

OHNMEISS, T. E., and BALDWIN, I. T. 2000. Optimal Defense Theory predicts the ontogeny of an induced nicotine defense. *Ecology* 81:1765–1783.

OPITZ, S., and SCHNEIDER, B. 2002. Organ-specific analysis of phenylphenalenone-related compounds in *Xiphidium caeruleum*. *Phytochemistry* 61:819–825.

OYEDEJI, O. A., and AFOLAYAN, A. 2005. Comparative study of the essential oil composition and antimicrobial activity of *Leonotis leonurus* and *L. ocymifolia* in the Eastern Cape, South Africa. *S. Afr. J. Bot.* 71:114–116.

OZER, H., SOKMEN, M., GULLUCE, M., ADIGUZEL, A., SAHIN, F., SOKMEN, A., KILIC, H., and BARIS, O. 2007. Chemical composition and antimicrobial and antioxidant activities of the essential oil and methanol extract of *Hippomarathrum microcarpum* (Bieb.) from Turkey. *J. Agric. Food Chem.* 55:937–942.

PORTER, A. J. R., MORTON, A. M., KIDDLE, G., DOUGHTY, K. J., and WALLSGROVE, R. M. 1991. Variation in the glucosinolate content of oilseed rape *Brassica napus* L. leaves I. Effect of leaf age and position. *Ann. Appl. Biol.* 118:461–468.

RHOADES, D. F. 1979. Evolution of plant chemical defense against herbivores, pp. 3–54, in G. A. Rosenthal, and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary Metabolites. Academic, New York.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDER, S. J., PARE, P. W., and HENNEBERRY, T. J. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27:679–695.

SCANLON, T. S., and WILLIS, E. D. 1985. Calculation of flame ionization detector relative response factors using the effective carbon number concept. *J. Chromatogr. Sci.* 23:333–340.

SCHMELZ, E. A., ALBORN, H. T., BANCHIO, E., and TUMLINSON, J. H. 2003. Quantitative relationships between induced jasmonic acid levels and volatile emission in *Zea mays* during Spodoptera exigua herbivory. *Planta* 216:665–673.

SEMBDNER, G., and PARTHIER, B. 1993. The biochemistry and the physiological and molecular actions of jasmonates. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 44:569–589.

STIPANOVIC, R. D., BELL, A. A., O'BRIEN, D. H., LUKEFAHR, M. J., and DRAWER, P. O. 1977. Heliocide H2: an insecticidal sesterterpenoid from cotton Gossypium. *Tetrahedron Lett.* 6:567–570.

STIPANOVIC, R. D., BELL, A. A., O'BRIEN, D. H., and LUKEFAHR, M. J. 1978a. Heliocide H1: a new insecticidal 25-carbon terpenoid from cotton *Gossypium hirsutum*. *J. Agric. Food Chem.* 26:115–118.

STIPANOVIC, R. D., BELL, A. A., O'BRIEN, D. H., and LUKEFAHR, M. J. 1978b. Heliocide H3: An insecticidal terpenoid from *Gossypium hirsutum*. *Phytochemistry* 17:151–152.

STIPANOVIC, R. D., ALTMAN, D. W., BEGIN, D. L., GREENBLATT, G. A., and BENEDICT, J. H. 1988. Terpenoid aldehydes in upland cottons analysis by aniline and HPLC methods. *J. Agric. Food Chem.* 36:509–515.

TRAW, M. B., and DAWSON, T. E. 2002. Differential induction of trichomes by three herbivores of black mustard. *Oecologia* 131:526–532.

TURLINGS, T. C. J., BERNASCONI, M., BERTOSSA, R., BIGLER, F., CALOZ, G., and DORN, S. 1998. The induction of volatile emissions in maize by three herbivore species with different feeding habits: Possible consequences for their natural enemies. *Biol. Control* 11:122–129.

VALKAMA, E., KORICHEVA, J., OSSIPOV, V., OSSIPOVA, S., HAUKIOJA, E., and PIHLAJA, K. 2005. Delayed induced responses of birch glandular trichomes and leaf surface lipophilic compounds to mechanical defoliation and simulated winter browsing. *Oecologia* 146:385–393.

VAN POECKE, R. M. P., and DICKE, M. 2002. Induced parasitoid attraction by Arabidopsis thaliana: involvement of the octadecanoid and the salicylic acid pathway. *J. Exp. Bot.* 53:1793–1799.

VAZQUEZ-FLOTA, F., CARRILLO-PECH, M., MINERO-GARCIA, Y., and MIRANDA-HAM, M. D. 2004. Alkaloid metabolism in wounded *Catharanthus roseus* seedlings. *Plant Physiol. Biochem.* 42:623–628.

WITTSTOCK, U., and GERSHENZON, J. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol.* 5:300–307.

ZHANG, J., MACE, M. E., STIPANOVIC, R. D., and BELL, A. A. 1993. Production and fungitoxicity of the terpenoid phytoalexins in cotton: inoculated with *Fusarium oxysporum* f. sp. vasinfectum. *J. Phytopathol.* 139:247–252.

ZIPFEL, C. 2007. The Effect of Bt-Transformation and Various Environmental Factors on the Volatile Emission of Maize: Potential Influence on Direct and Indirect Defense Against Herbivores. PhD dissertation, Friedrich-Schiller-University, Jena.

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Behavioral Responses of Adult *Sitophilus granarius* to Individual Cereal Volatiles

Giacinto S. Germinara · Antonio De Cristofaro · Giuseppe Rotundo

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Abstract The antennae of *Sitophilus granarius* (L.) (Coleoptera: Curculionidae) adults detect a wide variety of compounds in the odor blend of various cereal grains (Germinara et al., *Tec. Molit.*, 53:27–34, 2002). In the present study, we looked at the behavioral responses of the granary weevil to 20 of these individual volatiles (aliphatic alcohols, aldehydes, ketones, and aromatics) in a two-choice pitfall olfactometer, using the aggregation pheromone and propionic acid as the attractant and repellent controls, respectively. Five doses, ranging from 1 μg to 1 mg, of each compound were tested. At least one concentration of eight compounds attracted beetles but required doses 1,000- to 5,000-fold higher than the concentration of aggregation pheromone to elicit a response. Three compounds, while attractive at lower concentrations, acted as repellents at higher doses. Twelve compounds were repellent at concentrations similar to the quantity of propionic acid that significantly repelled beetles. The data show that granary weevil adults have the ability to respond behaviorally to a wide range of cereal volatiles and that responses may change as a function of concentration. The results suggest that host finding behavior of weevils will depend on the balance of positive and negative volatile stimuli from grain as the relative concentrations of volatiles may change during storage. An understanding of how the weevils respond to such changes could be useful for the development of effective integrated pest management strategies.

G. S. Germinara (✉) · A. De Cristofaro · G. Rotundo
Department of Animal, Plant and Environmental Sciences,
University of Molise,
Via De Sanctis,
86100 Campobasso, Italy
e-mail: germinara@unimol.it

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Introduction

The granary weevil, *Sitophilus granarius* (L.) (Coleoptera: Curculionidae), is a serious pest of stored grains worldwide. Infestations not only cause significant losses due to the consumption of grains; they also result in elevated temperature and moisture conditions that lead to an accelerated growth of molds, including toxigenic species (Sauer et al. 1984; Magan et al. 2003). Recent legislation that limits the use of fumigants and broad-spectrum contact insecticides, with increasing consumer demand for safe food, makes the control of such storage pests difficult. Furthermore, as larvae develop within the grains, any effective control strategy requires the early and accurate monitoring of adult weevils.

The use of semiochemicals has stimulated interest, as they may be used to improve current monitoring as well as direct means of controlling stored-product insect pests (Phillips 1997; Cox 2004). In the case of *S. granarius*, there is a male-produced aggregation pheromone, 1-ethylpropyl-(2*S*,3*R*)-3-hydroxy-2-methylpentanoate (Phillips et al. 1989; Chambers et al. 1996) that is attractive to both sexes (Faustini et al. 1982). Phytophagous insects also use volatiles from plant materials to locate suitable substrates (Dickens 1984; Visser 1986), and it has been shown that extracts from carob pods and peanuts (Collins et al. 2004, 2007; Wakefield et al. 2005) as well as specific components attract granary weevil adults (Collins et al. 2007). *S.*

granarius adults orient to volatile blends emitted by grains of several cereal species (Levinson and Kanaujia 1981; Rietdorf and Steidle 2002), and the presence of phagostimulatory compounds are considered crucial in the infestation process by this pest (Kanaujia and Levinson 1981). Male and female *S. granarius* respond to various extracts from stored winter wheat (Levinson and Kanaujia 1982) and to lipid fractions that consist mostly of triacylglycerols and fatty acids (Nawrot et al. 1995).

Despite this behavioral evidence and the identification of many volatiles emitted by grains of several cereal species (Maga 1978; Zhou et al. 1999; Sides et al. 2001), little attention has been given to the response of weevils to individual compounds. Chambers et al. (1996) showed that a wheat extract mixture was electroantennographically (EAG) active but showed no specific peaks of activity in using the combined gas chromatography–electroannographic detection approach. In light of the results, they suggested that the EAG response to the whole extract must be due to small contributions from a large number of components. In previous studies, we observed that both male and female *S. granarius* antennae responded to a wide range of synthetic compounds found in cereal volatiles (Germinara et al. 2002) and that adults were repelled by propionic acid (Germinara et al. 2007). In this study, we examined the behavioral response of granary weevil adults to different doses of individual, EAG-active compounds present in cereal volatiles in the search for candidate semiochemicals useful for weevil pest management.

Materials and Methods

Insects *S. granarius* were reared on whole wheat kernels for several generations in continuous dark at 23±2°C and 60±5% relative humidity (r.h.). Adult beetles, of mixed sex and age, were used for the experiments.

Odor Stimuli Test compounds (Table 1; Sigma-Aldrich, Milan, Italy) were selected based on their presence in cereal grains (Maga 1978; Zhou et al. 1999) and their EAG activity to granary weevil adults (Germinara et al. 2002). Solutions (decimal dilutions from 100 to 0.1 μg/μl) were prepared with mineral oil (Sigma-Aldrich) to reduce the rate of evaporation.

Solutions (0.1 ng/μl to 5 μg/μl) of the aggregation pheromone 1-ethylpropyl-(2*S*,3*R*)-3-hydroxy-2-methylpentanoate (Novapher, San Donato Milanese, Italy; chemical purity 91%) and propionic acid (100 μg/μl; Sigma-Aldrich; chemical purity >99.9%) were used as positive controls for attractant and repellent compounds, respectively. Solutions were stored at −20°C until needed.

Table 1 Chemical purity of synthetic compounds known to occur in natural cereal volatiles that were tested in a behavioral assay using the granary weevil, *S. granarius*

Compound	Purity (%)
Aliphatic alcohols	
1-Butanol	99
1-Pentanol	99
1-Hexanol	99
3-Methyl-1-butanol	99
Aliphatic aldehydes	
Butanal	99
Pentanal	99
Hexanal	98
Heptanal	95
(*E*)-2-Hexenal	95
(*E*,*E*)-2,4-Heptadienal	88
(*E*,*E*)-2,4-Nonadienal	85
(*E*,*E*)-2,4-Decadienal	85
Aliphatic ketones	
2-Pentanone	99
2-Hexanone	99
2-Heptanone	98
2,3-Butanedione	97
Aromatics	
3-Methoxy-2-methyl-4-pyrone (maltol)	99
Furfural	99
Phenylacetaldehyde	90
3-Methoxy-4-hydroxy-benzaldehyde (vanillin)	97

Pitfall Bioassays The behavioral response of *S. granarius* adults to individual odor stimuli was measured by using a two-choice pitfall bioassay, similar to those described by Phillips et al. (1993) and Pike et al. (1994). The test arena was a steel container (32 cm diameter×7 cm high) with two diametrically opposed holes (3 cm diameter) located 3 cm from the side wall. Test or control (10 μl) stimuli were adsorbed onto a filter paper disk (0.7 cm diameter) suspended at the center of each hole by a cotton thread taped to the lower surface of the arena. Glass flasks (500 ml) were positioned under each hole, and the inside surfaces of their necks were coated with mineral oil to prevent captured insects from returning to the arena. The floor of the arena was covered with filter paper (Whatman No. 1) to provide a uniform surface and to facilitate insect movements (Pike et al. 1994). Twenty insects, deprived of food for at least 4 h, were placed under an inverted Petri dish (3 cm diameter×1.2 cm high) at the center of the arena and allowed 30 min to acclimate. They were then released and tested for 3 h in the dark at 25±2°C and 60±5% r.h. Experiments were carried out between 10:00 A.M. and 4:00 P.M. During the assay, the arena was covered with a steel lid to prevent insects from escaping. Insects were given a choice between a specific dose of the test stimulus

(1, 10, 100, 500, and 1,000 μg) and mineral oil, used as a control. Propionic acid was tested only at 1,000 μg, as this concentration has a strong repellent effect to adult granary (Germinara et al. 2007). To determine the optimal dose for insect attraction to the aggregation pheromone in our experimental device, we tested 0.001, 0.005, 0.05, 0.1, 0.25, 0.5, 5.0, and 50 μg. There were six replicates of each assay, and insects were only used once.

In each trial, a response index (RI) was calculated by using $RI=[(T-C)/Tot]\times 100$, where T is the number responding to the treatment, C is the number responding to the control, and Tot is the total number of insects released (Phillips et al. 1993). Positive values of RI indicate attraction to the treatment, while negative ones indicate repellence. The significance of the mean RI in each treatment of the two-choice pitfall bioassay was evaluated by the Student's t test for paired comparisons (Phillips et al. 1993). Data were submitted to linear regression analysis to evaluate the effect of the dose on the insects' response. The most significant positive or negative mean values of RI were first analyzed by an analysis of variance and subsequently ranked by using the least significant difference (LSD) multiple range test ($P=0.05$).

Results

The control repellent propionic acid 1 mg dose gave a significant RI (−57.2±2.9; $P<0.001$). The aggregation pheromone elicited significant positive responses from 5 to 250 ng, whereas it induced a significant negative response at 50 μg (Fig. 1).

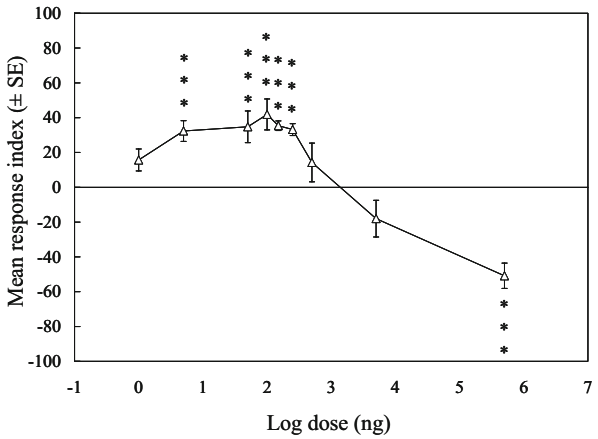

Fig. 1 Response of *S. granarius* to increasing doses of 1-ethylpropyl-(2*S*,3*R*)-3-hydroxy-2-methylpentanoate (aggregation pheromone) in two-choice pitfall bioassays. $N=6$. Significant response to experimental stimulus: *** $P<0.001$ (paired-sample t test)

Of the four aliphatic alcohols tested, 1-butanol and 3-methyl-1-butanol elicited significant positive responses at the 500 μg dose, while 1-hexanol elicited a significant negative response at the three highest doses tested (Table 2). 1-Pentanol was an attractant between 1 and 100 μg but was a repellent at 1 mg (Table 2). Pentanal was the only aliphatic aldehyde that caused significant attraction (at 100 and 500 μg), whereas six others had a repellent effect, particularly at high doses. (*E*,*E*)-2,4-Heptadienal was an attractant at 10 and 100 μg, whereas it elicited significant negative responses at the two highest doses (Table 2).

The four aliphatic ketones acted as significant repellents for granary weevil adults over the dose range tested, with the level of repellency generally increasing with an increase in dose (Table 2).

The aromatics 3-methoxy-4-hydroxy-benzaldehyde (vanillin), 3-methoxy-2-methyl-4-pyrone (maltol), and phenylacetaldehyde were attractants, but the concentrations that elicited a significant response varied depending on the compound (Table 2). In contrast, phenylacetaldeyde and furfural significantly repelled beetles at the highest dose tested.

In the case of compounds that induced significant positive or negative responses, regression analyses showed a dose-dependent relationship for eight of those with repellent properties but none for those that attracted *S. granarius* (Table 2). Both the highest positive ($F=3.57$; $df=8$; $P<0.01$; Table 3) and negative ($F=3.95$; $df=15$; $P<0.001$; Table 4) RI values differed among attractant and repellent compounds.

Discussion

Our results show that many cereal volatiles, known to be detected by antennal sensilla of *S. granarius* (Germinara et al. 2002), also elicit a significant behavioral response at one or more of the doses tested. Over the range of concentrations tested, five compounds (1-butanol, 3-methyl-1-butanol, pentanal, maltol, and vanillin) acted uniquely as attractants, while twelve [1-hexanol, butanal, hexanal, heptanal, (*E*)-2-hexenal, (*E*,*E*)-2,4-nonadienal, (*E*,*E*)-2,4-decadienal, 2,3-butanedione, 2-pentanone, 2-hexanone, 2-heptanone, and furfural) acted only as repellents. However, in the case of 1-pentanol, (*E*,*E*)-2,4-heptadienal, and phenylacetaldehyde, granary weevils were attracted to low concentrations but repelled by higher ones, thus showing the ability of the species to detect and respond differentially to variations in concentration of host volatiles.

The number of compounds present in the volatiles emitted from stored grains, as well as their concentrations,

Table 2 Response of adult *S. granarius* to increasing doses of some cereal volatiles in two-choice pitfall bioassays, determination coefficient (R^2) of log (dose)–response linear regression analysis and its significance (P)

Compound	Response Index (Mean±SE)[a]					R^2	P
	1 μg	10 μg	100 μg	500 μg	1,000 μg		
Aliphatic alcohols							
1-Butanol	−10.7±13.2	−16.8±8.9	11.4±6.8	15.0±2.7**	0.9±8.9	0.53	0.162
1-Pentanol	17.7±4.8*	14.2±2.0***	18.5±5.5*	11.3±8.1	−19.6±7.2*	0.41	0.247
1-Hexanol	0.7±9.5	11.2±5.8	−20.6±7.8*	−20.4±6.9*	−27.5±8.6*	0.72	0.070
3-Methyl-1-butanol	−8.5±12.5	2.5±6.2	4.6±11.1	23.7±5.8*	6.9±9.2	0.63	0.110
Aliphatic aldehydes							
Butanal	2.7±6.2	−11.4±9.2	−22.4±12.4	−29.3±10.1*	−28.6±7.7*	0.98	0.002
Pentanal	−1.1±6.3	−3.6±8.2	24.2±9.3*	21.0±3.7**	−0.9±7.5	0.20	0.450
Hexanal	1.0±9.0	−22.3±7.4*	−24.0±9.1*	−25.6±3.5***	−35.8±5.1***	0.82	0.034
Heptanal	−2.7±4.3	10.6±8.9	−11.2±7.6	−22.9±6.7*	−40.3±9.8**	0.67	0.091
(*E*)-2-Hexenal	−1.0±3.0	−15.8±5.6*	−17.4±4.8*	−62.7±7.2***	−52.2±6.9***	0.83	0.032
(*E*,*E*)-2,4-Heptadienal	6.2±5.1	21.8±7.1*	43.2±5.2***	−40.4±4.9**	−34.2±11.3*	0.30	0.341
(*E*,*E*)-2,4-Nonadienal	12.9±9.6	−10.1±8.6	−9.5±2.9	−34.5±7.7*	−63.0±6.4***	0.82	0.034
(*E*,*E*)-2,4-Decadienal	−14.4±7.5	−19.9±5.5*	−39.5±2.9***	−39.8±7.4**	−51.6±4.4***	0.93	0.008
Aliphatic ketones							
2-Pentanone	1.0±5.7	12.5±8.3	−34.8±3.9***	−35.7±7.3**	−53.6±8.1***	0.79	0.045
2-Hexanone	−5.0±8.8	9.9±4.0	−20.0±2.2***	−30.7±5.8**	−39.2±5.1***	0.71	0.072
2-Heptanone	7.6±12.9	−18.4±4.9*	−23.3±8.0*	−43.7±7.9**	−52.5±4.1***	0.95	0.005
2,3-Butanedione	9.0±6.4	4.1±7.4	−15.0±11.7	−26.8±9.9	−46.0±4.2***	0.90	0.014
Aromatics							
Maltol	−7.0±9.7	16.2±5.3*	19.7±6.1*	31.1±5.3**	11.9±4.6*	0.53	0.164
Furfural	6.8±8.0	8.6±4.7	−5.0±3.7	6.0±6.8	−48.0±6.9**	0.39	0.259
Phenylacetaldehyde	10.1±4.8	11.2±2.3**	21.2±5.0**	−7.0±4.1	−25.7±6.0**	0.42	0.237
Vanillin	7.0±5.9	4.7±3.2	9.8±7.9	34.7±6.9**	−12.2±6.0	0.003	0.926

*P<0.05; **P<0.01; ***P<0.001 (significant response to experimental stimulus; paired-sample t test)
[a] N=6

will change over time (Zhou et al. 1999; Sides et al. 2001). Thus, viewed from an ecological perspective, our results should be considered within the context of resource utilization by the granary weevil. Some of the volatiles that attract *S. granarius* have been identified in the aroma of freshly broken grains of several cereal species (Maga 1978) and, consequently, may act as kairomones. For example, pentanal, 1-pentanol, and phenylacetaldehyde, all of which attracted beetles, increase with time following storage (Heiniö et al. 2002). Similarly, the attractant vanillin could result as a biotransformation product of ferulic acid (Kavitha et al. 2005), while (*E*,*E*)-2,4-heptadienal could be an oxidation product of linolenic acid (Nielsen et al. 2004), a minor fatty acid in wheat (Morrison 1987). Thus, once these volatiles increase above a certain concentration, they act as attractants, which could explain the preference for aged wheat kernels over fresh ones (Levinson and Kanaujia 1981). It is of interest to note that, while higher concentrations of host volatiles are required to elicit a response from granary beetles, as seems also the case for *Oryzaephilus surinamensis* (L.) and *Oryzaephilus mercator* (Fauvel) (Pierce et al. 1990), the RIs for (*E*,*E*)-2,4-heptadienal, vanillin, and maltol at their most attractive dose were similar to that of the aggregation pheromone (Table 3). A number of

Table 3 Comparison of the highest mean response indexes of adult *S. granarius* to the attractant compounds

Compound	Dose (μg)	Response Index[a] (Mean±SE)
1-Pentanol	10	14.2±2.0 a
1-Butanol	500	15.0±2.7 a
Pentanal	500	21.0±3.7 ab
Phenylacetaldehyde	100	21.2±5.0 ab
3-Methyl-1-butanol	500	23.7±5.8 ab
Maltol	500	31.1±5.3 abc
Vanillin	500	34.7±6.9 bc
Aggregation pheromone	0.1	41.9±8.9 c
(*E*,*E*)-2,4-Heptadienal	100	43.2±5.2 c

Means with the same letter are not significantly different at P=0.05 by LSD test for multiple comparisons.
[a] N=6

Table 4 Comparison of the lowest mean response indexes of adult *S. granarius* to the repellent compounds

Compound	Dose (μg)	Response Index[a] (Mean±SE)
1-Pentanol	1,000	−19.6±7.2 a
Phenylacetaldehyde	1,000	−25.7±6.0 ab
1-Hexanol	1,000	−27.5±8.6 ab
Butanal	500	−29.3±10.1 ab
Hexanal	1,000	−35.8±5.1 abc
2-Hexanone	1,000	−39.2±5.1 bcd
Heptanal	1,000	−40.3±9.8 bcde
(*E*,*E*)-2,4-Heptadienal	500	−40.4±4.9 bcde
2,3-Butanedione	1,000	−46.0±4.2 cde
Furfural	1,000	−48.0±6.9 cdef
(*E*,*E*)-2,4-Decadienal	1,000	−51.6±4.4 cdef
2-Eptanone	1,000	−52.5±4.1 cdef
2-Pentanone	1,000	−53.6±8.1 def
Propionic acid	1,000	−57.2±2.9 ef
(*E*)-2-Hexenal	500	−62.7±7.2 f
(*E*,*E*)-2,4-Nonadienal	1,000	−63.0±6.4 f

Means with the same letter are not significantly different at $P=0.05$ by LSD test for multiple comparisons.
[a] $N=6$

these compounds may also be used as kairomones by other species: pentanal, vanillin, and maltol are attractive to *Sitophilus oryzae* (L.) (Phillips et al. 1993); pentanal attracts *O. surinamensis* and *O. mercator* (Pierce et al. 1990), whereas phenylacetaldehyde is an attractant for several Lepidopteran species (El-Sayed 2006), including *Ephestia cautella* (Walker) and *Plodia interpunctella* (Hübner) (Olsson et al. 2005), as well as *Ephestia kuehniella* Zeller (Olsson et al. 2006).

3-Methyl-1-butanol has been implicated as a kairomone for other stored-product insects, such as the larvae of *Tribolium castaneum* (Herbst) (Sinha 1990), adults of *O. surinamensis*, *O. mercator*, *Cryptolestes ferrugineus* (Stephens), *Ahasverus advena* (Waltl), *Cathartus quadricollis* (Guérin) (Pierce et al. 1991), and *E. kuehniella* (De Cristofaro et al. 2000). Unlike Sinha 1990, we also found that it attracted the granary weevil. This difference may be explained by the fact that we tested lower concentrations. This observation, together with the fact that certain compounds, similarly to the aggregation pheromone, are attractants at low concentrations but have a repellent effect at higher ones (Table 2), highlights the necessity of testing a range of doses.

Similarly, repellent compounds may serve as reliable cues of habitat quality for the granary weevil. The production of hexanal, commonly used as an indicator of lipid oxidation in cereals (Piggot et al. 1991), increases over time in stored native oats (Heiniö et al. 2002) and raw oat flour (Molteberg et al. 1996). There will also be an increase of (*E*,*E*)-2,4-decadienal and (*E*,*E*)-2,4-nonadienal due to the oxidation of linoleic acid, the major fatty acid of wheat lipids (Grosh and Schieberle 1991). Similarly, 2-heptanone is the major ketone formed when raw oat flour is stored (Molteberg et al. 1996) and, along with other ketones, is a typical metabolite of mold-infested cereals (Pasanen et al. 1996; Magan and Evans 2000). The idea that these could provide reliable cues for *S. granarius* is supported by the observed positive correlations between the concentration and repellency (Table 2). Furthermore, the optimal doses of (*E*,*E*)-2,4-nonadienal, (*E*)-2-hexenal, 2-pentanone, 2-heptanone, (*E*,*E*)-2,4-decadienal, and furfural were as effective as the repellent control, propionic acid.

The results reported here are of significance from a management perspective. Specific blends of synthetic attractants may be used to monitor and/or trap the granary weevil. These could be deployed alone or in combination with the aggregation pheromone of *S. granarius* as (1) synergistic or additive effects have been reported for several species (Landolt and Phillips 1997), including the congeners *Sitophilus zeamais* (Walgenbach et al. 1987) and *S. oryzae* (Phillips et al. 1993), and (2) some olfactory cells may respond to both host-plant volatiles and pheromones (De Cristofaro et al. 2004; Ansebo et al. 2005). An alternative method may be to use repellent compounds to mask and/or alter odors emitted from stored grains to reduce the granary beetle's ability to detect suitable food and oviposition sites.

The results of this study suggest that host finding by the granary weevil is a complex process that depends on the balance of positive and negative stimuli that change over time. Consequently, a clear understanding of the biological activity of different cereal volatiles, individually and in combination, will be essential for developing semiochemical-based granary weevil management strategies. Furthermore, as noted above, considerable attention will not only have to be given to the selection of the actual compounds but also to the specific concentration to be used. Furthermore, one must also consider the impact that these blends might have on other species that utilize the same resources to ensure that compounds to repel one pest is not an attractant for another. For example, while hexanal, heptanal, and (*E*,*E*)-2,4-nonadienal (at low doses) repel the granary weevil, they have been reported as attractants for *O. surinamensis* and *O. mercator*, two secondary pests of stored grain and processed cereals (Pierce et al. 1990). Such differential responses to the same semiochemicals that originate from a heterogeneous food substrate undoubtedly play an important role in niche partitioning within the stored-grain ecosystem (Phillips et al. 1993). However, a failure to understand these interactions when deploying lures to control one species may lead to an increase in the density of other pests.

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References

ANSEBO, L., IGNELL, R., LÖFQVIST, J., and HANSSON, B. 2005. Responses to sex pheromone and plant odours by olfactory receptor neurons housed in sensilla auricillica of the codling moth, *Cydia pomonella* (Lepidoptera: Tortricidae). *J. Insect. Physiol.* 51:1066–1074.

CHAMBERS, J., VAN WYK, C. B., WHITE, P. R., GERRARD, C. M., and MORI, K. 1996. Grain weevil, *Sitophilus granarius* (L.): antennal and behavioral responses to male-produced volatiles. *J. Chem. Ecol.* 22:1639–1654.

COLLINS, L. E., WAKEFIELD, M. E., CHAMBERS, J., and COX, P. D. 2004. Progress towards a multi-species lure: comparison of behavioural bioassay methods for multi-species attractants against three pests of stored grain. *J. Stored Prod. Res.* 40:341–353.

COLLINS, L. E., BRYNING, G. P., WAKEFIELD, M. E., CHAMBERS, J., and COX, P. D. 2007. Progress towards a multi-species lure: identification of components of food volatiles as attractants for three storage beetles. *J. Stored Prod. Res.* 43:53–63.

COX, P. D. 2004. Potential for using semiochemicals to protect stored products from insect infestation. *J. Stored Prod. Res.* 40:1–25.

DE CRISTOFARO, A., ROTUNDO, G., and GERMINARA, G. S. 2000. Electrophysiological and olfactory responses of *Ephestia kuehniella* Zeller adults to cereals' semiochemicals. *IOBC-WPRS Bull.* 23:189–194.

DE CRISTOFARO, A., IORIATTI, C., PASQUALINI, E., ANFORA, G., GERMINARA, G. S., VILLA, M., and ROTUNDO, G. 2004. Electrophysiological responses of codling moth, *Cydia pomonella* (L.) (Lepidoptera Tortricidae), to codlemone and pear ester ethyl (*E*,*Z*)-2,4-decadienoate: peripheral interactions in their perception. *Bull. Insectol.* 52:137–144.

DICKENS, J. C. 1984. Olfaction in the boll weevil *Anthonomus grandis* Boh (Coleoptera: Curculionidae): electroantennogram studies. *J. Chem. Ecol.* 10:1759–1785.

EL-SAYED, A. M. 2006. The Pherobase: Database of Insect Pheromones and Semiochemicals. <http://www.pherobase.com>.

FAUSTINI, D. L., GIESE, W. L., PHILLIPS, J. K., and BURKHOLDER, W. E. 1982. Aggregation pheromone of the male granary weevil, *Sitophilus granarius* (L.). *J. Chem. Ecol.* 8:679–687.

GERMINARA, G. S., ROTUNDO, G., DE CRISTOFARO, A., and GIACOMETTI, R. 2002. Risposte elettroantennografiche di *Sitophilus granarius* (L.) e *S. zeamais* Motschulsky a sostanze volatili dei cereali. *Tec. Molit.* 53:27–34.

GERMINARA, G. S., ROTUNDO, G., and DE CRISTOFARO, A. 2007. Repellence and fumigant toxicity of propionic acid against adults of *Sitophilus granarius* (L.) and *S. oryzae* (L.). *J. Stored Prod. Res.* 43:229–233.

GROSH, W., and SCHIEBERLE, P. 1991. Bread, pp. 41–77, in E. Maarse (ed.). Volatile Compounds in Food and Beverages. Marcel Dekker, New York.

HEINIÖ, R.-L., LEHTINEN, P., OKSMAN-CALDENTEY, K.-M., and POUTANEN, K. 2002. Differences between sensory profiles and development of rancidity during long-term storage of native and processed oat. *Cereal Chem.* 79:367–375.

KANAUJIA, K. R., and LEVINSON, H. Z. 1981. Phagostimulatory responses and oviposition behaviour of *Sitophilus granarius* L. to newly harvested and stored wheat grains. *Z. Angew. Entomol.* 91:417–424.

KAVITHA, S., SHYAMALA, H., MURALIKRISHNA, G., VARADARAJ, M. C., and RATI RAO, E. 2005. Starch and cell wall degrading enzymes from fungal organisms grown on cereal and millet brans. *Eur. Food Res. Technol.* 220:560–564.

LANDOLT, P. J., and PHILLIPS, T. W. 1997. Host plant influence on sex pheromone behavior of phytophagous insects. *Annu. Rev. Entomol.* 42:371–391.

LEVINSON, H. Z., and KANAUJIA, K. R. 1981. Phagostimulatory responses of male and female *Sitophilus granarius* to newly harvested and stored wheat grains. *Naturwissenschaften* 68:44.

LEVINSON, H. Z., and KANAUJIA, K. R. 1982. Feeding and oviposition behaviour of the granary weevil (*Sitophilus granarius* L.) induced by stored wheat, wheat extracts and dummies. *Z. Angew. Entomol.* 93:292–305.

MAGA, J. A. 1978. Cereal volatiles: a review. *J. Agric. Food Chem.* 26:175–178.

MAGAN, N., and EVANS, P. 2000. Volatiles as an indicator of fungal activity and differentiation between species, and the potential use of electronic nose technology for early detection of grain spoilage. *J. Stored Prod. Res.* 36:319–340.

MAGAN, N., HOPE, R., CAIRNS, V., and ALDRED, D. 2003. Post-harvest fungal ecology: impact of fungal growth and mycotoxin accumulation in stored grain. *Eur. J. Plant Pathol.* 109:723–730.

MOLTEBERG, E. L., MAGNUS, E. M., BJØRGE, J. M., and NILSSON, A. 1996. Sensory and chemical studies of lipid oxidation in raw and heat-treated oat flours. *Cereal Chem.* 73:579–587.

MORRISON, W. R. 1987. Wheat lipid composition. *Cereal Chem.* 55:548–558.

NAWROT, J., WINIECKI Z., SZAFRANEK J., MALINSKI E., and HARMATHA, J. 1995. Search for natural antifeedants and attractants for stored product pests, pp. 149–164, in Council of Europe (ed.). Proceedings of the International Forum on Stored Product Protection and Post-Harvest Treatment of Plant Products, Strasbourg, France.

NIELSEN, G. S., LARSEN, L. M., and POLL, L. 2004. Formation of volatile compounds in model experiments with crude leek (*Allium ampelprasum* var. Lancelot) enzyme extract and linoleic acid or linolenic acid. *J. Agric. Food Chem.* 52:2315–2321.

OLSSON, P.-O. C., ANDERBRANT, O., LÖFSTEDT, C., BORG-KARLSON, A.-K., and LIBLIKAS, I. 2005. Electrophysiological and behavioural responses to chocolate volatiles in both sexes of the pyralid moths, *Ephestia cautella* and *Plodia interpunctella*. *J. Chem. Ecol.* 31:2947–2961.

OLSSON, P.-O. C., ANDERBRANT, O., and LÖFSTEDT, C. 2006. Attraction and oviposition of *Ephestia kuehniella* induced by volatiles identified from chocolate products. *Entomol. Exp. Appl.* 119:137–144.

PASANEN, A.-L., LAPPALAINEN, S., and PASANEN, P. 1996. Volatile organic metabolites associated with some toxic fungi and their mycotoxins. *Analyst* 121:1949–1953.

PHILLIPS, T. W. 1997. Semiochemicals of stored-product insects: research and applications. *J. Stored Prod. Res.* 33:17–30.

PHILLIPS, J. K., CHONG, J. M., ANDERSEN, J. F., and BURKHOLDER, W. E. 1989. Determination of the enantiomeric composition of (*R**, *S**)-1-ethylpropyl 2-methyl-3-hydroxypentanoate, the male pro-duced aggregation pheromone of *Sitophilus granarius*. *Entomol. Exp. Appl.* 51:149–153.

PHILLIPS, T. W., JIANG, X.-L., BURKHOLDER, W. E., PHILLIPS, J. K., and TRAN, H. Q. 1993. Behavioral responses to food volatiles by two species of stored-product Coleoptera, *Sitophilus oryzae* (Curculionidae) and *Tribolium castaneum* (Tenebrionidae). *J. Chem. Ecol.* 19:723–734.

PIERCE, A. M., PIERCE, H. D. Jr, OEHLSCHLAGER, A. C., and BORDEN, J. H. 1990. Attraction of *Oryzaephilus surinamensis* (L.) and *Oryzaephilus mercator* (Fauvel) (Coleoptera: Cucujidae) to some common volatiles of food. *J. Chem. Ecol.* 16:465–475.

PIERCE, A. M., PIERCE, H. D., BORDEN, J. H., and OEHLSCHLAGER, A. C. 1991. Fungal volatiles: semiochemicals for stored-product beetles (Coleoptera: Cucujidae). *J. Chem. Ecol.* 17:581–597.

PIKE, V., SMITH, J. L., WHITE, R. D., and HALL, D. R. 1994. Studies on responses of stored-product pests, *Prostephanus truncatus* (Horn) and *Sitophilus zeamais* Motsch., to food volatiles, pp. 566–569, in E. Highley, E. J. Wright, H. J. Banks, and B. R. Champ (eds.). Proceedings of the Sixth International Working Conference on Stored Product Protection, Canberra, Australia-CAB International, Wallingford, Oxon.

PIGGOT, J. R., MORRISON, W. R., and CLYNE, J. 1991. Changes in lipids and in sensory attributes on storage of rice milled to different degrees. *Int. J. Food Sci. Technol.* 26:615–628.

RIETDORF, K., and STEIDLE, J. L. M. 2002. Was Hopkins right? Influence of larval and early adult experience on the olfactory response in the granary weevil *Sitophilus granarius* (Coleoptera, Curculionidae). *Physiol. Entomol.* 27:223–227.

SAUER, D. B., STOREY, C. L., and WALKER, D. E. 1984. Fungal populations in US farming-stored grain and their relationship to moisture, storage time, regions and insect infestation. *Phytopathology* 74:1050–1053.

SIDES, A., ROBARDS, K., HELLIWELL, S., and AN, M. 2001. Changes in the volatile profiles of oats induced by processing. *J. Agric. Food Chem.* 49:2125–2130.

SINHA, R. N. 1990. Role of odor volatiles in the stored grain ecosystem, pp. 3–13, in F. Fleurat Lessard, and P. Ducom (eds.). Proceedings of the Fifth International Working Conference on Stored Product Protection, Bordeaux, France. Imprimerie du Médoc, Bordeaux-Blanquefort, France.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 37:141–172.

WAKEFIELD, M. E., BRYNING, G. P., and CHAMBERS, J. 2005. Progress towards a lure to attract three stored product weevils, *Sitophilus zeamais* Motschulsky, *S. oryzae* (L.) and *S. granarius* (L.) (Coleoptera: Curculionidae). *J. Stored Prod. Res.* 41:145–161.

WALGENBACH, C. A., BURKHOLDER, W. E., CURTIS, M. J., and KHAN, Z. A. 1987. Laboratory trapping studies with *Sitophilus zeamais* (Coleoptera: Curculionidae). *J. Econ. Entomol.* 80:763–767.

ZHOU, M., ROBARDS, K., GLENNIE-HOLMES, M., and HELLIWELL, S. 1999. Analysis of volatiles compounds and their contribution to flavor in cereals. *J. Agric. Food Chem.* 47:3941–3953.

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Enantiospecific Effect of Pulegone and Pulegone-Derived Lactones on *Myzus persicae* (Sulz.) Settling and Feeding

Katarzyna Dancewicz · Beata Gabrys · Iwona Dams · Czesław Wawrzeńczyk

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Abstract The effect of pulegone chiral center configuration on its antifeedant activity to *Myzus persicae* was examined. Biological consequences of structural modifications of (*R*)-(+)- and (*S*)-(−)-pulegone, the lactonization, iodolactonization, and incorporation of hydroxyl and carbonyl groups were studied, as well. The most active compounds were (*R*)-(+)-pulegone (**1a**) and δ-hydroxy-γ-spirolactones (5*S*,6*R*,8*S*)-(−)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one (**5b**) and (5*R*,6*S*,8*S*)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one **(6b)** derived from (*S*)-(−)-pulegone (**1b**). The compounds deterred aphid probing and feeding at preingestional, ingestional, and postingestional phases of feeding. The preingestional effect of (*R*)-(+)-pulegone (**1a**) was manifested as difficulty in finding and reaching the phloem (i.e., prolonged time preceding the first contact with phloem vessels), a high proportion of probes not reaching beyond the mesophyll layer before first phloem phase, and/or failure to find sieve elements by 20% of aphids during the 8-hr experiment. The ingestional activity of (*R*)-(+)-pulegone (**1a**) and hydroxylactones **5b** and **6b** resulted in a decrease in duration of phloem sap ingestion, a decrease in the proportion of aphids with sustained sap ingestion, and an increase in the proportion of aphid salivation in phloem. δ-Keto-γ-spirolactone (5*R*,8*S*)-(−)-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2,6-dione **(8b)** produced a weak ingestional effect (shortened phloem phase). The postingestional deterrence of (*R*)-(+)-pulegone **(1a)** and δ-hydroxy-γ-spirolactones (5*R*,6*S*,8*R*)-(+)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]-decan-2-one (**5a**), **5b**, (5*S*,6*R*,8*R*)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one (**6a**), **6b**, and δ-keto-γ-spirolactone **8b** prevented aphids from settling on treated leaves. The *trans* position of methyl group CH_3–8 and the bond C5–O1 in lactone **6b** appeared to weaken the deterrent activity in relation to the *cis* diastereoisomer (**5b**).

Keywords Antifeedant · Pulegone · Chirality · Peach potato aphid · Probing behavior · EPG

K. Dancewicz · B. Gabrys (✉)
Department of Biology, University of Zielona Gora,
Szafrana 1,
65-516 Zielona Gora, Poland
e-mail: B.Gabrys@wnb.uz.zgora.pl

I. Dams · C. Wawrzeńczyk
Department of Chemistry,
Wroclaw University of Environmental and Life Sciences,
Norwida 25,
50–375 Wroclaw, Poland

Introduction

Substances that alter insect behavior have attracted much attention as potential crop protection compounds that, at least in part, might replace traditional pesticides (Unelius et al. 2006); they comprise repellents, oviposition inhibitors, antifeedants, etc. The most widely known antifeedants belong to different chemical groups and come from natural sources (Ley and Toogood 1990; Wawrzyniak 1996; Simmonds 1998). Plant terpenoids are one of the major classes of secondary metabolites synthesized by plants that are toxic, unpalatable, or at least repellent to herbivores. As such, they function as defensive agents (Pickett 1991; Harrewijn et al. 2001; Wittstock and Gershenzon 2002). Discovery of insect control substances include, among others, ajugarin, azadirachtin, and polygodial. The sesquiterpenoid polygodial was successfully applied in the field against bird cherry-oat aphid *Rhopalosiphum padi*. It gave results similar to those obtained with the broad-spectrum

pyrethroid cypermethrin (Pickett et al. 1994). Monoterpenoids are components of many plant essential oils and often show insecticidal, feeding deterrent, and repellent activities (Simmonds 1998). Citral appeared toxic to Mediterranean fruit fly *Ceratitis capitata*, American cockroach *Periplaneta americana*, and housefly *Musca domestica* (Salvatore et al. 2004; Choi et al. 2006; Price and Berry 2006) and repellent to termites *Coptotermes formosanus* (Zhu et al. 2001) and mosquitoes *Aedes aegypti* (Oyedele et al. 2002).

Annual world crop loss due to aphids is estimated at 2% of all losses attributed to insect feeding (Wellings et al. 1989). Apart from removing vital fluids from plant sieve elements, aphids are effective vectors of virus diseases: approximately 60% of all plant viruses are spread by aphids. According to Blackman and Eastop (1985), the peach aphid *Myzus persicae* (Sulz.) can infest plants belonging to over 40 different families that include many economically important ones, and it has the ability to transmit more than 100 plant viruses. Elimination or at least reduction of penetration of plant tissues by aphids may save plants from infection by pathogenic agents (Martin et al. 1997).

Aphids respond to many monoterpenoids. The repellent properties of linalool and α-terpineol to *Myzus persicae* were reported by Hori (1998, 1999). Gutierrez et al. (1997) found that geraniol inhibited *Myzus persicae* settling on host plants. (*S*)-Limonene restrained phloem sap ingestion and had other negative effects on the behavior of the peach potato aphid (Halarewicz-Pacan et al. 2003). Citral and linalool had repellent effects that were manifested in a significant decrease in time spent on leaves, decreased total and mean time of penetration, and lower numbers of probes compared to controls. Citral, linalool, (*S*)-limonene, α-ionone, and camphene reduced the total and mean probing time of aphids and settling on the leaves (Gabrys et al. 2005).

Our interest in pulegone and its likely antifeedant effect on *Myzus persicae* was inspired by reports on the compound's broad spectrum biological activity. Pugelone is repellent to birds, including the common starling *Sturnus vulgaris*, red-winged blackbird *Agelaius phoeniceus*, and Northern bobwhite *Colinus virginianus* (Avery et al. 1996; Mason and Epple 1998). It is also toxic to the German cockroach *Blatella germanica*, *Musca domestica*, and storage pests [rice weevil *Sitophilus oryzae*, red flour beetle *Tribolium castaneum*, and the sawtoothed grain beetle *Oryzaephilus surinamensis* (Franzios et al. 1997; Lee et al. 2003)]. Pulegone downgrades reproduction and development of the pea aphid *Acyrthosiphon pisum*, probably by killing aphid symbionts *Buchnera* sp., which is attributed to its bactericidal activity (Harrewijn et al. 2001). The fungicidal effect of pulegone has also been reported (Gata-Gonçalves et al. 2003).

Pulegone is the major component of essential oils of several species of *Mentha* (e.g., *M. pulegium*, *M. arvensis*, *M. longifolia*, and *M. spicata*) and occurs in two isomeric forms: (*R*)- and (*S*)-pulegone (Phatak and Heble 2002; Vetere et al. 2002; Conover and Lyons 2005). The enantiomers of a chiral compound may differ significantly in respect to their interaction with biological receptors, thus resulting in a different biological activity (Juza et al. 2000). The biological activity depends not only on the stereochemistry but is related also to the presence of various functional groups. Often, natural antifeedants are lactones with one or more additional functional groups (Ley and Toogod 1990; Wawrzeńczyk et al. 1997). From the practical point of view, the use of plant-derived antifeedants on a large scale is not justified economically. Synthetic analogs of natural compounds are more accessible for application.

The aim of this work was to assess the effect of pulegone chiral center configuration on its antifeedant activity to *Myzus persicae* and to study the biological consequences of structural modifications of (*R*)-(+)- and (*S*)-(−)-pulegone: the lactonization, iodolactonization, and incorporation of hydroxy and carbonyl groups. The biological study included various aspects of aphid host plant selection and acceptance processes. The behavioral responses of *Myzus persicae* to pure and structurally modified pulegone enantiomers were investigated to reveal the biological basis of their deterrent activity.

Methods and Materials

Chemicals (*R*)-(+)-Pulegone (>98%), (*S*)-(−)-pulegone (>99%), (1*S*,2*R*,5*S*)-(+)-isopulegol (>99%), and (1*R*,2*S*,5*R*)-(−)-isopulegol (>99%) were purchased from Aldrich and Fluka. Test lactones **3**–**8** (Fig. 1) were synthesized from optically pure isomers of pulegone and isopulegol. The synthetic methodology for compounds **3**–**6**, based on the orthoacetate modification of the Claisen rearrangement and iodolactonization of γ,δ-unsaturated acids or acidic lactonization of epoxy esters, was described earlier (Dams et al. 2004a,b). δ-Hydroxy-γ-spirolactones **5**–**6** were oxidized with pyridinium dichromate to the corresponding δ-keto-γ-spirolactones **7**–**8** (Dams et al. 2004b). The structures of synthesized lactones were determined on the basis of spectroscopic and crystallographic methods. The enantiomeric purity of the final products was higher than 97% as determined by gas chromatography by using chiral columns: CP-Cyclodextrin-β-2,3,6-m-19, 25 m×0.25 mm×0.25 μm and Chirasil-Val-L, 25 m×0.25 mm×25 μm. It was not possible to obtain pure enantiomers of **6a** and **6b**. To evaluate their activity, it was necessary to use **6a**/**6b** mixtures. The activity of **6a** was studied as a mixture **5a**/**6a**=28%:72% and **6b**–**5b**/**6b**=28%:72%.

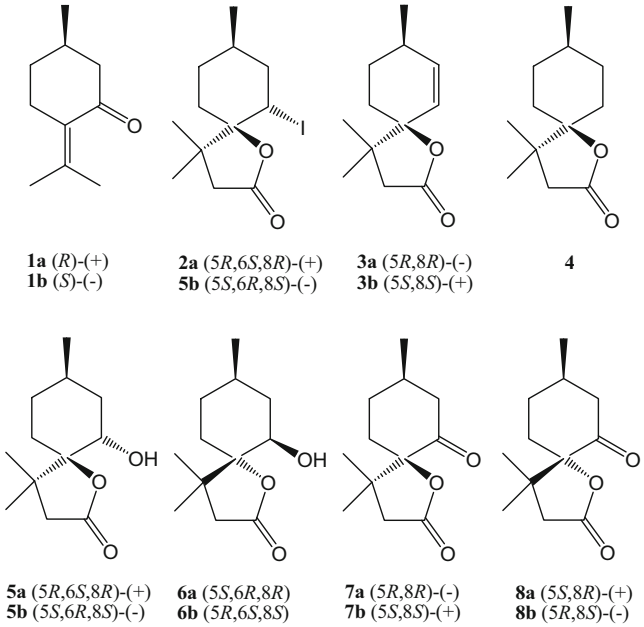

Fig. 1 Structures of pulegone and pulegone-derived lactones. **1a** (*R*)-(+)-pulegone, **1b** (*S*)-(−)-pulegone; **2a** (5*R*,6*S*,8*R*)-(+)-6-iodo-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one, **2b** (5*S*,6*R*,8*S*)-(−)-6-iodo-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one; **3a** (5*R*,8*R*)-(−)-4,4,8-trimethyl-1-oxaspiro[4.5]dec-6-en-2-one, **3b** (5*S*,8*S*)-(+)-4,4,8-trimethyl-1-oxaspiro[4.5]dec-6-en-2-one; **4** 4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one; **5a** (5*R*,6*S*,8*R*)-(+)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]-decan-2-one, **5b** – (5*S*,6*R*,8*S*)-(−)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro-[4.5]decan-2-one; **6a** (5*S*,6*R*,8*R*)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one, **6b** (5*R*,6*S*,8*S*)-6-hydroxy-4,4,8-trimethyl-1-oxaspiro[4.5]decan-2-one; **7a** (5*R*,8*R*)-(−)-4,4,8-trimethyl-1-oxaspiro[4.5]decane-2,6-dione, **7b** (5*S*,8*S*)-(+)-4,4,8-trimethyl-1-oxaspiro[4.5]decane-2,6-dione; **8a** (5*S*,8*R*)-(+)-4,4,8-trimethyl-1-oxaspiro[4.5]decane-2,6-dione, **8b** (5*R*,8*S*)-(−)-4,4,8-trimethyl-1-oxaspiro[4.5]decane-2,6-dione

Aphids, Plants, and Compound Application Aphids (*Myzus persicae*) and plants (Chinese cabbage *Brassica pekinensis*) were reared in laboratory at 20°C, 65% RH, and 16:8 L/D photoperiod. Compounds were applied to the adaxial surface of leaves as 0.1% ethanolic solutions, 0.01 ml/cm^2 of the leaf according to a method described by Polonsky et al. (1989). All biological assays were performed 1 hr after compound application to allow evaporation of the carrier solvent. Iodolactones were not studied in electronic penetration graph (EPG) experiments due to their instability.

Behavioral Responses of Aphids The antifeedant effect of pulegone and its structural analogs was assessed by watching the settling behavior of freely moving aphids and by electronically monitoring aphid stylet activities in plant tissues (probing behavior).

Settling of *Myzus persicae* was studied by using the "half-leaf test" (Polonsky et al. 1989). Compounds were applied to one half of a leaf; the other side of the midrib was coated with 0.1% ethanol and considered the "control." Aphids were offered a choice between equal areas of treated and control surfaces. Aphids that settled on each side of the midrib (i.e., aphids that did not move and had their antennae directed backwards, indicating probing) were counted at 15-, 30-min, 1-, 2-, and 24-hr intervals after access to the leaf (8 replicates, 20 viviparous apterous females/replicate). The relative index of deterrence was calculated after Powell et al. (1997): $R=(C-T)/(C+T)$. C represents the number of aphids settled on the control half of the leaf and T=the number of aphids settled on half of the leaf coated with the tested compound. R values may range from "−1" (indicating a good attractant) to "1" (indicating a good deterrent). Statistical differences in settling/no settling between treatment and control were tested for each substance by Student's *t*-test.

Myzus persicae probing behavior was monitored by using electronic registration of aphid stylet penetration in plant tissues referred to as EPG. This technique is commonly used in insect–plant studies. Aphids and plants become incorporated into an electric circuit; the circuit is closed when aphids insert their stylets into plant tissues. A weak voltage is applied through the circuit, and all changes in electrical properties are recorded as EPG waveforms that can be correlated with aphid activities and stylet position in plant tissues (Tjallingii 1994). The values of parameters derived from EPG recordings, e.g., the duration of probing, duration of phloem sap ingestion, number of probes, etc., reflect the suitability of a food source to the aphids (Mayoral et al. 1996). After attachment of the wire electrode, aphids were starved for 1 hr before the start of an experiment. The probing behavior of 16 apterous females was tested per substance and monitored continuously for 8 hr with a four-channel DC EPG recording device. Signals were analyzed with Probe 2.1 software provided by W.F. Tjallingii. The following EPG patterns were distinguished: np (non penetration—aphid stylets remained outside of the plant), A, B, C, F (pathway phase—penetration of non-phloem tissues), E1 (salivation into sieve elements), and E2 (ingestion of phloem sap). The E1/E2 transition patterns were included in E1. A number of sequential (i.e., describing the sequence of events during the recording) and non-sequential (i.e., referring to frequency, total, and average duration of patterns) parameters were calculated (Van Helden and Tjallingii 1993). Results were analyzed statistically with analysis of variance (Kruskal–Wallis test) by using Statistica 6.1 (StatSoft axxp505b946905ar28).

Results

(R)-(+)-Pulegone and its Analogs Significantly fewer aphids settled on the leaf halves coated with (*R*)-(+)-pulegone (**1a**), (+)-δ-iodo-γ-spirolactone (**2a**), δ-hydroxy-γ-spirolactones *cis*(5*R*,6*S*,8*R*)-(+)-**5a** and *trans*(5*S*,6*R*,8*R*)-**6a** than on

control leaf halves. The relative indices of deterrence after 24-hr observation were 0.43, 0.72, 0.25, and 0.31, respectively. The unsaturated γ-spirolactone (5*S*,8*R*)-(−)-γ-spirolactone (**3a)** showed strong deterrent properties during the first 2 hr of an experiment (R=0.82). However, this activity had ceased by 24 hr (Fig. 2).

The most distinctive modification of aphid behavior during probing (EPG experiments) was observed on plants treated with (*R*)-(+)-pulegone (**1a**) (Table 1, Fig. 2). Total time of probing was significantly shorter on treated leaf halves—72% of 8-hr experimental time—compared to 90% for control plants. Aphids penetrated mainly peripheral tissues (78% of penetration time compared to 47% on control tissue). The time before reaching phloem vessels was nearly twice as long with non-penetration accounting for one third of that time. On (*R*)-(+)-pulegone-treated plants, aphid probes that preceded the first phloem phase were numerous (2.6 times as many as on the control half of leaves), but the probing was short, usually not longer than 2–10 min (83% of all probes). Moreover, some aphids failed to reach phloem vessels. Among those that got through to the phloem, most did not ingest sap for longer than 10 min (i.e., there was no sustained sap ingestion in more than 30% of aphids). The high proportion of salivation during penetration of phloem vessels (18% of all activities in sieve elements) was noteworthy. In 44% of aphids, recurrent alterations between E1 and E2 during every period of phloem phase were observed (the E1/E2 transition patterns were included in E1; data not shown). In general, aphid behavior generally did not change on leaf halves treated with δ-hydroxy-γ-spirolactones **5a** and **6a** compared to control leaf halves. However, on these plants, as on plants treated with (*R*)-(+)-pulegone (**1a**), aphids usually did not show sustained sap ingestion during the first contact with phloem vessels (Table 1).

(S)-(−)-Pulegone and its Analogs Significantly fewer aphids settled on leaf halves treated with (5*S*,6*R*,8*S*)-(−)-δ-iodo-γ-spirolactone (**2b)**, δ-hydroxy-γ-spirolactones: *cis* (5*S*,6*R*,8*S*)-(−)-**5b**, *trans* (5*R*,6*S*,8*S*)-**6b**, and *trans* (*5R,8S*)-(−)-δ-keto-γ-spirolactone **(8b)** than on control leaf halves. The relative indices of deterrence after 24-hr were 0.34, 0.49, 0.48, and 0.67, respectively (Fig. 3).

Aphid probing on plants treated with (*S*)-(−)-pulegone (**1b**) was similar to that on control plants. The most noticeable changes occurred after application of hydroxylactone **5b** (Table 2). Although aphid stylet penetration was not inhibited on **5b**-treated leaf halves, the total duration of phloem activities was reduced by a half, and fewer aphids showed sustained sap ingestion compared to controls [62 vs. 100% on control leaf halves or 94% on (*S*)-(−)-pulegone-treated plants]. The time to reach phloem elements was doubled, the duration of first phloem sap ingestion period was half, and the proportion of salivation in all activities in sieve elements was five times greater than on the control half of the leaves. On **8b**-treated plants, 81% of aphids reached phloem vessels, but the duration of first phloem phase was half as long as on control plants. On **6b**-treated plants, the first phloem phase was delayed by more than 2 hr on average, 32% fewer aphids reached sieve elements, and the proportion of salivation in the phloem phase was six times greater compared to controls. None or few aphids showed sustained sap ingestion during first phloem phase on plants treated with (*S*)-(−)-pulegone and lactones **5b**, **8b**, and **6b**. Thirty-two percent (**6b**) and 38% (**5b**) of aphids failed to ingest phloem sap for longer than 10 min at a time (Table 2).

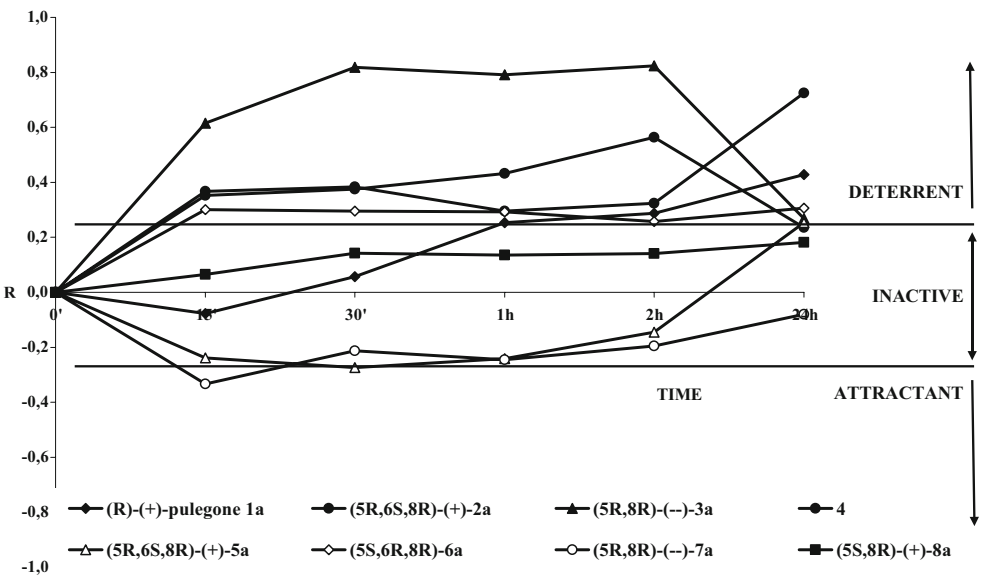

Fig. 2 Indices of deterrence of (*R*)-(+)-pulegone and (R)-(+)-pulegone-derived lactones

Table 1 Probing behavior of *Myzus persicae* (Sulz.) on plants treated with (*R*)-(+)-pulegone and its analogs[a]

Parameters	Control, mean (±SE)	(R)-(+)-pulegone, mean (±SE)	(5*R*,6*S*,8*R*)-5a, mean (±SE)	(5S,6R,8R)-6a, mean (±SE)	*P* value
Total penetration time (h)	7.2 (±0.2) a	5.8 (±0.4) b	7.1 (±0.1) ab	7.2 (±0.2) a	0.003
Total duration of phloem phase (h)	3.8 (±0.4) ab	1.9 (±0.5) b	2.9 (±0.5) ab	4.6 (±0.6) a	0.004
Proportion of phloem phase in penetration (%)	53.0 ab	32.5 b	40.2 ab	63.3 a	0.006
Time from 1st probe to 1st phloem phase (h)	1.2 (±0.3) a	3.0 (±0.5) b	1.5 (±0.2) ab	1.7 (±0.4) ab	0.009
Duration of non-penetration before 1st phloem phase (min)	15.0 (±4.3) a	64.0 (±12.9) b	18.7 (±3.9) a	21.8 (±7.5) a	0.002
Number of probes before 1st phloem phase (number)	6.0 (±1.3) a	15.8 (±2.3) b	7.0 (±1.2) ab	7.5 (±1.6) ab	0.039
Probes < 2 min (number)	2.6a	9.9b	1.9a	3.5 ab	0.001
Probes 2–10 min (number)	1.8a	5.8b	3.9ab	2.6 ab	0.002
Probes > 10 min (number)	1.6a	3.2a	1.2a	1.4 a	0.206
Proportion of aphids reaching phloem phase (%)	100	81.3	93.8	100	0.098
Proportion of aphids with sustained phloem sap ingestion (%)	100	68.8	81.3	87.5	0.108
Duration of 1st phloem sap ingestion time (h)	1.2 (±0.4)	1.4 (±0.6)	1.6 (±0.6)	2.6 (±0.5)	0.893
Proportion of salivation (E1) in phloem phase (E1 + E2) (%)	1.6	18.4	3.3	1.2	0.396

[a] Parameters derived from 8-hr EPG recording. Values followed by different letters in rows represent significant differences. *P* values of Kruskal–Wallis test

Discussion

Behavioral Aspects of Feeding Deterrent Activity of Pulegone and its Analogs Frazier and Chyb (1995) suggested that insect feeding can be inhibited at three levels: preingestional, immediate effect associated with host finding and host selection processes involving gustatory receptors; ingestional, related to food transport and production, release, and digestion by salivary enzymes; and postingestional, long-term effects involving various aspects of digestion and absorption of food. Aphids differ from chewing herbivores with respect to the location of their main gustatory receptors. Mouthparts lack external chemoreceptors, and the taste organ is located in the hypopharynx, i.e., the hypopharyngeal gustatory organ; hence the ingestion of sap is crucial for recognition and acceptance of a host plant (Wensler and Filshie 1969; Ponsen 1987; Harrewijn 1990). Before reaching the phloem vessels of host food plants, aphids ingest small samples of parenchyma cell contents for gustatory purposes (Martin et al. 1997). Gabrys and Tjallingii (2002) showed that aphids can distinguish a host from a non-host plant before reaching sieve elements. Because aphid-probing behavior cannot be observed directly, the parameters derived from EPG recordings are used as reliable indicators of preingestional and ingestional factors that affect feeding. Long penetration

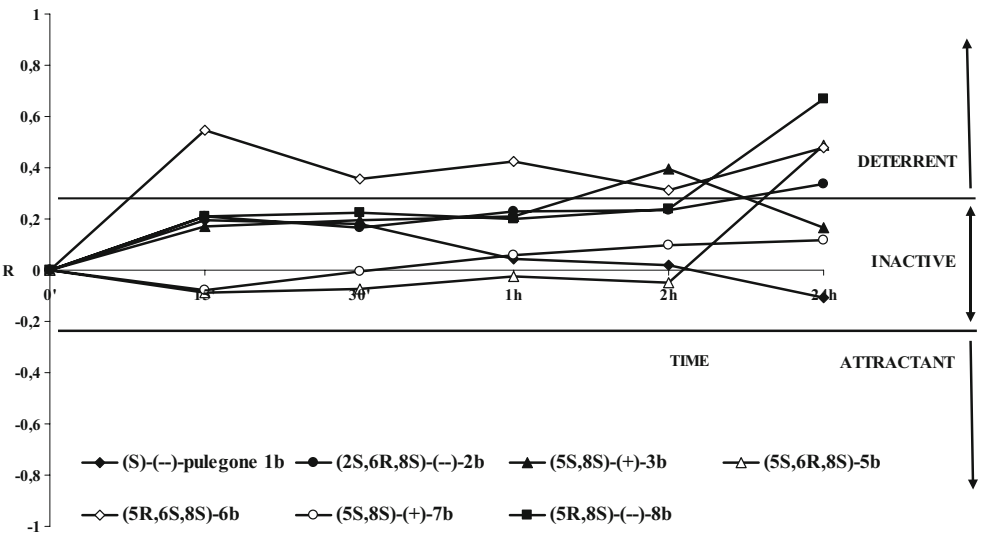

Fig. 3 Indices of deterrence of (*S*)-(−)-pulegone and (*S*)-(−)-pulegone-derived lactones

Table 2 Probing behavior of *Myzus persicae* (Sulz.) on plants treated with (*S*)-(−)-pulegone and its analogs[a]

Parameters	Control mean (±SE)	(*S*)-(−)-pulegone mean (±SE)	(5*S*,6*R*,8*S*)-5b mean (±SE)	(5*R*,6*S*,8*S*)-6b mean (±SE)	(5*R*,8*S*)-8b mean (±SE)	*P* value
Total penetration time (h)	7.2 (±0.2)	7.2 (±0.2)	6.7 (±0.2)	6.7 (±0.3)	6.8 (±0.1)	0.199
Total duration of phloem phase (h)	3.8 (±0.4) a	3.4 (±0.4) ab	1.8 (±0.4) b	2.7 (±0.7) ab	2.2 (±0.4) ab	0.019
Proportion of phloem phase in penetration (%)	53.0 a	47.7 ab	26.8 b	39.9 ab	32.8 ab	0.019
Time from 1st probe to 1st phloem phase (h)	1.2 (±0.3) a	2.8 (±0.5) ab	2.4 (±0.4) ab	3.4 (±0.5) b	1.2 (±0.2) a	0.005
Duration of non-penetration before 1st phloem phase (min)	15.0 (±4.3)	34.1 (±8.2)	29.4 (±7.5)	44.9 (±11.3)	19.0 (±5.8)	0.111
Number of probes before 1st phloem phase (number)	6.0 (±1.3)	13.3 (±3.4)	13.0 (±2.7)	13.5 (±2.9)	9.3 (±2.0)	0.167
Probes <2 min (number)	2.6 (±0.6)	6.1 (±2.1)	5.5 (±1.5)	6.7 (±2.0)	5.3 (±1.6)	0.627
Probes 2–10 min (number)	1.8 (±0.5)	4.9 (±1.2)	4.3 (±1.1)	3.6 (±0.8)	3.1 (±0.5)	0.187
Probes >10 min (number)	1.6 (±0.5) a	2.3 (±0.6) a	3.2 (±0.9) a	3.2 (±0.5) ac	0.9 (±0.2) ab	0.028
Proportion of aphids reaching phloem phase (%)	100	93.8	100	93.8	100	0.551
Proportion of aphids with sustained phloem sap ingestion (%)	100 a	93.8 a	62.5 b	68.8 ab	81.3 ab	0.032
Duration of 1st phloem sap ingestion (h)	1.2 (±0.4) a	1.9 (±0.5) ac	0.5 (±0.3) b	1.6 (±0.3) abc	0.6 (±0.3) ab	0.005
Proportion of salivation (E1) in phloem phase (E1 + E2) (%)	1.6 a	2.3 a	8.1 b	9.4 ab	4.4 ab	0.002

[a] Parameters derived from 8-hr EPG recording. Values followed by different letters in rows represent significant differences. *P* values of Kruskal–Wallis test

times of non-phloem tissues compared to total penetration time, a large number of short vs. long probes before the first phloem phase, a relatively long time to first phloem phase within a probe, and a failure to find sieve elements may be interpreted as preingestional effects of antifeedants on aphids that restrain probing at the level of non-phloem tissues. Short (<10 min) probes are limited either to the epidermis (<2 min; Van Hoof 1958) or do not reach beyond the mesophyll layer (2−10 min; Gabrys et al. 1997). Similarly, the short total and mean duration of phloem sap ingestion and the high proportion of salivation during penetration of phloem vessels may point to feeding deterrence at the ingestional level (Mayoral et al. 1996; Gabrys and Pawluk 1999). Particularly, prolonged salivation is characteristic of aphid behavior on resistant plant cultivars or non-host plants (Van Helden and Tjallingii 1993; Wilkinson and Douglas 1998; Gabrys and Pawluk 1999).

In this study, pulegone and some of its structural analogs were found to deter aphid probing and feeding at the preingestional [(*R*)-(+)-pulegone], ingestional [(*R*)-(+)-pulegone, δ-hydroxy-γ-spirolactones **5b** and **6b**, δ-keto-γ-spirolactone **8b**], and postingestional [(*R*)-(+)-pulegone (**1a**), δ-hydroxy-γ-spirolactones **5a**, **5b**, **6a**, **6b**, and δ-keto-γ-spirolactone **8b**] levels.

The preingestional effect of (*R*)-(+)-pulegone (**1a**) was characterized by aphid difficulties in finding and reaching phloem tissues. These difficulties were manifested in: prolonged time preceding the first contact with phloem vessels (2.5 times as long as on controls), greater number of probes not reaching beyond mesophyll layers before first phloem phase (3.6 times as many as on controls), and a failure to find sieve elements by 20% of aphids during 8-hr experiments. Azadirachtin- and *S*-limonene-derived lactone have similar effects on *Myzus persicae* during the pre-phloem phase (Nisbet et al. 1993; Halarewicz-Pacan et al. 2003). On non-host plants or resistant varieties of crop plants, aphid probing may also be impeded at the level of peripheral tissues. Caillaud (1999) showed that *Acyrthosiphon pisum* did not reach or ingest phloem sap from non-host plants. There were only short probes to the mesophyll. Likewise, *Aphis gosypii* on resistant lines of *Cucumis melo* showed a high number of short probes that never reached phloem vessels (Garzo et al. 2004). Pathway activities of cabbage aphid *Brevicoryne brassicae* were suppressed on the non-host plant *Vicia faba* and accidental host plants *Thlaspi arvense* and *Lunaria annua* (Gabrys and Pawluk 1999).

The ingestional effect of (*R*)-(+)-pulegone (**1a**) was characterized by a decreased duration of phloem sap ingestion (by as much as 50% compared to controls),

decreased proportion of aphids with sustained sap ingestion (by 30%), and increased proportion of salivation in aphid activities in phloem (about 12 times). Furthermore, the ingestional effect of feeding deterrence was observed after application of δ-hydroxy-γ-spirolactones **5b** and **6b**. There was a decrease in the total duration of phloem phase, the duration of first phloem phase, and the proportion of phloem phase in activities related to stylet penetration (**5b**). Between 30–40% of aphids failed to ingest sap for longer than 10 min on **6b**- and **5b**-coated leaves, respectively, and the duration of salivation into sieve elements was five times longer than on control leaves. Weak ingestional effects occurred with δ-keto-γ-spirolactone **8b**-treated leaves and were manifested in shortened phloem phase. Ingestion of the phloem sap for longer than 10 min reflects the acceptance of a plant by an aphid (Tjallingii and Mayoral 1992). The mechanism of resistance of plant varieties or cultivars to aphids is manifested in the reduction of time in sap ingestion (Cole et al. 1993; Sauge et al. 1998; Powell 2004). The presence of azadirachtin in the phloem sap results in the termination of feeding (Nisbet et al. 1993). A decrease in the duration of sap ingestion is a good indicator of the deterrent properties of specific chemical compounds—shown via the use of artificial diets by Givovich and Niemeyer (1995). Frequent interruptions of E2 in phloem phases and the relatively high proportion of salivation in aphid activities in phloem are possible responses of aphids to resistance mechanism in plants (Tjallingii 2001). Increased salivation duration is often reported in aphids that feed on resistant varieties of crop plants (Van Helden and Tjallingii 1993; Mayoral et al. 1996; Wilkinson and Douglas 1998). Sauge et al. (1998) found frequent alterations between E1 and E2 patterns during the phloem phase of *Myzus persicae* on resistant varieties of peach, and Van Helden and Tjallingii (1993) in *Nasonovia ribis-nigri* on lettuce. In the present study, such interesting phenomenon occurred after the application of (*R*)-(+)-pulegone (**1a**). Miles (1990) and Miles and Oertli (1993) suggest that aphid saliva may neutralize plant resistance factors in the phloem. Tjallingii and Cherqui (1999) showed that the watery saliva secreted into phloem elements contained various proteins, including detoxification enzymes. Leszczynski (2001) is of the opinion that the enzymes present in the saliva may help to metabolize toxic allelochemicals encountered during sap ingestion.

The postingestional deterrence by pulegone and some of its analogs may be assumed because of their influence on aphid settling. The effect of (*R*)-(+)-pulegone (**1a**) was long-term and discouraged aphids from settling on plants 24 hr after application, suggesting a role in postingestional activity. The δ-hydroxy-γ-spirolactones **5a** and **6a** derived from (*R*)-(+)-pulegone (**1a**) were probably also postingestional antifeedants. Application of these compounds did not affect aphid behavior during the pre-pholem or phloem phase of stylet penetration; however, aphids refused to settle on leaf halves treated with these compounds or with **5b**, **6b**, or **8b** within 24 hr after treatment. The postingestional effect of these terpenoids will require a separate study into their influence on digestive physiology and/or metabolism in aphids.

Structure–Activity Relationships The starting point for our experiments were two optical isomers of natural monoterpenoid pulegone: (*R*)-(+)- and (*S*)-(−)-pulegone. We found that only (*R*)-(+)-pulegone (**1a**) was deterrent to *Myzus persicae*; (*S*)-(−)-pulegone (**1b**) had no effect on aphid probing or feeding behavior. (*R*)-(+)-pulegone **(1a)** appears to be a feeding deterrent with the broadest spectrum of activity of the compounds studied. It affected aphid behavior during the initial, intermediate, and final (sap ingestion) phases of probing. (*R*)-(+)- and (*S*)-(−)-pulegone were the initial substrates for the synthesis of the bicyclic lactones with a *p*-mentane system, various functional groups, and different configuration of chiral centers. The incorporation of iodine into the bicyclic spirolactones (i.e., **2a** and **2b)** had the most dramatic impact on activity of all of the structural modifications. The deterrence indices of **2a** and **2b** were twice and four times as high as those of (*R*)-(+)-pulegone (**1a**) and (*S*)-(−)-pulegone (**1b**), respectively. Lactones with hydroxy and carbonyl groups were also active (**5a**, **5b**, **6a**, **6b**, and **8b**); however, there were differences in the activity levels among the enantiomers. Generally, lactones synthesized from (*S*)-(−)-pulegone (**1b**) were more active (e.g., hydroxylactones **5b** and **6b**) and showed broad spectrum deterrence while **5a** and **6a** deterred aphid settling but had no effect during the preingestive and ingestive stages of aphid probing. There was also a difference in activity between two diastereoisomeric hydroxylactones **5b** and **6b**, with the **5b** having broader spectrum of activity. The *trans* position of methyl group CH_3–8 and the bond C5–O1 in the lactone ring of **6b** appeared to weaken the deterrent activity in relation to diastereoisomer **5b** with *cis* configuration.

Our comparative study of the biological activity of enantiomeric pairs of pulegone and pulegone-derived lactones shows a correlation between chiral center configuration and expression of biological activity. Chirality is a unique character of most known biological processes, and enantiomers of bioactive molecules show different activities toward living organisms (Avalos et al. 2000). These studies confirm other reports that suggest that (*R*)-(+)-pulegone is a more bioactive compound than the (*S*)-isomer. For example, the hepatotoxicity and pneumotoxicity of pulegone is attributed to (*R*)-configuration of pulegone (Chan 2001). Addition of a lactone moiety and a hydroxyl group to (*S*)-

(−)-pulegone introduced antifeedant properties not possessed by the inactive substrate, thus confirming the importance of the lactone moiety to expression of biological activity. Similar effects were obtained in feeding deterrence studies of pulegone and pulegone-derived spirolactones toward the Colorado potato beetle *Leptinotarsa dacemlineata* Say, lesser mealworm *Alphitobius diaperinus* Panzer, confused flour beetle *Tribolium confusum* Duv., grain weevil *Sitophilus granarius* L., and khapra beetle *Trogoderma granarium* Ev. (Szczepanik et al. 2005; Wawrzenczyk et al. 2005).

References

AVALOS, M., BABIANO, R., CINTAS, P., JIMENEZ, J. L., and PALACIOS, J. C. 2000. From parity to chirality: chemical implications revisited. *Tetrahedron: Asymmetry.* 11:2845–2874.

AVERY, M. L., DECKER, D. G., HUMPHREY, J. S., and LAUKERT, C. C. 1996. Mint plant derivatives as blackbird feeding deterrents. *Crop. Prot.* 15:461–464.

BLACKMAN, R. L., and EASTOP, V. F. 1985. Aphids on the World's Crops: An Identification Guide. Wiley, New York.

CAILLAUD, M. C. 1999. Behavioural correlates of genetic divergence due to host specialization in the pea aphid *Acyrthosiphon pisum*. *Entomol. Exp. Appl.* 91:227–232.

CHAN, K. K. 2001. Quantitation of monoterpenoid compounds with potential medicinal use in biological fluids. *J. Chromatogr. A.* 936:47–57.

CHOI, W., PARK, B., LEE, Y., JANG, D. Y., YOON, H. Y., and LEE, S. 2006. Fumigant toxicities of essential oils and monoterpenes against *Lycoriella mali* adults. *Crop. Prot.* 25:398–401.

COLE, R. A., RIGALL, W., and MORGAN, A. 1993. Electronically monitored feeding behaviour of the lettuce root aphid (*Pemphigis bursarius*) on resistant and susceptible lettuce varieties. *Entomol. Exp. Appl.* 68:179–185.

CONOVER, M. R., and LYONS, K. S. 2005. Will free-ranging predators stop depredating untreated eggs in pulegone-scented gull nests after exposure to pulegone-injected eggs? *Appl. Anim. Behav. Sci.* 93:135–145.

DAMS, I., BIAŁOŃSKA, A., CIUNIK, Z., and WAWRZEŃCZYK, C. 2004a. Lactones.21. Synthesis and odoriferous properties of lactones with the *p*-menthane system. *J. Agric. Food Chem.* 52:1630–1634.

DAMS, I., BIAŁOŃSKA, A., CIUNIK, Z., and WAWRZEŃCZYK, C. 2004b. Lactones. 22. Synthesis of terpenoid lactones with the *p*-menthane system. *Eur. J. Org. Chem.* 2004:2662–2668.

FRAZIER, J. L., and CHYB, S. 1995. Use of feeding inhibitors in insect control, pp. 364–381, in R. F. Chapman, and G. de Boer (eds.). Regulatory Mechanisms in Insect FeedingChapman & Hall, New York.

FRANZIOS, G., MIROTSOU, M., HATZIAPOSTOLOU, E., KRAL, J., SCOURAS, Z. G., and MAVRAGANI-TSIPIDOU, P. 1997. Insecticidal and genotoxic activities of mint essential oils. *J. Agric. Food Chem.* 45:2690–2694.

GABRYS, B., DANCEWICZ, K., HALAREWICZ-PACAN, A., and JANUSZ, E. 2005. Effect of natural monoterpenes on behaviour of the peach potato aphid *Myzus persicae* (Sulz.). *IOBC/WPRS Bull.* 28:29–34.

GABRYS, B., and PAWLUK, M. 1999. Acceptability of different species of Brassicaceae as hosts for the cabbage aphid. *Entomol. Exp. Appl.* 91:105–109.

GABRYS, B., and TJALLINGII, W. F. 2002. The role of sinigrin in host plant recognition by aphids during initial plant penetration. *Entomol. Exp. Appl.* 104:89–93.

GABRYS, B., TJALLINGII, W. F., and VAN BEEK, T. 1997. Analysis of EPG recorded probing by cabbage aphid on host plant parts with different glucosinolate contents. *J. Chem. Ecol.* 23:1661–1673.

GARZO, E., PALACIOS, I., and FERERES, A. 2004. Characterization of melon germplasm resistant to *Aphis gossypii* Glover, pp. 441–447, in J. J. Simon, C. A. Dedryver, C. Rispe, and M. Hulle (eds.). Aphids in a New MillenniumINRA, Paris.

GATA-GONÇALVES, L., NOGUEIRA, J. M. F., MATOS, O., and DE SOUSA, R. B. 2003. Photoactive extracts from *Thevetia peruviana* with antifungal properties against *Cladosporium cucumerinum*. *J. Photochem. Photobiol. B.* 70:51–54.

GIVOVICH, A., and NIEMEYER, H. 1995. Comparison of the effect of hydroxamic acids from wheat on five species of cereal aphids. *Entomol. Exp. Appl.* 74:115–119.

GUTIERREZ, C., FERERES, A., EINA, M., CABRERA, R., and GONZAES-COLOMA, A. 1997. Behavioral and sublethal effects of structurally related lower terpenes on *Myzus persicae*. *J. Chem. Ecol.* 23:1641–1650.

HALAREWICZ-PACAN, A., GABRYŚ, B., DANCEWICZ, K., and WAWRZEŃCZYK, C. 2003. Enantiospecific effect of limonene and limonene-derived bicyclic lactones on settling and probing behaviour of the peach potato aphid *Myzus persicae* (Sulz.). *J. Plant Prot. Res.* 43:133–142.

HARREWIJN, P. 1990. Resistance mechanisms of plant genotypes to various aphid species, pp. 117–130, in R. K. Campbell, and R. D. Eikenbary (eds.). Aphid–Plant Genotype InteractionsAmsterdam, Elsevier.

HARREWIJN, P., VAN OOSTEN, A. M., and PIRON, P. G. M. 2001. Natural Terpenoids as Messengers. Kluwer, Dordrecht, The Netherlands.

HORI, M. 1998. Repellency of rosemary oil against *Myzus persicae* in a laboratory and in a screenhouse. *J. Chem. Ecol.* 24:1425–1432.

HORI, M. 1999. Antifeeding, settling inhibitory and toxic activities of labiate essential oils against the green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae). *Appl. Entomol. Zool.* 34:113–118.

JUZA, M., MAZZONTI, M., and MORBIDELLI, M. 2000. Simulated moving-bed chromatography and its application to chirotechnology. *Trends Biotechnol.* 18:108–118.

LEE, S., PETERSON, C. J., and COATS, J. R. 2003. Fumigation toxicity of monoterpenoids to several stored product insects. *J. Stored. Prod. Res.* 39:77–85.

LESZCZYNSKI, B. 2001. Rola allelozwiązków w oddziaływaniach owady–rośliny, pp. 61–86, in W. Oleszek, K. Głowniak, and B. Leszczyński (eds.). Biochemiczne oddziaływania środowiskoweAkademia Medyczna, Lublin.

LEY, S. V., and TOOGOOD, P. L. 1990. Insect antifeedants. *Chem. Br.* 1:31–35.

MARTIN, B., COLLAR, J. L., TJALLINGII, W. F., and FERERES, A. 1997. Intracellular ingestion and salivation by aphids may cause the acquisition and inoculation of non-persistently transmitted plant viruses. *J. Gen. Virol.* 78:2701–2705.

MASON, J. R., and EPPLE, G. 1998. Evaluation of bird repellent additives to a simulated pesticide carrier formation. *Crop Prot.* 17:657–659.

MAYORAL, A. M., TJALLINGII, W. F., and CASTANERA, P. 1996. Probing behaviour of *Diuraphis noxia* on five cereal species with different hydroxyamic acid levels. *Entomol. Exp. Appl.* 78:341–348.

MILES, P. W. 1990. Aphid salivary secretions and their involvement in plant toxicoses, pp. 131–147, in R. K. Campbell, and R. D. Eikenbary (eds.). Aphid-Plant Genotype interactionsElsevier Science, Amsterdam.

MILES, P. W., and OERTLI, J. J. 1993. The significance of antioxidants in the aphid-plant interaction: the redox hypothesis. *Entomol. Exp. Appl.* 67:275–283.

NISBET, A. J., WOODFORD, J. A. T., and CONNOLLY, J. D. 1993. Systemic antifeedant effects of azadirachtin on the peach-potato aphid *Myzus persicae*. *Entomol. Exp. Appl.* 68:87–98.

OYEDELE, A. O., GBOLADE, A. A., SOSAN, M. B., ADEWOYIN, F. B., SOYELU, O. L., and ORAFIDIYA, O. O. 2002. Formulation of an effective mosquito-repellent topical product from Lemongrass oil. *Phytomedicine.* 9:259–262.

PHATAK, S. V., and HEBLE, M. R. 2002. Organogenesis and terpenoid synthesis in *Mentha arvensis*. *Fitoterapia.* 73:32–39.

PICKETT, J. A. 1991. Lower terpenoids as natural insect control agents, pp. 297–313, in J. B. Harborne, and F. A. Tomas-Barberan (eds.). Ecological Chemistry and Biochemistry of Plant TerpenoidsClarendon, Oxford.

PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1994. Attempts to control aphid pests by integrated use of semiochemicals, pp 1239−1246. Brighton Crop Protection Conference—Pests and Diseases. British Crop Protection Council, Thornton Heath, UK.

POLONSKY, J., BHATNAGAR, S. C., GRIFFITHS, D. C., PICKETT, J. A., and WOODCOCK, C. M. 1989. Activity of qassinoids as antifeedants against aphids. *J. Chem. Ecol.* 15:933–998.

PONSEN, M. B. 1987. Alimentary tract, pp. 79–97, in A. K. Minks, and P. Harrewijn (eds.). Aphids, Their Biology, Natural Enemies and Control. Vol. AElsevier, Amsterdam.

POWELL, G. 2004. Sieve element salivation and the transmission to ingestion, pp. 479–483, in J. J. Simon, C. A. Dedryver, C. Rispe, and M. Hulle (eds.). Aphids in a New MillenniumINRA, Paris.

POWELL, G., HARDIE, J., and PICKETT, J. A. 1997. Laboratory evaluation of antifeedant compounds for inhibiting settling by cereal aphids. *Entomol. Exp. Appl.* 84:189–193.

PRICE, D. N., and BERRY, M. S. 2006. Comparison of effects of octopamine and insecticidal essential oils on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches. *J. Insect Physiol.* 52:309–319.

SALVATORE, A., BORKOSKY, S., WILLINK, E., and BARDON, A. 2004. Toxic effects of lemon peel constituents on *Ceratitis capitata*. *J. Chem. Ecol.* 30:323–333.

SAUGE, M. H., KERVELLA, J., and AHBE, Y. 1998. Probing behaviour of the green peach aphid *Myzus persicae* on resistant *Prunus* genotypes. *Entomol. Exp. Appl.* 89:223–232.

SIMMONDS, M. S. J. 1998. Chemoecology. The legacy left by Tony Swain. *Phytochemistry.* 49:1183–1190.

SZCZEPANIK, M., DAMS, I., and WAWRZENCZYK, C. 2005. Feeding deterrent activity of terpenoid lactones with the p-menthane system against the Colorado potato beetle (Coleoptera: Chrysomelidae). *Environ. Entomol.* 34:1433–1440.

TJALLINGII, W. F. 1994. Sieve element acceptance by aphids. *Eur. J. Entomol.* 91:47–52.

TJALLINGII, W. F. 2001. Plant penetration by aphids as revealed by electrical penetration graphs. *Aphids and other Homopterous Insects.* 8:105–120.

TJALLINGII, W. F., and CHERQUI, A. 1999. Aphid saliva and aphid-plant interactions. *Exp. Appl. Entomol.* 10:169–174.

TJALLINGII, W. F., and MAYORAL, A. M. 1992. Criteria for host plant acceptance by aphids, pp. 280–282, in S. B. J. Menken, J. H. Visser, and P. Harrewijn (eds.). Proc. 8th Int. Symp. Insect-Plant RelationshipsKluwer, Dordrecht.

UNELIUS, C. R., NORDLANDER, G., NORDENHEM, H., HELLQVIST, C., LEGRAND, S., and BORG-KARLSON, A-K. 2006. Structure–activity relationships of benzoic acid derivatives as antifeedants for the pine weevil, *Hylobius abietis*. *J. Chem. Ecol.* 32:2191–2203.

VAN HELDEN, M., and TJALLINGII, W. F. 1993. Tissue localisation of lettuce resistance to the aphid *Nasonovia ribisnigri* using electrical penetration graphs. *Entomol. Exp. Appl.* 68:269–278.

VAN HOOF, H. A. 1958. An Investigation of the Biological Transmission of a Non-Persistent Virus, p. 112, in A. Van Putten and B. Oortmeier (eds.). Meded. Inst. Planteziektenkundig. Wageningen Agricultural University, Alkmaar.

VETERE, V., SANTORI, G. F., MOGLIONI, A., MOLTRASIO IGLESIAS, G. Y., CASELLA, M. L., and FERRETTI, O. A. 2002. Hydrogenation of (−)-menthone, (+)-isomenthone, and (+)-pulegone with platinum/tin catalysts. *Catal. Letters* 84:251–257.

WAWRZENCZYK, C., DAMS, I., SZUMNY, A., SZCZEPANIK, M., NAWROT, J., PRADZYNSKA, A., GABTYS, B., DANCEWICZ, K., MAGNUCKA, E., GAWDZIK, B., OBARA, R., and WZOREK, A. 2005. Synthesis and evaluation of antifeedant, antifungal and atibacterial activity of isoprenoid lactones. *Pol. J. Environ. Stud.* 14:Suppl69–84.

WAWRZEŃCZYK, C., PARUCH, E., OLEJNICZAK, T., SALETRA, A., NAWROT, J., PRĄDZYŃSKA, A., HALAREWICZ-PACAN, A., and GABRYŚ, B. 1997. Lactones 4. The effect of the compound configuration on the feeding deterrent activity of some terpenoid lactones. Proc. 2nd International Conference on Insects: Chemical, Physiological and Environmental Aspects, September 14–19, Lądek Zdrój, Poland: 222–227.

WAWRZYNIAK, M. 1996. The effect of selected plant extracts on the cabbage butterfly, *Pieris brassicae* L. (Lepidoptera). *Pol. J. Ent.* 65:93.

WELLINGS, P. W., WARD, S. A., DIXON, A. F. G., and ABBINGE, R. 1989. Crop loss assessment, pp. 49–64, in A. K. Minks, and P. Harrewijn (eds.). Aphids, Their Biology, Natural Enemies and Control. Vol. CElsevier, Amsterdam.

WENSLER, R. J. D., and FILSHIE, B. K. 1969. Gustatory sense organs in the food canal of aphids. *J. Morphol.* 129:473–492.

WILKINSON, T. L., and DOUGLAS, A. E. 1998. Plant penetration by pea aphids (*Acyrthosiphon pisum*) of different plant range. *Ent. Exp. Appl.* 87:43–50.

WITTSTOCK, U., and GERSHENZON, J. 2002. Constitutive plant toxins and theirs role in defense against herbivores and pathogens. *Curr. Opin. Plant. Biol.* 5:1–8.

ZHU, B. C. R., HENDERSON, G., CHEN, F., FEI, H., and LAINE, R. A. 2001. Evaluation of vetiver oil and seven insect-active essential oils against the Formosan subterranean termite. *J. Chem. Ecol.* 27:1617–1625.

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Effects of *p*-Vinylphenyl Glycosides and Other Related Compounds on the Oviposition Behavior of *Ceratitis capitata*

Cecilia Socolsky · Mirta L. Fascio · Norma B. D'Accorso · Analia Salvatore · Eduardo Willink · Yoshinori Asakawa · Alicia Bardon

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Abstract Elaphoside-A [*p*-vinylphenyl (β-D-glucopyranosyl)-(1→3)-β-D-allopyranoside], a Mediterranean fruit fly oviposition deterrent, was previously isolated from an Argentine collection of the fern *Elaphoglossum piloselloides*. In order to establish the structural requirements for the observed oviposition inhibition, we synthesized and characterized 4 known and 21 new aromatic glycosides structurally related to elaphoside-A. Their effects on the oviposition behavior of *Ceratitis capitata* females are discussed.

Keywords Oviposition deterrence · *Ceratitis capitata* · Aromatic glycosides · *Elaphoglossum piloselloides*

C. Socolsky · A. Bardon (✉)
Facultad de Bioquímica, Química y Farmacia,
Universidad Nacional de Tucumán,
Ayacucho 471,
Tucumán 4000, Argentina
e-mail: alisan@unt.edu.ar

M. L. Fascio · N. B. D'Accorso
CIHIDECAR, Departamento de Química Orgánica,
Facultad de Ciencias Exactas y Naturales,
Universidad de Buenos Aires,
Ciudad Universitaria, Pabellón 2, Tercer piso,
Buenos Aires 1428, Argentina

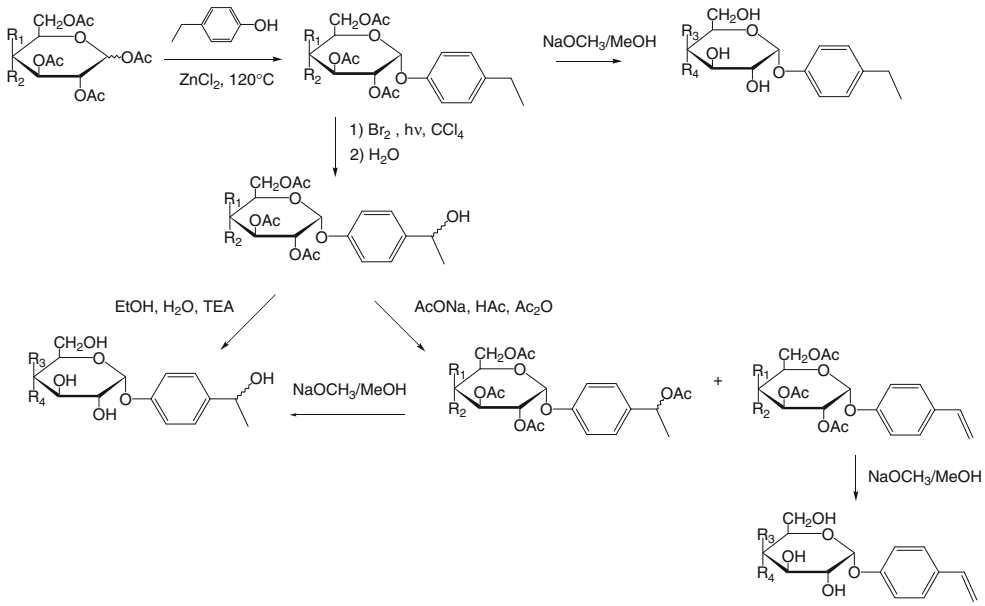
A. Salvatore · E. Willink
Estación Experimental Agroindustrial Obispo Colombres,
Av. William Cross 3150,
Tucumán 4101, Argentina

Y. Asakawa
Faculty of Pharmaceutical Sciences, Tokushima Bunri University,
Yamashiro-cho,
Tokushima 770-8514, Japan

Introduction

The influence of chemical agents on the ovipositional behavior of insects can be used to control pests as well as vector insects. Some natural products display oviposition-deterrent activity against different insects. A chromene from *Blepharispermum subsessile* (Compositae) is an oviposition deterrent for *Phthorimacea operculella* (Kulkarni et al. 1987). Essential oils from medicinal plants have been successfully tested as oviposition deterrents on *Thrips tabaci* (Koschier and Sedy 2003) and the *Anopheles stephensi*, *Aedes aegypti*, and *Culex quinquefasciatus* mosquito species (Prajapati et al. 2005). The ovipositional response of the fruit fly *Anastrepha suspensa* (Loew) to stimulants and deterrents was studied by Szentesi et al. (1979). Factors that affect the oviposition behavior of some tephritid fruit flies include host-plant odor as well as the physical and chemical characteristics of the oviposition substrate, such as size, shape, color, and presence of deterrent chemicals.

The Mediterranean fruit fly *Ceratitis capitata* (Tephritidae) causes important economic damage in the north of Argentina and other countries; therefore, substances that affect their behavior can be used in the development of pest-control agents. Previously, it was reported that elaphoside-A [*p*-vinylphenyl (β-D-glucopyranosyl)-(1→3)-β-D-allopyranoside], a bitter-tasting styrene glycoside isolated from the methanol extract of the fern *Elaphoglossum piloselloides*, partially inhibited the oviposition of *C. capitata* when incorporated into the surface of an artificial fruit at a concentration of 6 μg/cm^2. The number of eggs laid on treated artificial fruit was 48% lower than that oviposited on control (nontreated) fruit at the mentioned dose (Socolsky et al. 2003). In order to determine the structural requirements



Scheme 1 Synthesis of α-ANOMERS. When R_1=OAc, R_2=H, R_3=OH and R_4=H, the scheme is illustrated for α-D-galactopyranosyl derivatives. When R_1=H, R_2=OAc, R_3=H and R_4=OH, α-D-glucopyranosyl derivatives are obtained, and when R_1=H, R_2=*O*-2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl, R_3=H and R_4=*O*-β-D-galactopyranosyl, α-lactose derivatives are obtained

for activity, a synthetic scheme (Scheme 1) was designed by varying the sugar moiety and the alkyl substituent at the *para* position of the aromatic ring to obtain 25 glycosides (**1**–**25**) structurally related to the active natural product (Fig. 1). In addition, four compounds (**26**–**29**) were obtained by chemical modification and degradation of elaphoside-A. Compounds **1**–**10**, **12**–**22**, and **24**–**29** were tested for their capacity to inhibit the oviposition of *C. capitata* in comparison with elaphoside-A and the commercial *p*-vinylanisole (**30**).

Methods and Materials

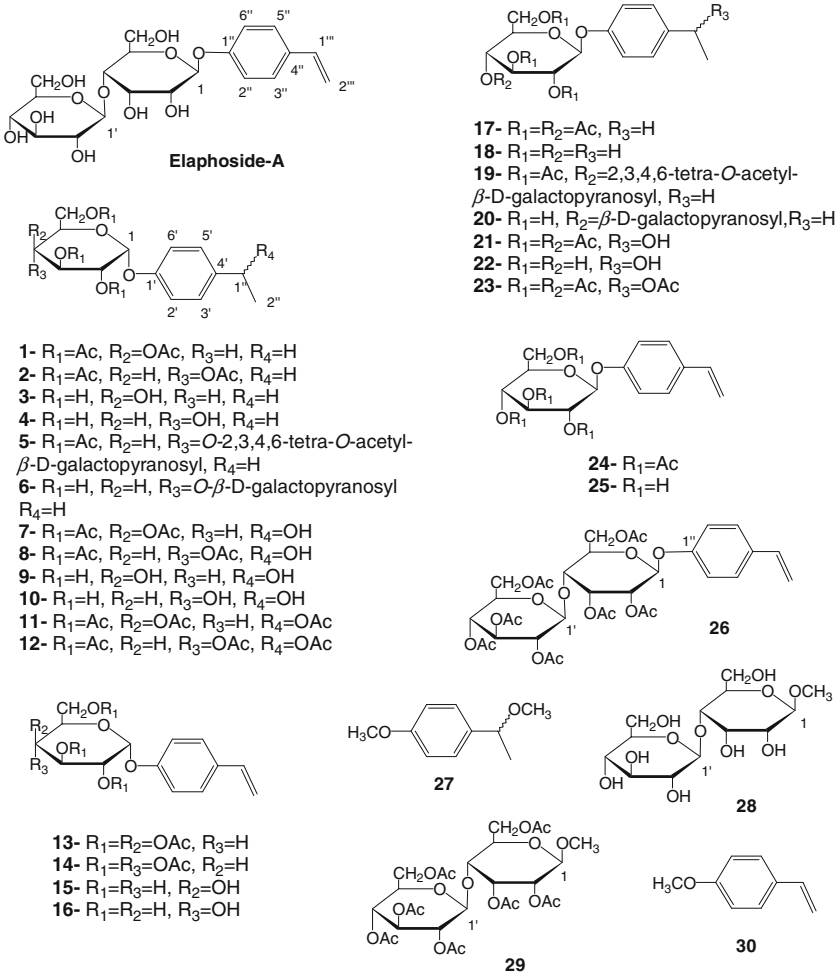
General Infrared spectra were recorded on a Shimadzu FT/IR-8400S. Optical activities were determined on a JASCO P-1030 polarimeter. Nuclear magnetic resonance (NMR) spectra were recorded on a Varian Gemini 200, Varian Unity 300, Bruker 500 or Varian Unity 600, with tetramethylsilane (TMS) as internal standard. Low- and high-resolution mass spectra were measured on a JEOL JMS AX-500 spectrometer. Column chromatography was performed over silica gel (230–400 Mesh, Merck) with hexane-AcOEt or toluene-EtOH gradients as mobile phases. For thin-layer chromatography, silica gel precoated aluminum plates (Kiesel gel 60 F254, Merck) were employed. The visualization of spots was accomplished by spraying the plates with a H_2SO_4 solution followed by heating. High-performance liquid chromatography (HPLC) separations were performed using C8 and C18 Phenomenex columns (*Luna*, 5 μm, 10×250 mm) with pure methanol or methanol–water mixtures as mobile phases.

Insects A colony of *C. capitata* was initiated with pupae obtained from infested oranges from the northwest of Argentina. Adults were fed on an artificial diet made of water and a mixture of sugar and yeast hydrolysate (3:1). They were maintained in a rearing room with a photoperiod 12L:12D, at 24±2°C and 60±10% relative humidity.

Oviposition-Deterrent Activity Artificial fruits (oviposition substrates) were prepared by boiling a mixture of peach juice (500 ml), agar (15 g), and sodium benzoate (one teaspoonful) as preservative (Fig. 2). This agar solution was poured into cylindrical molds, allowed to gel, and sliced. The agar cylinders were then wrapped in plastic wrap Rolopac to avoid dehydration. The surface of the wrapped cylinder was pricked with a needle and treated with an acetone or methanol solution of the sample to be tested. An amount of 15 μg/cm^2 of the test compound was deposited. Control cylinders were impregnated only with the solvent that was then removed in vacuo. Three groups of *C. capitata* adults were selected from the laboratory colony. Each group, consisting of seven male–female pairs, was placed in a small cage and covered with voile (a light, almost transparent cloth made of silk). Two agar cylinders (sample and control) were placed over the voile, and females oviposited on one or the other according to their preference (Fig. 2). After 4 days, eggs were gently rinsed from the agar and counted.

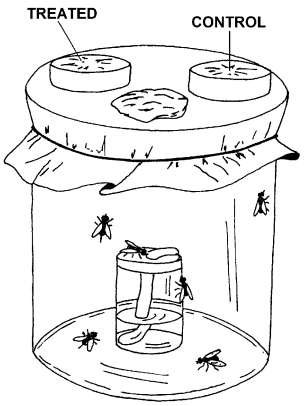
Statistical Analysis Results are reported as mean±SEM. Differences in the mean values were evaluated using the *t* test for all pairwise comparisons. In all statistical analyses, *P* values>0.05 were considered not significant.

Fig. 1 Chemical structures of the compounds evaluated for oviposition-deterrent activity



Synthesis of Aromatic Glycosides All synthetic compounds were prepared by previously reported methods (Conchie et al. 1957; Clingman 1964; Cannizzaro et al. 1998; Burger et al. 2004). The selected sugars were the monosaccharides D-glucose, D-galactose, and the disaccharide lactose. The synthetic scheme for the α-anomeric compounds is presented in Scheme 1. To obtain the β-anomeric compounds of glucose, *p*-toluenesulfonic acid was used as catalyst for the first reaction step instead of $ZnCl_2$. The β-anomeric glycoside of lactose was obtained by the Paulsen condensation method (Burger et al. 2004).

Fig. 2. Experimental setup for oviposition bioassay. Agar cylinders with peach juice were employed as artificial fruits. One of them contains the compound of interest on the surface (*treated*) and the other does not (*control*). They are placed over the cage containing the flies



Aromatic Glycosides Tested in Bioassays The synthetic compounds **1**–**25** (Fig. 1) were purified by reverse-phase HPLC, and their structures were established by spectroscopic methods (1 and 2D NMR, high-resolution mass spectrometry (MS), and infrared (IR)). NMR data are compiled (see Appendix).

Compounds **26**–**29** Compound **26,** *p*-vinylphenyl (2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-2,4,6-tri-*O*-acetyl-β-D-allopyranoside was obtained by acetylation of elaphoside-A (Socolsky et al. 2003). Compounds **27**, *p*-(1-metoxyethyl) anisole obtained as a racemic mixture, and **28,** methyl (β-D-glucopyranosyl)-(1→3)-β-D-allopyranoside, were products of methanolysis of elaphoside-A. Finally, compound **29**, methyl (2′,3′,4′,6′-tetra-*O*-acetyl-β-D-glucopyranosyl)-(1→3)-2,4,6-

tri-*O*-acetyl-β-D-allopyranoside, was obtained by acetylation of the methylated sugar.

Results and Discussion

For all compounds, the ^{1}H NMR spectra showed two doublet signals characteristic of a *para*-disubstituted benzene ring. One of them was located between 7.38 and 7.10 ppm (2H, d, $J\approx8$ Hz), and the other between 7.12 and 6.92 ppm (2H, d, $J\approx8$ Hz). The ethyl group of compounds **1**–**6** and **17**–**20** produced a quartet located between δ 2.60 and 2.47 (2H, q, $J\approx8$ Hz), and a triplet in the 1.21–1.04 ppm range (3H, t, $J\approx8$ Hz). The signals for the 1″-hydroxyethyl substituent of compounds **7**–**10**, **21**, and **22** were located in the δ 4.88–4.75 range (1H, q, $J\approx6.5$ Hz) and 1.48–1.32 (3H, d, $J\approx6.5$ Hz). Regarding the vinyl group of compounds **13**–**16**, **24**, and **25**, three characteristic signals were observed in the proton spectrum at δ 6.66 (1H, dd, $J\approx18$, 11 Hz), 5.65–5.63 (1H, dd, $J\approx18$, 1 Hz), and 5.18–5.10 ppm (1H, dd, $J\approx11$, 1 Hz). Finally, the 1″-acetoxyethyl substituent of compounds **11**, **12**, and **23** showed three signals at δ 5.86 5.83 (1H, q, $J=6.5$ Hz), δ 2.17–2.04 (3H, s), and δ 1.52–1.51 (3H, d, $J=6.5$ Hz). The proton NMR spectra of compounds **1**–**25** showed characteristic signals for the sugar moieties glucose, galactose, and lactose. Compounds **7**–**12** and **21**–**23** were obtained as mixtures of C-1″ epimers; therefore, in their ^{1}H and ^{13}C NMR spectra, some signals were duplicated. As the diastereomers' mixtures could not be resolved by high-resolution chromatographic methods, these compounds were tested as a 1:1 mixture of both epimers; therefore, the final concentration of each epimer was 7.5 μg/cm^2.

The previously described synthetic glycosides **1**–**10**, **12**–**22**, **24**, and **25** (Fig. 1) were evaluated in the bioassay (Fig. 2) in order to determine the structural requirements for oviposition-deterrent activity, taking into account that elaphoside-A is a very active compound. Our aim was to investigate how the activity is influenced by hydroxylation of the vinyl group and different sugar residues attached to

Table 1 Effect of aromatic glycosides on the oviposition-behavior of *C. capitata*

Compound	Number of Eggs Laid on the Control Fruit [a]	Number of Eggs Laid on the Treated Fruit [a]	Io=(100*T*/*C*)−100
Elaphoside-A	897.3±120.7a	464.7±73.0b	−47.9
1	979.0±51.6a	751.3±41.0b	−23.3
2	1081.7±137.4a	1091.0±123.4a	0.9
3	786.3±98.0a	744.7±89.7a	−5.3
4	1023.3±78.0a	657.3±83.8b	−35.8
5	395.3±47.6a	390.0±55.3a	−1.3
6	530.3±46.6a	356.3±63.7b	−32.8
7	1029.3±109.9a	666.7±47.2b	−35.2
8	1072.7±73.5a	990.3±17.9a	−7.7
9	1159.0±17.6a	894.0±85.1b	−22.9
10	1212.7±80.8a	810.3±83.1b	−33.2
12	1068.0±69.7a	1036.7±64.0a	−2.9
13	1140.3±127.6a	986.0±21.6a	−13.5
14	1090.3±76.6a	1014.3±87.9a	−7.0
15	976.7±23.6a	697.3±23.5b	−28.6
16	991.7±36.9a	687.7±29.3b	−30.6
17	1126.7±67.6a	1136.7±140.3a	0.9
18	353.0±35.8a	319.3±58.4a	−9.5
19	352.3±45.3a	242.0±35.0b	−31.3
20	1135.3±87.1a	1050.7±148.8a	−7.4
21	977.3±7.5a	820.0±52.6b	−16.0
22	697.0±61.6a	752.0±101.5a	7.9
24	1028.7±77.6a	963.0±61.8a	−6.4
25	757.3±52.0a	633.7±45.9b	−16.3
26	505.7±99.6a	458.0±96.7a	−9.4
27	645.3 ±34.4a	369.0±39.9b	−42.8
28	520.7±11.0a	432.0±43.6a	−17.0
29	511.7±29.9a	412.3±20.0b	−10.0
30	571.7±26.4a	312.0±15.1b	−45.4

Numbers represent mean±SEM, $n=3$. Means within a row followed by the same letter are not significantly different ($P>0.05$, paired *t* test)

the styrene moiety and acetylation of the sugars. We also tested the activity of products **26**–**29** obtained by derivatization and degradation of elaphoside-A (Fig. 1). The effects of elaphoside-A and *p*-vinylanisole (**30**) were evaluated under the same experimental conditions for comparison.

Oviposition-Deterrent Activity The results are summarized in Table 1. To facilitate their interpretation, an oviposition index was defined as Io=(100*T*/*C*)−100, where *T* is the number of eggs laid in the treated artificial fruit, and *C* is the number of eggs deposited in the control fruit. This index takes negative values for oviposition deterrents and positive values for oviposition attractants.

As shown (Table 1), the only compounds that inhibit oviposition at a rate close to that of the natural product elaphoside-A are **27** (racemic mixture) and **30**, indicating that the presence of a methoxy group attached to the aromatic ring confers activity to the compound. It is noted that the effect of each enantiomer in mixture **27** could not be assessed since the racemate could not be resolved.

None of the synthetic glycosides was as active as elaphoside-A, although the α-anomeric compounds **4**, **6**, **7**, **10**, and **16**, as well as the β-anomeric peracetylated glycoside of the disaccharide lactose (**19**), were fairly active, with Io<−30. Acetylation of elaphoside-A led to a loss of activity. An Io=−47.9 was determined for elaphoside-A, while nonsignificant effects were displayed by the peracetylated analog **26**. Accordingly, pairwise comparison of effects produced by the peracetylated glycosides **2**, **5**, **8**, **13**, **14**, and **24** (Io, 0.9, −1.3, −7.7, −13.5, −7.0, and −6.4, respectively) with those of the corresponding non-acetylated analogs **4**, **6**, **10**, **15**, **16**, and **25** (Io, −35.8, −32.8, −33.2, −28.6, −30.6, and −16.3, respectively), indicated that acetylation produced decrease or loss of the activity. In contrast, pairwise comparison of the effects produced by the α-anomers of glucose **4**, **10**, and **16** (Io, −35.8, −33.2, and −30.6, respectively) with those of the corresponding β-anomers **18**, **22**, and **25** (Io, −9.5, 7.9, −16.3, respectively) indicates that the former are more deterrent than the latter.

To evaluate the changes in activity when the vinyl group, present in the natural product elaphoside-A, was chemically modified, glycosides carrying the substituents ethyl (**1**–**6**, and **7**–**20**), 1″-hydroxyethyl (**7**–**10**, **21**, and **22**), and 1″-acetoxyethyl (**12**) were evaluated in the bioassay. As shown (Table 1), different effects were observed in each case, and thus, no structure–activity relationships could be derived.

While elaphoside-A (Io=−47.9) and the most active synthetic glycoside, *p*-ethylphenyl α-D-glucopyranoside **4** (Io=−35.8), are bitter-tasting substances, their peracetylated derivatives **26** (Io=−9.4) and **2** (Io=+0.9), respectively, had no apparent taste. Thus, taste may play a role in the election of the oviposition substrate by *C. capitata*.

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Appendix

p-Ethylphenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Galactopyranoside (**1**)

High-resolution fast atom bombardment mass spectrum (HR-FAB-MS) *m/z*: 475.1599 $[M + Na]^+$, calculated for $C_{22}H_{28}O_{10}Na$: 475.1580; $[\alpha]_D$ +251.8 ($CHCl_3$, c 1.0 g/dl, 19.4°C); IR ν_{max} [cm^{-1}] (neat), 1,747, 1,608, 1,510, 1,371, 1,217, 1,070; ^1H NMR (500 MHz, $CDCl_3$), δ 6.98 (d, *J*=8.8 Hz, H-2′, H-6′), 7.13 (d, *J*=8.5 Hz, H-3′, H-5′), 2.60 (q, *J*=7.5 Hz, H-1″), 1.21 (t, *J*=7.5 Hz, H-2″), 5.73 (d, *J*=3.6 Hz, H-1), 5.28 (dd, *J*=10.9, 3.7 Hz, H-2), 5.58 (dd, *J*=11.0, 3.4 Hz, H-3), 5.53 (dd, *J*=3.4, 1.1 Hz, H-4), 4.37 (t, *J*=6.6 Hz, H-5), 4.13 (dd, *J*=11.2, 6.3 Hz, H-6a), 4.07 (dd, *J*=11.2, 7.2 Hz, H-6b), 2.16, 2.07, 2.02, 1.94 (s, CH_3C=O); ^{13}C NMR (125 MHz, $CDCl_3$): δ 154.4 (C-1′), 116.8 (C-2′, C-6′), 128.9 (C-3′, C-5′), 139.0 (C-4′), 28.1 (C-1″), 15.8 (C-2″), 95.1 (C-1), 67.9 (C-2), 67.6 (C-3), 68.0 (C-4), 67.1 (C-5), 61.5 (C-6), 170.4, 170.3, 170.2, 170.0 (C=O), 20.74, 20.69, 20.64, 20.58 (CH_3C=O).

p-Ethylphenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Glucopyranoside (**2**)

High-resolution chemical ionization mass spectrum (HR-CI-MS) *m/z*: 453.1775 $[M + H]^+$, calculated for $C_{22}H_{29}O_{10}$: 453.1761; ^1H NMR (500 MHz, $CDCl_3$): δ 7.00 (d, *J*=8.6 Hz, H-2′, H-6′), 7.13 (d, *J*=8.4 Hz, H-3′, H-5′), 2.60 (q, *J*=7.6 Hz, H-1″), 1.21 (t, *J*=7.6 Hz, H-2″), 5.70 (d, *J*=3.8 Hz, H-1), 5.03 (dd, *J*=10.2, 3.6 Hz, H-2), 5.70 (t, *J*=9.8 Hz, H-3), 5.15 (t, *J*=9.9 Hz, H-4), 4.14 (ddd, *J*=10.3, 4.5, 2.2 Hz, H-5), 4.25 (dd, *J*=12.3, 4.6 Hz, H-6a), 4.06 (dd, *J*=12.3, 2.3 Hz, H-6b), 2.06, 2.05, 2.04, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 154.1 (C-1′), 116.5 (C-2′, C-6′), 128.8 (C-3′, C-5′), 139.0 (C-4′), 28.0 (C-1″), 15.7 (C-2″), 94.3 (C-1), 70.4 (C-2), 70.1 (C-3), 68.3 (C-4), 67.8 (C-5), 61.6 (C-6), 170.5, 170.1, 169.6 (C=O), 20.66, 20.59, 20.57, 20.54 (CH_3C=O).

p-Ethylphenyl α-D-Galactopyranoside (**3**)

High-resolution electron impact mass spectrum (HR-EI-MS) *m/z*: 284.1258 $[M]^+$, calculated for $C_{14}H_{20}O_6$: 284.1260; $[\alpha]_D$ +222.5 (MeOH, c 1.0 g/dl, 20.3°C); IR ν_{max} [cm^{-1}] (neat), 3,366, 1,609, 1,511, 1,228, 1,080, 1,032; ^{1}H NMR (500 MHz, MeOH-d_4): δ 6.97 (d, *J*=8.9 Hz, H-2′, H-6′), 7.11 (d, *J*=8.9 Hz, H-3′, H-5′), 2.58 (q, *J*=7.6 Hz, H-1″), 1.19 (t, *J*=7.6 Hz, H-2″), 5.43 (d, *J*=3.2 Hz, H-1), 3.96–3.91 (overlapping signals, H-2, H-3, H-5), 3.98 (dd, *J*=2.8, 1.1 Hz, H-4), 3.71 (dd, *J*=11.4, 5.7 Hz, H-6a), 3.67 (dd, *J*=11.4, 6.6 Hz, H-6b); ^{13}C NMR (50 MHz, MeOH-d_4): δ 156.9 (C-1′), 118.4 (C-2′, C-6′), 129.6 (C-3′, C-5′), 139.5 (C-4′), 29.1 (C-1″), 16.4 (C-2″), 100.0 (C-1), 70.1 (C-2)[1], 71.4 (C-3)[1], 70.8 (C-4), 73.0 (C-5), 62.5 (C-6).

p-Ethylphenyl α-D-Glucopyranoside (**4**)

HR-FAB-MS *m/z*: 307.1174 $[M + Na]^+$, calculated for $C_{14}H_{20}O_6Na$: 307.1158; ^{1}H NMR (500 MHz, D_2O): δ 6.98 (d, *J*=8.7 Hz, H-2′, H-6′), 7.13 (d, *J*=8.7 Hz, H-3′, H-5′), 2.47 (q, *J*=7.5 Hz, H-1″), 1.04 (t, *J*=7.5 Hz, H-2″), 5.46 (d, *J*=3.6 Hz, H-1), 3.58 (dd, *J*=9.8, 3.6 Hz, H-2), 3.78 (t, *J*=9.4 Hz, H-3), 3.38 (t, *J*=9.6 Hz, H-4), 3.65 (ddd, *J*=9.7, 4.7, 2.5 Hz, H-5), 3.63–3.60 (overlapping signals, H-6a, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 154.8 (C-1′), 118.2 (C-2′, C-6′), 129.9 (C-3′, C-5′), 140.5 (C-4′), 28.2 (C-1″), 16.0 (C-2″), 98.2 (C-1), 71.9 (C-2), 73.8 (C-3), 70.2 (C-4), 73.2 (C-5), 61.1 (C-6).

p-Ethylphenyl 2,3,6,2′,3′,4′,6′-Hepta-*O*-Acetyl-α-Lactoside (**5**)

HR-EI-MS *m/z*: 740.2523 $[M]^+$, calculated for $C_{34}H_{44}O_{18}$: 740.2527; $[\alpha]_D$ +83.3 ($CHCl_3$, c 1.0 g/dl, 20.1°C); IR ν_{max} [cm^{-1}] (neat), 1,747, 1,510, 1,369, 1,217, 1,047; ^{1}H NMR (600 MHz, $CDCl_3$): δ 7.00 (d, *J*=8.5 Hz, H-2″, H-6″), 7.12 (d, *J*=8.5 Hz, H-3″, H-5″), 2.60 (q, *J*=7.6 Hz, H-1‴), 1.21 (t, *J*=7.6 Hz, H-2‴), 5.60 (d, *J*=3.6 Hz, H-1), 4.95 (dd, *J*=10.3, 3.7 Hz, H-2), 5.69 (dd, *J*=10.2, 9.6 Hz, H-3), 3.84 (t, *J*=9.7 Hz, H-4), 4.05 (ddd, *J*=10.1, 4.6, 1.9 Hz, H-5), 4.42 (dd, *J*=12.0, 1.9 Hz, H-6a), 4.14 (dd, *J*=12.1, 4.8 Hz, H-6b), 4.50 (d, *J*=7.9 Hz, H-1′), 5.12 (dd, *J*=10.3, 7.9 Hz, H-2′), 4.96 (dd, *J*=10.4, 3.5 Hz, H-3′), 5.36 (dd, *J*=3.6, 1.1 Hz, H-4′), 3.89 (td, *J*=7.3, 1.1 Hz, H-5′), 4.16 (dd, *J*=11.2, 6.4 Hz, H-6′a), 4.09 (dd, *J*=11.2, 7.3 Hz, H-6′b), 2.17, 2.09, 2.08, 2.05, 2.04, 2.02, 1.96 (s, C*H*$_3$C=O); ^{13}C NMR (150 MHz, $CDCl_3$): δ 154.2 (C-1″), 116.5 (C-2″, C-6″), 128.8 (C-3″, C-5″), 138.9 (C-4″), 28.0 (C-1‴), 15.8 (C-2‴), 94.3 (C-1), 70.68 (C-2), 69.8 (C-3), 76.2 (C-4), 68.7 (C-5), 61.7 (C-6), 101.0 (C-1′), 69.1 (C-2′), 71.0 (C-3′), 66.6 (C-4′), 70.73 (C-5′), 60.9 (C-6′) 170.32, 170.30, 170.1, 170.0, 169.7, 169.6, 169.1 (C=O), 20.8, 20.7, 20.62, 20.58, 20.57, 20.4 (*C*H$_3$C=O).

p-Ethylphenyl α-Lactoside (**6**)

HR-EI-MS *m/z*: 446.1790 $[M]^+$, calculated for $C_{20}H_{30}O_{11}$: 446.1788; $[\alpha]_D$ +126.3 (MeOH, c 1.0 g/dl, 20.0°C); IR ν_{max} [cm^{-1}] (neat), 3,387, 1,610, 1,510, 1,373, 1,227, 1,070, 1,020; ^{1}H NMR (600 MHz, MeOH-d_4): δ 7.05 (d, *J*=8.7 Hz, H-2″, H-6″), 7.11 (d, *J*=8.8 Hz, H-3″, H-5″), 2.58 (q, *J*=7.6 Hz, H-1‴), 1.19 (t, *J*=7.6 Hz, H-2‴), 5.42 (d, *J*=3.7 Hz, H-1), 3.62 (dd, *J*=9.8, 3.7 Hz, H-2), 3.98 (dd, *J*= 9.7, 8.8 Hz, H-3), 3.67 (dd, *J*=9.9, 8.7 Hz, H-4), 3.79 (t, *J*=3.3 Hz, H-5), 3.87 (dd, *J*=12.2, 3.6 Hz, H-6a), 3.74 (dd, *J*=12.3, 3.5 Hz, H-6b), 4.38 (d, *J*=7.7 Hz, H-1′), 3.56 (dd, *J*=9.7, 7.7 Hz, H-2′), 3.49 (dd, *J*=9.8, 3.3 Hz, H-3′), 3.82 (dd, *J*=3.3, 0.7 Hz, H-4′), 3.60 (ddd, *J*=7.5, 4.7, 0.9 Hz, H-5′), 3.80 (dd, *J*=11.4, 7.5 Hz, H-6′a), 3.71 (dd, *J*=11.5, 4.6 Hz, H-6′b); ^{13}C NMR (150 MHz, MeOH-d_4): δ 156.6 (C-1″), 118.1 (C-2″, C-6″), 129.7 (C-3″, C-5″), 139.6 (C-4″), 29.1 (C-1‴), 16.5 (C-2‴), 99.2 (C-1), 73.1 (C-2), 73.4 (C-3), 80.6 (C-4), 77.2 (C-5), 62.6 (C-6), 105.2 (C-1′), 72.7 (C-2′)[1], 74.9 (C-3′), 70.4 (C-4′), 72.6 (C-5′)[1], 61.6 (C-6′).

p-(1″-Hydroxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Galactopyranoside (Mixture of Epimers **7**)

HR-FAB-MS *m/z*: 491.1509 $[M + Na]^+$, calculated for $C_{22}H_{28}O_{11}Na$: 491.1529; IR ν_{max} [cm^{-1}] (neat), 3,481, 1,745, 1,608, 1,510, 1,371, 1,217, 1,066; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.04 (d, *J*=8.6 Hz, H-2′, H-6′), 7.32 (d, *J*=8.6 Hz, H-3′, H-5′), 4.87 (q, *J*=6.4 Hz, H-1″), 1.48/1.47 (d, *J*=6.5 Hz, H-2″)[2], 5.76 (d, *J*=3.2 Hz, H-1), 5.28 (dd, *J*=10.9, 3.6 Hz, H-2), 5.58 (dd, *J*=10.9, 3.4 Hz, H-3), 5.53 (dd, *J*=3.4, 1.0 Hz, H-4), 4.35 (t, *J*=6.6 Hz, H-5), 4.12 (dd, *J*=11.3, 6.3 Hz, H-6a), 4.07 (dd, *J*=11.3, 6.6 Hz, H-6b)/4.07 (dd, *J*=11.2, 6.6 Hz, H-6b)[2], 2.17, 2.08, 2.03, 1.95 (s, C*H*$_3$CO); ^{13}C NMR (50 MHz, $CDCl_3$): δ 155.6 (C-1′), 116.7 (C-2′, C-6′), 126.7 (C-3′, C-5′), 140.5 (C-4′), 69.8/69.7 (C-1″)[2], 25.18/25.16 (C-2″)[2], 95.0 (C-1), 67.78 (C-2), 67.5 (C-3), 67.83 (C-4), 67.1 (C-5), 61.4 (C-6), 170.4, 170.3, 170.2, 170.0 (C=O), 20.7, 20.63, 20.59, 20.55 (*C*H$_3$C=O).

p-(1″-Hydroxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Glucopyranoside (Mixture of Epimers **8**)

HR-FAB-MS *m/z*: 491.1509 $[M + Na]^+$, calculated for $C_{22}H_{28}O_{11}Na$: 491.1529; IR ν_{max} [cm^{-1}] (neat), 3,500, 1,751, 1,608, 1,508, 1,369, 1,219, 1,043; ^{1}H NMR

[1] Interchangeable signals.

[2] Duplicated signals due to the presence of the two C-1 epimers.

(500 MHz, $CDCl_3$): δ 7.07 (d, J=8.6 Hz, H-2′, H-6′), 7.32 (d, J=8.6 Hz, H-3′, H-5′), 4.88 (q, J=6.4 Hz, H-1″), 1.48/1.48 (d, J=6.5 Hz, H-2″)[3], 5.73 (d, J=3.8 Hz, H-1)/5.73 (d, J=4.0 Hz, H-1)[3], 5.04 (dd, J=10.2, 3.7 Hz, H-2), 5.71 (t, J=9.9 Hz, H-3), 5.16 (dd, J=10.2, 9.5 Hz, H-4), 4.12 (ddd, J=10.3, 4.4, 2.2 Hz, H-5), 4.25 (dd, J=12.3, 4.5 Hz, H-6a), 4.05/4.04 (dd, J=12.3, 1.9 Hz, H-6b)[3], 2.06, 2.05, 2.04, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.3 (C-1′), 116.5 (C-2′, C-6′), 126.7 (C-3′, C-5′), 140.6 (C-4′), 69.7/69.6 (C-1″)[3], 25.2 (C-2″), 94.3 (C-1), 70.4 (C-2), 70.0 (C-3), 68.2 (C-4), 67.9 (C-5), 61.5 (C-6), 170.5, 170.14, 170.12, 169.6 (C=O), 20.7, 20.61, 20.57, 20.55 (*C*H_3C=O).

p-(1″-Hydroxyethyl)-Phenyl α-D-Galactopyranoside (Mixture of Epimers **9**)

HR-EI-MS *m/z*: 300.1210 $[M]^+$, calculated for $C_{14}H_{20}O_7$: 300.1209; ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.12 (d, J=8.5 Hz, H-2′, H-6′), 7.28 (d, J=8.5 Hz, H-3′, H-5′), 4.77 (q, J=6.5 Hz, H-1″), 1.40 (d, J=6.5 Hz, H-2″), 5.47 (d, J=3.0 Hz, H-1)/5.47 (d, J=2.5 Hz, H-1)[3], 3.96–3.93 (overlapping signals, H-2, H-3), 3.97 (dd, J=2.5, 1.3 Hz, H-4), 3.92 (t, J=6.3 Hz, H-5), 3.69 (dd, J=11.3, 5.7 Hz, H-6a), 3.66/3.66 (dd, J=11.4, 6.7 Hz, H-6b)[3]; ^{13}C NMR (125 MHz, MeOH-d_4): δ 157.9 (C-1′), 118.2 (C-2′, C-6′), 127.6 (C-3′, C-5′), 141.5 (C-4′), 70.44/70.40 (C-1″)[3], 25.5 (C-2″), 99.8 (C-1), 70.0 (C-2)[4], 71.4 (C-3)[4], 70.8 (C-4), 73.0 (C-5), 62.4 (C-6).

p-(1″-Hydroxyethyl)-Phenyl α-D-Glucopyranoside (Mixture of Epimers **10**)

HR-EI-MS *m/z*: 300.1212 $[M]^+$, calculated for $C_{14}H_{20}O_7$: 300.1209; ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.12 (d, J=8.5 Hz, H-2′, H-6′), 7.28 (d, J=8.6 Hz, H-3′, H-5′), 4.77 (q, J=6.4 Hz, H-1″), 1.40 (d, J=6.5 Hz, H-2″), 5.46 (d, J=3.6 Hz, H-1), 3.56 (dd, J=9.7, 3.7 Hz, H-2), 3.85 (t, J=9.3 Hz, H-3), 3.43 (t, J=9.3 Hz, H-4), 3.65 (ddd, J=9.6, 4.6, 2.5 Hz, H-5), 3.72 (dd, J=11.7, 2.1 Hz, H-6a), 3.68 (dd, J=11.8, 4.6 Hz, H-6b); ^{13}C NMR (125 MHz, MeOH-d_4): δ 157.7 (C-1′), 118.0 (C-2′, C-6′), 127.7 (C-3′, C-5′), 141.5 (C-4′), 70.41/70.37 (C-1″)[3], 25.5 (C-2″), 99.4 (C-1), 73.3 (C-2), 74.9 (C-3), 71.5 (C-4), 74.3 (C-5), 62.3 (C-6).

p-(1″-Acetoxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Galactopyranoside (Mixture of Epimers **11**)

HR-CI-MS *m/z*: 510.1757 $[M]^+$, calculated for $C_{24}H_{30}O_{12}$: 510.1737; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.04 (d, J=8.5 Hz, H-2′, H-6′)/7.03 (d, J=8.7 Hz, H-2′, H-6′)[3], 7.30 (d, J=8.5 Hz, H-3′, H-5′), 5.84/5.84 (q, J=6.5 Hz, H-1″)[3], 1.52/1.51 (d, J=6.5 Hz, H-2″)[3], 5.77/5.76 (d, J=3.6 Hz, H-1)[3], 5.28 (dd, J=10.9, 3.7 Hz, H-2)/5.27 (dd, J=11.0, 3.6 Hz, H-2)[3], 5.57 (dd, J=10.9, 3.5 Hz, H-3), 5.52 (d, J=3.4 Hz, H-4), 4.33 (t, J=6.8 Hz, H-5), 4.12 (dd, J=11.2, 6.5 Hz, H-6a), 4.07 (dd, J=11.2, 6.9 Hz, H-6b), 2.17, 2.06, 2.05, 2.05, 2.03 (s, CH_3CO); ^{13}C NMR (50 MHz, $CDCl_3$): δ 155.8 (C-1′), 116.6 (C-2′, C-6′), 127.6 (C-3′, C-5′), 136.3 (C-4′), 71.74/71.69 (C-1″)[3], 22.0 (C-2″), 94.7 (C-1), 67.7 (C-2), 67.4 (C-3), 67.8 (C-4), 67.1 (C-5), 61.3 (C-6), 170.3, 170.2, 170.1, 170.0 (C=O), 21.3, 20.59, 20.55, 20.47 (*C*H_3C=O).

p-(1″-Acetoxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Glucopyranoside (Mixture of Epimers **12**)

HR-CI-MS *m/z*: 510.1708 $[M]^+$, calculated for $C_{24}H_{30}O_{12}$: 510.1737; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.07/7.06 (d, J=8.8 Hz, H-2′, H-6′)[3], 7.30 (d, J=8.8 Hz, H-3′, H-5′), 5.84/5.83 (q, J=6.5 Hz, H-1″)[3], 1.51 (d, J=6.7 Hz, H-2″), 5.73/5.72 (d, J=3.7 Hz, H-1)[3], 5.04 (dd, J=10.3, 3.6 Hz, H-2)/5.03 (dd, J=10.2, 3.7 Hz, H-2)[3], 5.70 (t, J=9.9 Hz, H-3), 5.16 (dd, J=10.1, 9.4 Hz, H-4)/5.15 (dd, J=10.2, 9.5 Hz, H-4)[3], 4.10 (ddd, J=10.2, 4.3, 2.1 Hz, H-5), 4.25/4.24 (dd, J=12.3, 4.3 Hz, H-6a)[3], 4.05 (dd, J=12.4, 2.0 Hz, H-6b), 2.17, 2.06, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.7/155.6 (C-1′)[3], 116.5 (C-2′, C-6′), 127.6 (C-3′, C-5′), 136.39/136.37 (C-4′)[3], 71.8/71.7 (C-1″)[3], 22.04/22.02 (C-2″)[3], 94.20/94.15 (C-1)[3], 70.4 (C-2), 70.0 (C-3), 68.3 (C-4), 68.0 (C-5), 61.5 (C-6), 170.5, 170.2, 170.11, 170.08, 169.5 (C=O), 21.3, 20.65, 20.58, 20.55 (*C*H_3C=O).

p-Vinylphenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Galactopyranoside (**13**)

HR-EI-MS *m/z*: 450.1522 $[M]^+$, calculated for $C_{22}H_{26}O_{10}$: 450.1526; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.02 (d, J=8.7 Hz, H-2′, H-6′), 7.35 (d, J=8.7 Hz, H-3′, H-5′), 6.66 (dd, J=17.8, 10.9 Hz, H-1″), 5.65 (dd, J=17.6, 0.7 Hz, H-2″*trans*), 5.18 (dd, J=10.9, 0.5 Hz, H-2″*cis*), 5.78 (d, J=3.7 Hz, H-1), 5.29 (dd, J=10.7, 3.7 Hz, H-2), 5.58 (dd, J=10.8, 3.3 Hz, H-3), 5.53 (dd, J=3.3, 1.0 Hz, H-4), 4.34 (td, J=7.0, 0.9 Hz, H-5), 4.12 (dd, J=11.3, 6.3 Hz, H-6a), 4.06 (dd, J=11.3, 7.1 Hz, H-6b), 2.17, 2.08, 2.03, 1.94 (s, CH_3C=O); ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.9 (C-1′), 116.8 (C-2′, C-6′), 127.4 (C-3′, C-5′), 132.7 (C-4′), 135.9 (C-1″), 112.9 (C-2″), 94.8 (C-1), 67.8 (C-2), 67.5 (C-3), 67.9 (C-4), 67.2 (C-5), 61.4 (C-6), 170.4, 170.3, 170.2, 170.0 (C=O), 20.7, 20.65, 20.61, 20.5 (*C*H_3C=O).

p-Vinylphenyl 2,3,4,6-Tetra-*O*-Acetyl-α-D-Glucopyranoside (**14**)

HR-EI-MS *m/z*: 450.1534 $[M]^+$, calculated for $C_{22}H_{26}O_{10}$: 450.1526; ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.05 (d, J=

[3] See footnote 2.
[4] See footnote 1.

8.7 Hz, H-2′, H-6′), 7.36 (d, *J*=8.7 Hz, H-3′, H-5′), 6.66 (dd, *J*=17.5, 10.9 Hz, H-1″), 5.65 (dd, *J*=17.5, 0.7 Hz, H-2″*trans*), 5.19 (dd, *J*=10.8, 0.7 Hz, H-2″*cis*), 5.74 (d, *J*= 3.7 Hz, H-1), 5.04 (dd, *J*=10.3, 3.6 Hz, H-2), 5.70 (t, *J*= 9.9 Hz, H-3), 5.16 (t, *J*=9.4 Hz, H-4), 4.11 (ddd, *J*= 10.3, 4.4, 2.2 Hz, H-5), 4.25 (dd, *J*=12.3, 4.6 Hz, H-6a), 4.05 (dd, *J*=12.3, 2.3 Hz, H-6b), 2.06, 2.05, 2.04, 2.04 (s, C*H*$_3$CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 155.7 (C-1′), 116.6 (C-2′, C-6′), 127.4 (C-3′, C-5′), 132.7 (C-4′), 135.8 (C-1″), 112.9 (C-2″), 94.2 (C-1), 70.4 (C-2), 70.0 (C-3), 68.3 (C-4), 68.0 (C-5), 61.6 (C-6), 170.5, 170.1, 169.6 (C=O), 20.7, 20.62, 20.60, 20.57 (*C*H$_3$C=O).

p-Vinylphenyl α-D-Galactopyranoside (**15**)

HR-EI-MS *m/z*: 282.1097 $[M]^+$, calculated for $C_{14}H_{18}O_6$: 282.1103; $[\alpha]_D$ +275.8 (MeOH, c 1.0 g/dl, 20.1°C); IR ν_{max} [cm^{-1}] (neat), 3,389, 1,629, 1,605, 1,508, 1,234, 1,082, 1,033; ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.12 (d, *J*=8.8 Hz, H-2′, H-6′), 7.35 (d, *J*=8.8 Hz, H-3′, H-5′), 6.66 (dd, *J*=17.7, 11.0 Hz, H-1″), 5.63 (dd, *J*=17.5, 0.9 Hz, H-2″*trans*), 5.10 (dd, *J*=11.0, 0.9 Hz, H-2″*cis*), 5.49 (d, *J*=2.2 Hz, H-1), 3.89–3.84 (overlapping signals, H-2, H-3), 3.97 (dd, *J*=1.8, 1.1 Hz, H-4), 3.91 (t, *J*=6.3 Hz, H-5), 3.70 (dd, *J*=11.4, 5.6 Hz, H-6a), 3.66 (dd, *J*=11.4, 6.6 Hz, H-6b); ^{13}C NMR (125MHz, MeOH-d_4): δ 158.5 (C-1′), 118.3 (C-2′, C-6′), 128.3 (C-3′, C-5′), 133.4 (C-4′), 137.5 (C-1″), 112.4 (C-2″), 99.7 (C-1), 70.0 (C-2)[5], 71.4 (C-3)[5], 70.8 (C-4), 73.1 (C-5), 62.4 (C-6).

p-Vinylphenyl α-D-Glucopyranoside (**16**)

HR-EI-MS *m/z*: 282.1102 $[M]^+$, calculated for $C_{14}H_{18}O_6$: 282.1103; ^{1}H NMR (500 MHz, MeOH-d_4): δ 7.11 (d, *J*= 8.8 Hz, H-2′, H-6′), 7.35 (d, *J*=8.5 Hz, H-3′, H-5′), 6.66 (dd, *J*=17.7, 11.0 Hz, H-1″), 5.64 (dd, *J*=17.7, 0.8 Hz, H-2″*trans*), 5.11 (dd, *J*=11.0, 0.7 Hz, H-2″*cis*), 5.47 (d, *J*= 3.6 Hz, H-1), 3.56 (dd, *J*=9.7, 3.7 Hz, H-2), 3.84 (t, *J*= 9.3 Hz, H-3), 3.42 (t, *J*=9.4 Hz, H-4), 3.64 (ddd, *J*=9.8, 4.7, 2.5 Hz, H-5), 3.73 (dd, *J*=11.9, 2.5 Hz, H-6a), 3.68 (dd, *J*=11.9, 4.7 Hz, H-6b); ^{13}C NMR (50 MHz, MeOH-d_4): δ 158.3 (C-1′), 118.1 (C-2′, C-6′), 128.3 (C-3′, C-5′), 133.4 (C-4′), 137.5 (C-1″), 112.4 (C-2″), 99.2 (C-1), 73.3 (C-2), 74.9 (C-3), 71.5 (C-4), 74.4 (C-5), 62.3 (C-6).

p-Ethylphenyl 2,3,4,6-Tetra-*O*-Acetyl-β-D-Glucopyranoside[6] (**17**)

HR-EI-MS *m/z*: 452.1654 $[M]^+$, calculated for $C_{22}H_{28}O_{10}$: 452.1682; $[\alpha]_D$ −22.2 ($CHCl_3$, c 1.0 g/dl, 20.7°C); IR ν_{max} [cm^{-1}] (neat), 1,751, 1,608, 1,510, 1,367, 1,221, 1,047; ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.92 (d, *J*=8.7 Hz, H-2′, H-6′), 7.12 (d, *J*=8.7 Hz, H-3′, H-5′), 2.60 (q, *J*=7.6 Hz, H-1″), 1.21 (t, *J*=7.6 Hz, H-2″), 5.05 (d, *J*=7.6 Hz, H-1), 5.26 (dd, *J*=9.2, 7.5 Hz, H-2), 5.29 (t, *J*=9.2 Hz, H-3), 5.17 (t, *J*= 9.5 Hz, H-4), 3.85 (ddd, *J*=10.0, 5.3, 2.5 Hz, H-5), 4.29 (dd, *J*=12.3, 5.3 Hz, H-6a), 4.17 (dd, *J*=12.3, 2.4 Hz, H-6b), 2.08, 2.06, 2.05, 2.04 (s, C*H*$_3$C=O); ^{13}C NMR (150 MHz, $CDCl_3$): δ 154.9 (C-1′), 116.9 (C-2′, C-6′), 128.7 (C-3′, C-5′), 139.2 (C-4′), 28.0 (C-1″), 15.7 (C-2″), 99.3 (C-1), 71.1 (C-2), 72.7 (C-3), 68.2 (C-4), 71.8 (C-5), 61.9 (C-6), 170.5, 170.2, 169.3, 169.2 (C=O), 20.61, 20.55, 20.53, 20.50 (*C*H$_3$C=O).

p-Ethylphenyl β-D-Glucopyranoside (**18**)

HR-FAB-MS *m/z*: 307.1156 $[M + Na]^+$, calculated for $C_{14}H_{20}O_6Na$: 307.1158; $[\alpha]_D$ −65.4 (MeOH, c 1.0 g/dl, 19.9°C); IR ν_{max} [cm^{-1}] (neat), 3,335, 1,611, 1,511, 1,233, 1,070; ^{1}H NMR (500 MHz, D_2O): δ 6.96 (d, *J*=8.5 Hz, H-2′, H-6′), 7.14 (d, *J*=8.5 Hz, H-3′, H-5′), 2.49 (q, *J*=7.6 Hz, H-1″), 1.06 (t, *J*=7.5 Hz, H-2″), 4.96 (d, *J*=7.6 Hz, H-1), 3.43 (dd, *J*=9.4, 7.6 Hz, H-2), 3.48 (t, *J*=9.1 Hz, H-3), 3.36 (t, *J*=9.3 Hz, H-4), 3.48 (ddd, *J*=9.9, 5.7, 2.0 Hz, H-5), 3.80 (dd, *J*=12.3, 1.8 Hz, H-6a), 3.62 (dd, *J*=12.4, 5.7 Hz, H-6b); ^{13}C NMR (50 MHz, MeOH-d_4): δ 157.1 (C-1′), 117.7 (C-2′, C-6′), 129.6 (C-3′, C-5′), 139.4 (C-4′), 29.0 (C-1″), 16.4 (C-2″), 102.5 (C-1), 74.8 (C-2), 77.9 (C-3)[5], 71.3 (C-4), 78.0 (C-5)[5], 62.4 (C-6).

p-Ethylphenyl 2,3,6,2′,3′,4′,6′-Hepta-*O*-Acetyl-β-Lactoside (**19**)

HR-FAB-MS *m/z*: 763.2396 $[M + Na]^+$, calculated for $C_{34}H_{44}O_{18}Na$: 763.2425; $[\alpha]_D$ −22.4 ($CHCl_3$, c 1.0 g/dl, 22.4°C); IR ν_{max} [cm^{-1}] (neat), 3,028, 1,751, 1,608, 1,508, 1,369, 1,219, 1,057; ^{1}H NMR (600 MHz, $CDCl_3$): δ 6.89 (d, *J*=8.6 Hz, H-2″, H-6″), 7.10 (d, *J*=8.7 Hz, H-3″, H-5″), 2.60 (q, *J*=7.6 Hz, H-1‴), 1.20 (t, *J*=7.6 Hz, H-2‴), 5.01 (d, *J*=7.8 Hz, H-1), 5.16 (dd, *J*=9.3, 7.8 Hz, H-2), 5.27 (t, *J*=9.1 Hz, H-3), 3.89 (t, *J*=9.4 Hz, H-4), 3.77 (ddd, *J*=9.9, 5.8, 2.2 Hz, H-5), 4.50 (dd, *J*=12.3, 2.6 Hz, H-6a), 4.15 (dd, *J*=12.0, 5.6 Hz, H-6b), 4.51 (d, *J*=8.0 Hz, H-1′), 5.13 (dd, *J*=10.4, 7.9 Hz, H-2′), 4.97 (dd, *J*=10.4, 3.5 Hz, H-3′), 5.36 (dd, *J*=3.4, 1.0 Hz, H-4′), 3.90 (t, *J*=7.1 Hz, H-5′), 4.13 (dd, *J*=11.0, 6.8 Hz, H-6′a), 4.10 (dd, *J*=11.2, 7.3 Hz, H-6′b), 2.16, 2.09, 2.08, 2.07, 2.06, 1.97 (s, C*H*$_3$C=O); ^{13}C NMR (150 MHz, $CDCl_3$): δ 154.8 (C-1″), 116.8 (C-2″, C-6″), 128.7 (C-3″, C-5″), 139.2 (C-4″), 28.0 (C-1‴), 15.7 (C-2‴), 99.0 (C-1), 71.5 (C-2), 72.8 (C-3), 76.2 (C-4), 72.7 (C-5), 62.0 (C-6), 101.0 (C-1′), 69.0 (C-2′), 70.9 (C-3′), 66.6 (C-4′), 70.7 (C-5′), 60.8 (C-6′) 170.41, 170.38, 170.3, 170.2,

[5] See footnote 1.

[6] Known compound (Helferich and Höfmann, 1952; Ojika et al. 1984).

170.1, 169.6, 169.0 (C= O), 20.9, 20.8, 20.67, 20.65, 20.62, 20.59, 20.50 (CH_3C=O).

p-Ethylphenyl β-Lactoside (**20**)

^{1}H NMR (300 MHz, pyridin-d_5): δ 7.26 (d, *J*=8.7 Hz, H-2″, H-6″), 7.43 (d, *J*=8.7 Hz, H-3″, H-5″), 2.64 (q, *J*= 6.8 Hz, H-1‴), 1.26 (t, *J*=6.8 Hz, H-2‴), 5.73 (d, *J*= 7.5 Hz, H-1), 4.81–4.20 (overlapping signals H-2, H-3, H-4, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6′a, H-6′b), 4.24 (ddd, *J*=9.3, 6.3, 2.7 Hz, H-5), 5.31 (d, *J*=8.1 Hz, H-1′); ^{13}C NMR (75 MHz, pyridin-d_5): δ 156.7 (C-1″), 117.1 (C-2″, C-6″), 129.2 (C-3″, C-5″), 138.0 (C-4″), 28.3 (C-1‴), 16.2 (C-2‴), 102.0 (C-1), 74.6 (C-2), 76.8 (C-3), 81.8 (C-4), 76.8 (C-5), 61.9 (C-6), 105.9 (C-1′), 70.2 (C-2′), 72.6 (C-3′), 77.4 (C-4′), 75.3 (C-5′), 62.1 (C-6′).

p-(1″-Hydroxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-β-D-Glucopyranoside[7] (Mixture of Epimers **21**)

HR-CI-MS *m/z*: 469.1716 $[M + H]^+$, calculated for $C_{22}H_{29}O_{11}$: 469.1710; ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.97 (d, *J*=8.6 Hz, H-2′, H-6′), 7.31 (d, *J*=8.6 Hz, H-3′, H-5′), 4.88 (q, *J*=5.8 Hz, H-1″), 1.48 (d, *J*=6.5 Hz, H-2″), 5.07 (d, *J*=7.6 Hz, H-1)/5.07 (d, *J*=7.4 Hz, H-1)[8], 5.27 (t, *J* =9.1 Hz, H-2), 5.30 (t, *J*=9.0 Hz, H-3), 5.17 (t, *J*=9.4 Hz, H-4), 3.86 (ddd, *J*=9.9, 5.3, 2.3 Hz, H-5), 4.29 (dd, *J*=12.3, 5.2 Hz, H-6a), 4.17 (dd, *J*=12.3, 2.2 Hz, H-6b)/4.17 (dd, *J*= 12.2, 2.2 Hz, H-6b)[8], 2.08, 2.06, 2.05, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.2 (C-1′), 117.06/117.04 (C-2′, C-6′)[8], 126.7 (C-3′, C-5′), 140.9 (C-4′), 69.9/69.8 (C-1″)[8], 25.23/25.22 (C-2″)[8], 99.2 (C-1), 71.2 (C-2), 72.7 (C-3), 68.3 (C-4), 72.1 (C-5), 62.0 (C-6), 170.5, 170.2, 169.4, 169.3 (C=O), 20.7, 20.64, 20.63, 20.60 (CH_3C=O).

p-(1″-Hydroxyethyl)-Phenyl β-D-Glucopyranoside (Mixture of Epimers **22**)

HR-EI-MS *m/z*: 300.1198 $[M]^+$, calculated for $C_{14}H_{20}O_7$: 300.1209; IR ν_{max} [cm^{-1}] (neat), 3,367, 1,610, 1,510, 1,230, 1,068; ^{1}H NMR (500 MHz, D_2O): δ 7.00 (d, *J*=8.8 Hz, H-2′, H-6′), 7.26 (d, *J*=8.8 Hz, H-3′, H-5′), 4.75 (q, *J*=6.5 Hz, H-1″), 1.32 (d, *J*=6.5 Hz, H-2″), 4.98 (d, *J*=7.5 Hz, H-1), 3.43 (dd, *J*=8.9, 7.5 Hz, H-2), 3.48 (t, *J*=8.9 Hz, H-3), 3.36 (dd, *J*=9.8, 8.9 Hz, H-4), 3.48 (ddd, *J*=9.8, 5.5, 2.4 Hz, H-5), 3.79 (dd, *J*=12.4, 2.1 Hz, H-6a), 3.62 (dd, *J*=12.4, 5.7 Hz, H-6b); ^{13}C NMR (125 MHz, D_2O): δ 156.7 (C-1′), 117.5 (C-2′, C-6′), 128.0 (C-3′, C-5′), 140.6 (C-4′), 70.10/70.06 (C-1″)[8], 24.3 (C-2″), 101.1 (C-1), 73.8 (C-2), 76.9 (C-3), 70.3 (C-4), 76.4 (C-5), 61.4 (C-6).

[7] See footnote 6.

[8] See footnote 2.

p-(1″-Acetoxyethyl)-Phenyl 2,3,4,6-Tetra-*O*-Acetyl-β-D-Glucopyranoside (Mixture of Epimers **23**)

HR-EI-MS *m/z*: 510.1736 $[M]^+$, calculated for $C_{24}H_{30}O_{12}$: 510.1737; ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.99 (d, *J*= 8.7 Hz, H-2′, H-6′), 7.31 (d, *J*=8.6 Hz, H-3′, H-5′), 5.86 (q, *J*=6.5 Hz, H-1″), 1.51 (d, *J*=6.5 Hz, H-2″), 5.08/5.07 (d, *J*= 7.6 Hz, H-1)[8], 5.29 (dd, *J*=9.3, 7.5 Hz, H-2), 5.32 (t, *J*= 9.1 Hz, H-3), 5.19 (t, *J*=9.5 Hz, H-4), 3.87 (ddd, *J*=10.0, 5.3, 2.5 Hz, H-5), 4.31 (dd, *J*=12.3, 5.3 Hz, H-6a), 4.18/4.17 (dd, *J*=12.3, 2.3 Hz, H-6b)[8], 2.08/2.08[8], 2.06, 2.05, 2.05, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.4 (C-1′), 116.9 (C-2′, C-6′), 127.57/127.55 (C-3′, C-5′)[8], 136.7 (C-4′), 71.8 (C-1″), 22.1/22.0 (C-2″)[8], 99.0 (C-1), 71.1 (C-2), 72.7 (C-3), 68.3 (C-4), 72.0 (C-5), 61.9 (C-6), 170.6, 170.3, 170.2, 169.4, 169.3 (C=O), 21.3, 20.7, 20.62, 20.60, 20.58 (CH_3C=O).

p-Vinylphenyl 2,3,4,6-Tetra-*O*-Acetyl-β-D-Glucopyranoside[7] (**24**)

HR-EI-MS *m/z*: 450.1518 $[M]^+$, calculated for $C_{22}H_{26}O_{10}$: 450.1526; $[\alpha]_D$ −20.8 ($CHCl_3$, c 1.0 g/dl, 21.2°C); IR ν_{max} [cm^{-1}] (neat), 3,022, 1,755, 1,630, 1,606, 1,508, 1,367, 1,221, 1,047; ^{1}H NMR (500 MHz, $CDCl_3$): δ 6.95 (d, *J*=8.7 Hz, H-2′, H-6′), 7.34 (d, *J*= 8.7 Hz, H-3′, H-5′), 6.66 (dd, *J*=17.8, 10.9 Hz, H-1″), 5.65 (dd, *J*=17.5, 0.7 Hz, H-2″*trans*), 5.19 (dd, *J*=10.9, 0.7 Hz, H-2″*cis*), 5.08 (d, *J*=7.5 Hz, H-1), 5.27 (dd, *J*= 9.1, 7.5 Hz, H-2), 5.30 (t, *J*=9.0 Hz, H-3), 5.17 (t, *J*= 9.5 Hz, H-4), 3.86 (ddd, *J*=10.0, 5.2, 2.5 Hz, H-5), 4.29 (dd, *J*=12.3, 5.5 Hz, H-6a), 4.17 (dd, *J*=12.3, 2.5 Hz, H-6b), 2.08, 2.06, 2.05, 2.04 (s, CH_3CO); ^{13}C NMR (125 MHz, $CDCl_3$): δ 156.4 (C-1′), 117.0 (C-2′, C-6′), 127.3 (C-3′, C-5′), 133.0 (C-4′), 135.8 (C-1″), 113.0 (C-2″), 99.1 (C-1), 71.1 (C-2), 72.7 (C-3), 68.3 (C-4), 72.0 (C-5), 61.9 (C-6), 170.5, 170.2, 169.3, 169.2 (C=O), 20.63, 20.58, 20.56, 20.54 (CH_3C=O).

p-Vinylphenyl β-D-Glucopyranoside[7] (**25**)

HR-EI-MS *m/z*: 282.1097 $[M]^+$, calculated for $C_{14}H_{18}O_6$: 282.1103; $[\alpha]_D$ −81.5 (MeOH, c 1.0 g/dl, 21.0°C); IR ν_{max} [cm^{-1}] (neat), 3,318, 1,627, 1,606, 1,509, 1,245, 1,076, 1,046; ^{1}H NMR (600 MHz, MeOH-d_4): δ 7.04 (d, *J*= 8.5 Hz, H-2′, H-6′), 7.38 (d, *J*=8.5 Hz, H-3′, H-5′), 6.66 (dd, *J*=17.6, 10.9 Hz, H-1″), 5.64 (dd, *J*=17.3, 0.9 Hz, H-2″*trans*), 5.11 (dd, *J*=11.0, 0.9 Hz, H-2″*cis*), 4.90 (d, *J*= 7.6 Hz, H-1), 3.46–3.36 (overlapping signals, H-2, H-3, H-4, H-5), 3.89 (dd, *J*=12.1, 2.0 Hz, H-6a), 3.68 (dd, *J*=12.1, 5.6 Hz, H-6b); ^{13}C NMR (150 MHz, MeOH-d_4): δ 158.8 (C-1′), 117.7 (C-2′, C-6′), 128.3 (C-3′, C-5′), 133.5 (C-4′), 137.5 (C-1″), 112.4 (C-2″), 102.2 (C-1), 74.9 (C-2), 78.2 (C-3), 71.4 (C-4), 78.0 (C-5), 62.5 (C-6).

References

BURGER, K., KLUGE, M., KOKSCH, B., FEHN, S., BÖTTCHER, C., HENNIG, L., and MÜLLER, G. 2004. Hexafluoroacetone as protecting and activating reagent: a new approach to *O*-glycosides. *Heterocycles.* 64:143–52.

CANNIZZARO, C. E., THIEL, I. M. E., and D'ACCORSO, N. B. 1998. Synthesis and debenzoylation products of two perbenzoylated 2-substituted 5-D-galactosyl-1,3,4-oxadiazoles. *J. Heterocyclic Chem.* 35:481–4.

CLINGMAN, A. L. 1964. *p*-Vinylphenyl glycosides of cellobiose and maltose. *J. Med. Chem.* 7:242–3.

CONCHIE, J., LEVVY, G. A., and MARSH, C. A. 1957. Methyl and phenyl glycosides of the common sugars. *Adv. Carbohydr. Chem.* 12:157–87.

HELFERICH, B., and HÖFMANN, H. J. 1952. *p*-Oxy-styrol-β-D-glucosid. *Chem. Ber.* 85:175–80.

KOSCHIER, E. H., and SEDY, K. A. 2003. Labiate essential oils affecting host selection and acceptance of *Thrips tabaci* lindeman. *Crop Prot.* 22:929–34.

KULKARNI, M. M., NAGASAMPAGI, B. A., DESHPANDE, S. G., and SHARMA, R. N. 1987. Five chromenes from *Blepharispermum subsessile*. *Phytochemistry* 26:2969–71.

OJIKA, M., WAKAMATSU, K., NIWA, H., YAMADA, K., and HIRONO, I. 1984. Isolation and structures of two new *p*-hydroxystyrene glycosides, ptelatoside-A and ptelatoside-B from bracken, *Pteridium aquilinum* var. *latiusculum*, and synthesis of ptelatoside-A. *Chem. Lett.* 3:397–400.

PRAJAPATI, V., TRIPATHI, A. K., AGGARWAL, K. K., and KHANUJA, S. P. S. 2005. Insecticidal, repellent and oviposition-deterrent activity of selected essential oils against *Anopheles stephensi*, *Aedes aegypti* and *Culex quinquefasciatus*. *Bioresour. Technol.* 96:1749–57.

SOCOLSKY, C., SALVATORE, A., ASAKAWA, Y., and BARDÓN, A. 2003. Bioactive new bitter-tasting *p*-hydroxystyrene glycoside and other constituents from the fern *Elaphoglossum spathulatum*. *Arkivoc* 2003:347–55.

SZENTESI, A., GREANY, P. D., and CHAMBERS, D. L. 1979. Oviposition behavior of laboratory-reared and wild Caribbean fruit flies (*Anastrepha suspensa*; Diptera; Tephritidae): I. Selected chemical influences. *Entomol. Exp.* & *Appl.* 26:227–38.

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A Floral-Derived Compound Attractive to the Tephritid Fruit Fly Parasitoid *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae)

Eric Rohrig · John Sivinski · Peter Teal · Charles Stuhl · Martin Aluja

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Abstract Many adult hymenopteran parasitoids, even host-feeding species, consume the nectar of flowering plants. Previous field studies had identified plants attractive (*Lobularia maritima* L.) and unattractive (*Spermacoce verticillata* L) to certain opiine braconids (Hymenoptera). Under laboratory conditions, *Diachasmimorpha longicaudata* (Ashmead), a parasitoid of tephritid fruit fly larvae and representative opiine, responded in flight tunnels to *L. maritima* but not to *S. verticillata.* Volatile chemicals of the two flowers were collected and analyzed by using capillary gas liquid chromatography and mass spectral analysis. Acetophenone was isolated from *L. maritima* but not from *S. verticillata.* In flight tunnels, *D. longicaudata* were exposed to 10 concentrations (doses) of acetophenone. Female parasitoids showed a significant attraction to several acetophenone doses, with concentrations of 25 and 50 ng the most attractive. No odor source, either floral or floral-derived, was attractive to male parasitoids. Reliable trapping systems for parasitoid species, particularly species such as *D. longicaudata* used for augmentative biological control, would be a valuable monitoring tool. At present, there are few, if any, florally derived synthetic lures for attracting hymenopteran parasitoids.

Keywords Acetophenone · Opiinae · Traps · Biological control · Flowers

E. Rohrig
Department of Entomology and Nematology,
University of Florida,
Bldg. 970, Natural Area Dr.,
Gainesville, FL 32611, USA

E. Rohrig · J. Sivinski (✉) · P. Teal · C. Stuhl
USDA-ARS Center for Medical,
Agricultural and Veterinary Entomology,
1660/1700 SW 23rd Dr.,
Gainesville, FL 32604, USA
e-mail: jsivinski@gainesville.usda.ufl.edu

M. Aluja
Instituto de Ecología,
A.C., Apartado Postal 63,
91000 Xalapa, Veracruz, México

Introduction

Understanding the population dynamics of target pests and their natural enemies is critically important in Integrated Pest Management (IPM) (Kogan 1998; Suckling et al. 2002). Attraction and trapping techniques are among the simplest means of determining pest and natural enemy distributions and densities in the field, and this information, combined with known insect biology, can help determine what controls should be applied, when and where to use them, and the status of non-target organisms that might be affected (Prokopy 1985). Among the controls best timed and placed through the use of trapping-demographic data is the inundative release of beneficial insects (Jewitt and Carpenter 2001). It is particularly important to monitor the dispersal and survival of the mass-released predators/parasitoids so that adjustments can be made to the releases, and their efficacies estimated.

At present, most pest-insect attractants are based on sex pheromones, and majority of these are female-derived chemicals that capture males (Foster and Harris 1997). Sex pheromones are also emitted by female, and more rarely male, parasitic Hymenoptera (Sivinski and Petersson 1997; Kainoh 1999), and can also be used as attractants (Rice and Jones 1982; Jewett and Carpenter 2001; Suckling et al. 2002). However, compounds associated with hosts, or

environments where hosts might be found, may be more generally attractive to a presumably largely non-virgin and sexually unresponsive female parasitoid population. For example, among tephritid fruit fly parasitoids, *Fopius arisanus* (Sonan) and *Diachasmimorpha longicaudata* (Ashmead) females are attracted to fruit volatiles (Messing and Jang 1992; Eben et al. 2000; Altuzar et al. 2004), the latter particularly to decaying fruit and associated fungi (Greany et al. 1977). Yet another tephritid parasitoid *Psyttalia fletcheri* Silvestri responds to decaying fruits and leaves of pumpkins and cucumbers (Messing et al. 1996). Nishida (1956) earlier found that stem tissues of cucurbits are attractive to *P. fletcheri*, and Messing et al. (1996) suggest that the attractiveness to leaf and stem tissues may be due in part to "green leaf volatiles", a suite of common leaf-derived compounds known to be attractive to other braconid species (Whitman and Eller 1990).

Flowers of many species of plants produce odors that are also highly attractive to various natural enemies (Andersen 1987; Maini and Burgio 1999; Ventura et al. 2000; Landolt et al. 2001). Although nectar feeding, and to a lesser extent pollen consumption, is common in the parasitic Hymenoptera (Jervis et al. 1993), there has been little if any use of floral-derived compounds as attractants. However, there may be advantages to the use of such compounds. Like host odors, but unlike male-produced sex pheromones, flower volatiles might be more consistently appealing to the mated females that likely constitute the bulk of many parasitoid populations.

In the course of a previous field comparison, flowers that were attractive (*Lobularia maritima* L.) and unattractive (*Spermacoce verticillata* L.) to an economically significant subfamily of Braconidae, the Opiinae, were preliminarily identified (Rohrig 2006). In the present experiments, the flight tunnel responses of *D. longicaudata,* a widely used tephritid biological control agent and representative opiine, to the two flowering plants and to a compound uniquely identified from *L. maritima* volatiles were determined. These plants and the model insect are described below.

Spermacoce verticillata, "shrubby false buttonweed" (Rubiaceae) is native to the West Indies, but can be found in Florida and Texas as well as west Africa, the tropical Americas, and the south Pacific. Habitats consist of open or disturbed sandy zones and pinelands where it grows as a shrub. *Spermacoce verticillata* is a perennial dicot whose small white flowers form dense clusters at the upper stem nodes. Flowers have a mean corolla depth of 1.5 mm, a width of 1.0 mm, and possess a honey guard at the interior base of the corolla (Sivinski et al. 2006). The nectar is a major food source for the mole cricket ectoparasitoids *Larra bicolor* F. and *Larra analis* F. (Hymenoptera: Sphecidae) in Florida (Frank and Parkman 1999).

Lobularia maritima, "alyssum" (Brassicaceae), was introduced from the Mediterranean region and now ranges throughout most of the United States including Hawaii. This plant is a hardy, non-weedy, annual dicot herb that flowers consistently from fall through spring in subtropical areas. Small cruciform-stalked white flowers grow in clusters randomly throughout the plant that attract large numbers of parasitic Hymenoptera (Chaney 1998). On average, corollas are 0.67 mm wide by 1.4 mm deep (Sivinski et al. 2006).

Lobularia maritima nectar increases the longevity of several ichneumonoid parasitoids, both in the laboratory and in the field (Johanowicz and Mitchell 2000; Berndt and Wratten 2005). While *L. maritima* flowers increase the longevity and realized fecundity of the egg parasitoid *Trichogramma carverae* Oatman and Pinto (Begum 2004), this is only true of the white flower variety (Begum et al. 2004). This was the variety used in the present experiments.

Due to availability and its wide use as a biological control agent, *D. longicaudata* was chosen to examine the response of a representative opiine to the floral volatile. While a variety of chalcidoids, diapriids, figitids, and ichneumonoids parasitize Tephritidae (e.g., Sivinski et al. 2000), braconids of the subfamily Opiinae are typically the most numerous and diverse members of the guilds that attack frugivorous species (Purcell 1998). Opiinae are solitary, koinobiont, larval/egg-prepupal endoparasitoids of Cyclorrhapha Diptera (Bess et al. 1961; Lopez et al. 1999; Wharton 1999). Several species are considered important regulators of fruit fly populations (Wharton 1989) and have been introduced, and frequently established, throughout the world (Ovruski et al. 2000).

Diachasmimorpha longicaudata is one of the most widely used of these opiine tephritid biological control agents (Ovruski et al. 2000). Adult females use their relatively long ovipositor to parasitize a number of second- and third-instar fruit fly species' larvae in a wide variety of host fruits (Wharton 1989; Sivinski et al. 2000). The species was originally discovered in the Indo-Philippine region where it attacked *Bactrocera* spp. (Wharton and Marsh 1978), and in 1947 was introduced into Hawaii for the control of oriental fruit fly, *Bactrocera dorsalis* (Hendel) (Clancy and Dressner 1952). In 1972, *D. longicaudata* was established in Florida to control the Caribbean fruit fly, *Anastrepha suspensa* (Loew), and it subsequently reduced populations by ~40% (Baranowski et al. 1993).

In addition to wide-spread introductions, *D. longicaudata* has been mass-reared and inundatively released, either alone or in combination with sterile male flies, for the control of *Ceratitis capitata* (Wong et al. 1991), and various *Anastrepha* spp. (Sivinski et al. 1996; Montoya et al. 2000). In Florida, such releases suppressed *A. suspensa*

populations by as much as 96% and have been considered as a means of supporting "fly-free" zones to facilitate citrus exports.

Methods and Materials

Diachasmimorpha longicaudata were obtained from a >5-yr-old colony at the USDA-CMAVE and reared as described by Sivinski et al. (1996). Parasitoids were maintained in a climate-controlled room with a temperature range of 21–24°C and a relative humidity of 65–80%.

Chemical Analysis Preliminary chromatographic examinations of volatile chemicals collected from cut and attached flower were similar. Therefore, due to the handling ease, flowers used for compound identification were cut from the plant stem as close to floral tissue as possible, and the cut end was surrounded with cotton wool soaked in water. Flower heads were placed in a glass volatile collection chamber (Heath and Manukian 1992). This system consists of a chamber (30 cm long and 4 cm outside diameter [OD]) that has a sintered glass frit at the upwind end and a joint outlet with a single-port collector base. Humidified charcoal filtered air was pushed into one end of the chambers and over the flower heads. Air was pulled out the other end via a vacuum system. Air exiting the chamber passed through a volatile collection filter that contained 50 mg of Super-Q® (Refined Technologies, Woodland, TX, USA) to collect and hold any volatiles. Afterwards, collection filters were eluted with three aliquots of 100 µl methylene dichloride to remove volatile compounds.

Extracts of samples were analyzed by capillary gas liquid chromatography (GC) using a Hewlett Packard 5890 (Palo Alto, CA, USA) equipped with a cool on-column injector and flame ionization detector. The column, a 30 m×0.25 mm (i.d.) SE-30 capillary column (Alltech Assoc., New Gloucester, ME, USA), was attached to a 10-m length of 0.25-mm (id) deactivated fused silica as a retention gap, which was in turn attached to a 10-cm length of 0.5 mm id deactivated fused silica in the injector. Helium (linear flow velocity 18 cm/sec) was used as a carrier gas. The oven temperature and injector temperatures were programmed to go from 60°C (held for 5 min) to 200 °C at a rate of 10°C/min. Mass spectral analysis was accomplished with an HP6890 GC equipped with a DB-5 MS® column (30 m×0.25 mm ID×0.25 µm,) linked to an HP 5973 mass spectrometer. Both electron impact (70 eV) and chemical ionization (isobutene reagent gas) spectra were obtained. Helium was the carrier gas, and a splitless injector (injector temperature of 240°C, split valve delay of 0.5 min) was used. The oven temperature was held at 35°C for 1 min, then programmed to increase at a rate of 10°C/min to 230°C, which was held for 10 min. The ion source temperature was 230°C. Tentative identifications were made by comparison of fragmentation patterns with patterns available in the NIST-MS library and libraries developed at USDA-CMAVE (Gainesville, FL, USA). Identifications were confirmed by comparison of chromatographic retention times and mass spectra of natural compounds with those of commercially available standards analyzed on the same instrument.

Electroantennagrams To determine if male and female *D. longicaudata* had a sensory response to *L. maritima* volatiles (see results), extracts were analyzed with a GC interfaced to both flame ionization (FID) and electroantennograph detectors. In this manner, antennal responses were matched with FID signals for compounds eluting from the GC. Volatile extracts were prepared in the manner described above, and 1-µl aliquots were analyzed on a Hewlett-Packard (HP) 5890 Series II gas chromatograph equipped with an HP-5 column (30 m×0.32 mm ID×0.25 mm) (Agilent, Palo Alto, CA, USA). The oven temperature was held at 40 °C for 5 min, then programmed to increase to 10°C /min to 220°C and held at this temperature for 5 min. Helium was used as a carrier gas at a flow rate of 2.0 ml/min. A humidified air stream was delivered over the antenna is at 1 ml/min.

The antennae were excised by grasping the scape with jeweler's forceps (No. 5, Miltex Instrument Company Inc, Switzerland) at the base, and the antennae were removed from the head of either male or female wasps. The extreme distal and proximal ends of the antennae were held between gold electrodes in conductivity gel (Syntech, Hilversum, The Netherlands). The electroantennal detector (EAD) and FID signals were concurrently recorded with a GC-EAD program (Syntech GC-EAD 2000, Hilversum, The Netherlands), which analyzed the amplified signals on a PC.

Flight Tunnel Bioassays To determine the response of *D. longicaudata* to various odor sources, parasitoids were observed in three identical flight tunnels. These flight tunnels were constructed of clear Plexiglas and measured 128 cm long×32×32 cm, and were located inside two climate-controlled greenhouses. Natural sunlight illuminated the chambers. Although the greenhouses were climate controlled, extremes of outside temperatures caused the internal temperature to fluctuate between 21° and 27°C and relative humidity between 60% and 85%. Airflow and air speed were maintained at 0.3 to 0.4 m/s by using a variable speed fan at the downwind end. This was the speed that stimulated the most flight in *D. longicaudata* in previous investigations (Messing et al. 1997). Odors were released into the anterior end of the tunnel. Two separate chambers that contained either an odor source or a blank-air control

were housed outside of the flight tunnel. Chambers were constructed from 114-l all-glass aquaria (31×61×61 cm). Lids were constructed of clear Plexiglas and fit tightly to prevent outside air from entering the chambers. Purified air was passed through the sample chambers at 0.3 m/s regulated with adjustable flow meters (Aalborg Instruments, Monsey, NY, USA) and into the flight tunnel. Each chamber was connected to a trap inside the front end of the tunnel. The traps were located midway between the top and bottom of the tunnel, and were constructed from clear cylindrical plastic vials (9×5 cm) placed horizontally. Both cylinders had orange colored snap tight lids and had a 1.3-cm hole in the center to allow air to exit. This provided an opening for the parasitoids to enter in search of the odor source.

Traps, washed initially with a mild detergent, were changed with each new odor source, and the tubing connecting the traps to volatile-source container was washed with detergent after each replication.

In all flight tunnel experiments, 25 presumably mated (sexes held together for 3–7 d) parasitoids were used per day. All parasitoids had access to water, but food was withheld for 24 hr before use in experiments to enhance response to potential food sources. Flight tunnels were checked hourly from 0900 to 1700 hours. A parasitoid inside a trap was recorded as a positive response, and was removed and returned to the tunnel after counting. Each day, the tubes connecting the odor chambers to the traps were switched to prevent positional effects.

Ripe mango fruit, *Mangifera indica* L., and the flowering plants were tested as odor sources. Mangos are attractive to *D. longicaudata* in flight tunnel tests (Eben et al. 2000) and were used as a positive control. Flowering plants were in full bloom and contained in 2-l plastic pots. Each of the three odor sources was tested individually against a blank control of clean odorless air. There were 10 replicates for males alone and 10 for females alone, each replicate lasting a day. In addition, there were 10-d-long replicates of 25 females and 25 males together with *L. maritima* as the odor source. This was done to ensure that any sexual dimorphism in response was not due to undetermined environmental factors present during separate-sex replications.

Acetophenone was loaded on 11-mm sleeve stopper natural red rubber septa by dissolving the synthetic material at various doses in methylene chloride and filling the large well of the septa with 100 µl of solution. Septa were air-dried for 24 hr and stored in a freezer, except during use in the wind tunnels. During flight tunnel tests, septa were placed in glass airtight containers (2 l) that served as the odor chambers.

All volatile loaded septa were run against blank septa loaded with 100 µl of methylene chloride and air-dried as a control. To find the dose that elicited the greatest response, various concentrations were exposed to parasitoids. An initial dose of 1 mg did not produce a response, and increasingly lower doses were tested until there was no significant response. Each dose was initially run for 3 d in sequential decreasing order from highest to lowest. Only females were in the wind tunnel. Acetophenone doses used were as follows: 1 mg, 100 µg, 50 µg, 10 µg, 1 µg, 100 ng, 50 ng, 25 ng, 10 ng, 1 ng. If any response was seen, then that volatile dose was run for 10 d with females and 10 d with males.

Statistical Analyses Statistical analyses were conducted by using SAS programming (SAS Institute Inc. 2001). Polynomial regression analysis (PROC GLM) was used to examine flight responses over time and the interactions of "time of day ×volatile source" on response. Analysis of variance (PROC ANOVA) followed by means separation through the Waller test was employed to compare the mean responses to various doses. Note that the protocol for all wind tunnel experiments included the return of captured females to the population inside the tunnel. While this was a simple means of controlling for insect density and environmental history, the summed captures could include multiple responses by a particular insect. This led us, when comparing a particular volatile to its control, to employ the most conservative method available, the nonparametric "paired sign test" (Zar 1974) where a consistently greater response over 10 replicates to a particular source yields a P value of 0.01. Mean and variance data should be interpreted in terms of "responses" and not "insects responding".

Results

Volatile Identification Gas chromatographic analysis of volatiles from the flowers of each plant revealed the presence of surprisingly few compounds (Fig. 1a). After subtraction of compounds present in volatiles from both species, only three were unique to volatiles collected from *L. maritima*, and only a single compound was common to those collected from intact flowers and from excised flowers. Mass spectral analysis (both electron impact (EI) spectra and chemical ionization (CI) spectra) of the unique compound indicated a mass of 120 amu with significant losses of 15 (CH_3), 28 (CH_2O). Tentative identifications were made by comparison of fragmentation patterns with patterns available in the NIST-MS library and libraries developed at USDA-CMAVE (Fig. 1a). Acetophenone (Fig. 1b) (8.96 min; C8H8O, molecular weight 120.15 g/mol [US EPA, 1987]) showed a large peak in *L. maritima* volatiles and was not present in those of *S. verticillata*.

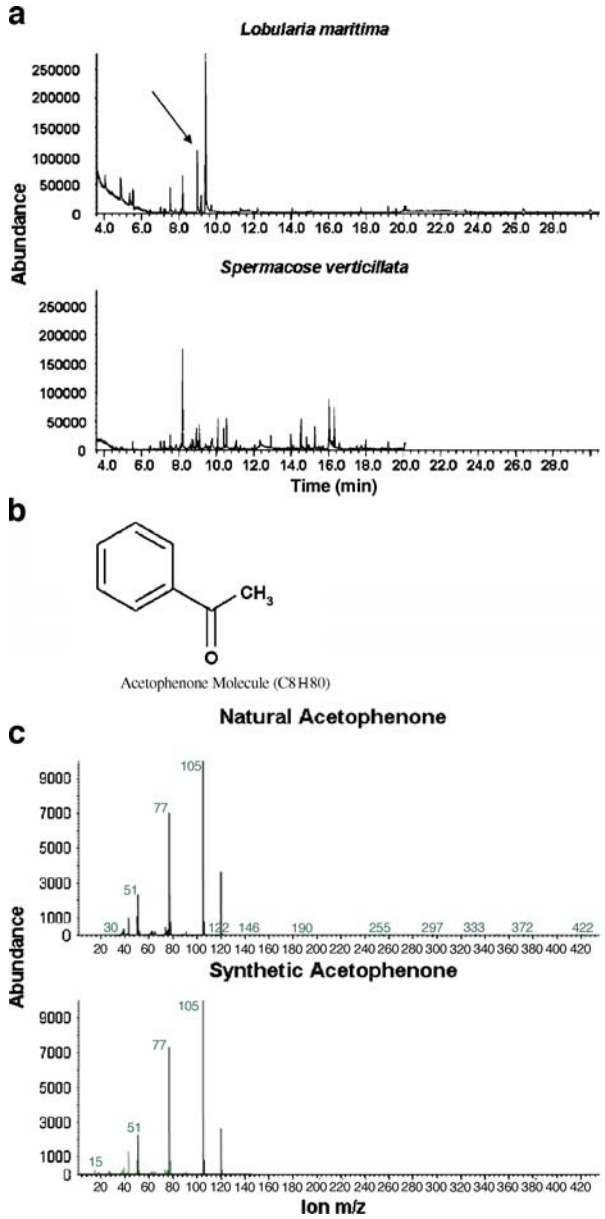

Fig. 1 (**a**) GC analysis of chemical constituents of two flowering plant, *Lobularia maritima* and *Spermacoce verticillata*, volatiles as revealed by mass spectra. The arrow indicates the unique presence of acetophenone in *L. maritima.* (**b**) Acetophenone molecule. (**c**) A mass spectroscopy comparison between the putative acetophenone collected from *L. maritima* and a known synthetic standard

Identification was confirmed by comparison of GC retention times and MS of natural compounds with those of commercially available standards analyzed on the same instruments (Fig. 1c).

Electroantennagrams Both male and female *D. longicaudata* registered a neuronal response that correlated to the putative acetophenone peak present in *L. maritima* volatile extracts (Fig. 2). Of the three females tested, their average responses over three exposures to volatiles were areas (mV/time) of 1.2, 0.7, and 1.5. Of the three males tested, their average responses over three exposures were 0.4, 0.6, and 1.1.

Flight Tunnel Bioassays Males showed no significant response to any odor source. In several instances, they were caught in isolation traps, but in general, they exhibited occasional walking and flying behaviors at the rear of the flight tunnel away from the odor source.

Females had different responses to various volatiles (F=240.5, df=9, 1430, P=0.001), and were more likely to be trapped by some volatiles than by their corresponding controls (F=35.8, df=1, 1430, P=0.001). Responses differed over time in a non-linear fashion (F=67.3, df=1, 1430, P=0.001) (Fig. 3). However, the responses over time were similar among treatments, i.e., there was no significant interaction between type of volatile and the temporal pattern of response (F=0.68, df=1, 1430, P=0.41).

Because it was impractical to control the amount of volatiles emitted by fruit and flowers, there was no direct comparison of these non-formulated volatile sources. Both mango and *L. maritima* significantly elicited female responses, but *S. verticillata* did not (Fig. 4). These tests

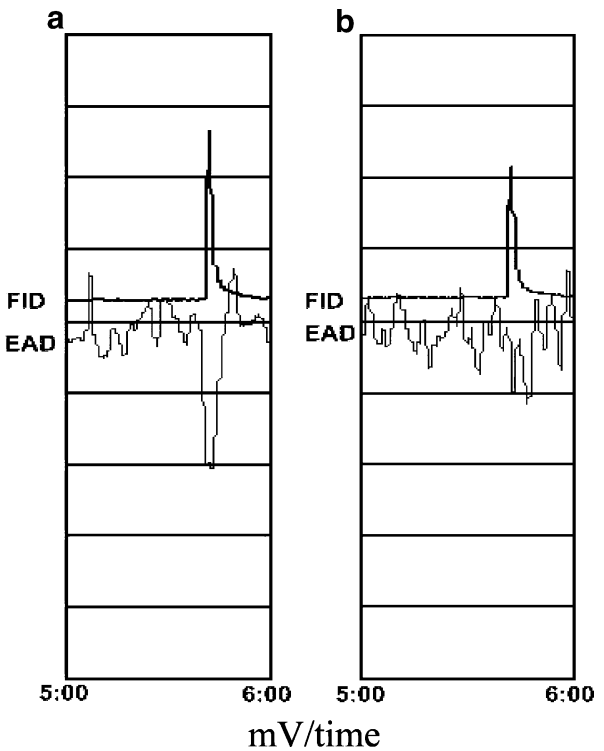

Fig. 2 Neuronal responses of (**a**) female and (**b**) male *Diachasmimorpha longicaudata* to the acetophenone present in volatile extracts of *Lobularia maritima* flowers. The darker line represents the presence of acetophenone as revealed by gas chromatography and the lighter line the response of neurons to the same volatile sample

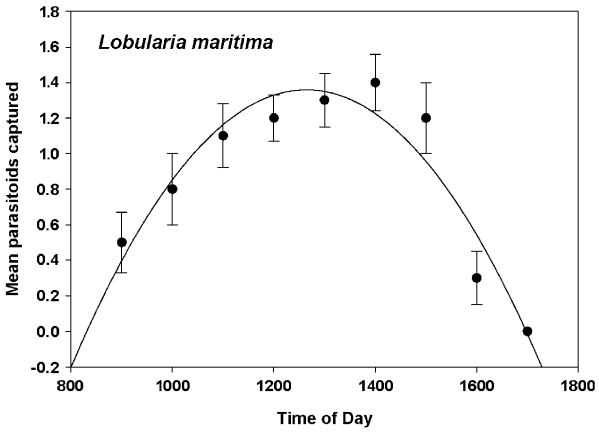

Fig. 3 Mean (SE) windtunnel captures of female *Diachasmimorpha longicaudata* over time in response to flowering *Lobularia maritima*

were followed by exposures of parasitoids to formulated doses of acetophenone, the compound present in *L. maritima*, but absent in *S. verticillata*. Of the 10 doses tested, four elicited a response from parasitoids (Fig. 4). Acetophenone concentrations of 100, 50, 25, and 10 ng all showed significant attraction, with 25- and 50-ng doses being the most attractive (F=3889, df=3, P=0.003; Fig. 5). Acetophenone doses of 1 mg, 100, 50, 10, and 1 µg, and 1 ng elicited no response in female parasitoids in the wind tunnel.

When both males and females were simultaneously exposed in the wind tunnel to *L. maritima* flower volatiles, no males entered the isolation chambers, but female response was similar to the single-sex experiment with *L. maritima* (mean of 0.9 [SE 0.07] responses/hourly observation period; paired sign test, greater response to volatile source equal to 10 of 10; P=0.01).

Discussion

These experiments provide additional evidence that not all flowers are equally attractive to any one particular species of parasitoid (Jervis et al. 1993). In the field, *L. maritima* caught more Braconidae, specifically unidentified Opiinae, than did *S. verticillata* (Rohrig 2006). This difference was substantiated in flight tunnel tests that found a significant flight response by the opiine *D. longicaudata* to *L. maritima* but not to *S. verticillata*. In addition to this corroborative evidence in support of the generality that there is variance in the diversity of parasitoids attracted to flowers, there were several novel/unusual findings. These included: (1) the suggestion that there may be broad phylogenetic preferences for certain flowers, i.e., the common response of at least two allopatric opiines to the pair of flower species (Rohrig 2006); (2) the identification of a compound from a flower volatile that is attractive to a hymenopteran parasitoid; and (3) the sexual dimorphic response to *L. maritima*, with only females attracted to either the complete flower volatiles or the isolated compound unique to *L. maritima*.

Direct comparisons of parasitoid olfactory response to different live flowers can lead to ambiguous results. Although the sizes of the plants used and the overall area of flowers present were similar (Rohrig 2006), the amounts of volatiles released may have been different. Even within a species, odor concentration can vary among flower parts and change diurnally as well as seasonally (Bergstrom et al. 1995). The identification of the attractive compounds present in floral odors can help overcome this obstacle. Once isolated, compounds can be presented in fixed amounts, thereby reducing variation in the odors encountered by the test insects. Thus, the identification of

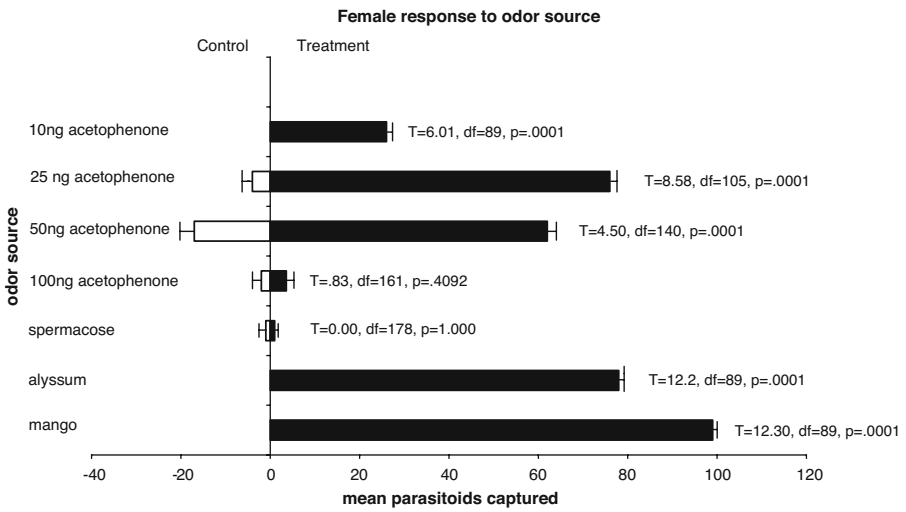

Fig. 4 Mean (SE) of summed captures/replicate of females *Diachasmimorpha longicaudata* exposed to various volatiles and their corresponding blank air controls

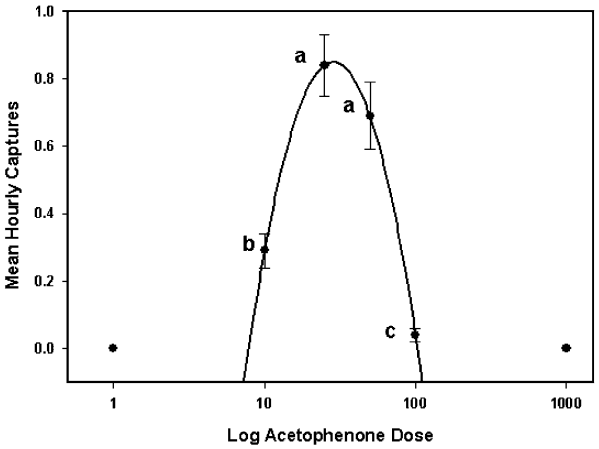

Fig. 5 Mean (SE) captures of female *Diachasmimorpha longicaudata* exposed to various doses of acetophenone. The regression line fits only those doses that elicited a significant response. Means that share a letter are not significantly different

acetophenone as a compound both unique to *L. maritima* and attractive to *D. longicaudata* supports the difference in attractiveness observed between the two whole plants. The isolation of acetophenone also allowed estimation of optimal release rates.

Diachasmimorpha longicaudata had a sexually dimorphic response to all volatiles that elicited flight; male parasitoids were not attracted to any. This was true when the sexes were tested both separately and together, so that unnoticed environmental cues present during single-sex experiments cannot be responsible for the sexual difference. Males did exhibit oriented flight and entered the isolation traps in response to virgin females + fruit + honey solutions in experiments conducted in the same wind tunnels (C. S., unpublished data). Thus, the lack of response in the present experiments was due to the volatiles used and not because of male inability to perform in the flight tunnel environment.

Messing and Jang (1992) had previously found that female *D. longicaudata* responded to various host fruit stimuli to a greater extent than did males. Similar sexually dimorphic responses to plant materials are not restricted to hymenopteran parasitoids. For instance, females of the tachinid *Eucelatoria bryani* Sabrosky are attracted to many more plant volatiles, including those of flowers, than are males (Martin et al. 1990).

There are several possible explanations of why female, and not male *D. longicaudata*, are attracted to acetophenone. For instance, females may have unique nutritional needs that flower nectar provides. Acetophenone possess a flowery smell and is used in the perfume industry in fragrances such as vanilla, honeysuckle, and jasmine (USEPA 1987). It is emitted as a volatile by several flowers including *Centaurea scabiosa* L., a species frequently visited by butterflies (Andersson et al. 2002), *Elsholtzia argyi* Dong (Peng and Yang 2005), and *Calanthe sieboldi* Decne (Awano et al. 1997). In the laboratory, *D. longicaudata* feeds avidly on juices of fruits that would be commonly encountered while searching fallen fruit for hosts (Sivinski et al. 2006). Ground-foraging female parasitoids, however, would also be in the general vicinity of flowers and could visit them to obtain additional other nutrients (Koptur 2005). The relatively ambiguous laboratory evidence that flowers are an important adult food sources for *D. longicaudata* does not offer strong support for this conclusion (Sivinski et al. 2006). Maximum longevities were significantly extended in the presence of flowers (including the presently examined species), but there was no difference between the mean longevities of parasitoids provided with flowers and those given only water.

Females might also mistake acetophenone for a host-fruit volatile or a male-produced pheromone. It has been isolated from Rambutan fruit (*Nephelium lappaceum* L.; Ong et al. 1998) and guava pulp (*Psidium guajava* L.) (Idstein and Scherier 1985), and is also produced by a number of diverse insects as a semiochemical (Kohnle et al. 1987; Schulz et al. 1993; Aldrich et al. 1995; Birkett et al. 2004). The latter hypothesis likely can be discarded since neither acetophenone nor closely related compounds are present in the male-produced sex pheromone (Nancy Epsky, unpublished data).

However, female *D. longicaudata* might mistake the flower volatile for a fruit cue. Tephritids use odor to locate fruit, and the same ability would be useful to fruit fly parasitoids (Eben et al. 2000). Males of both fruit flies and their natural enemies may not have been subject to the same selection pressures as females to develop sensitivity to fruit odor stimuli. For example, when 4-ethyl-acetophenone in the peel of navel oranges (*Citrus sinensis* Osbeck) was used as an odor source in electroantennogram studies of *Ceratitis capitata* (Weid.), the Mediterranean fruit fly, it elicited a significant voltage spike in females, but not in males (Hernandez et al. 1996). Levinson et al. (2003) found that female *C. capitata* sensilla were significantly more responsive to orange odor than male sensilla. Electroantennagrams showed that male *D. longicaudata* sensed acetophenone, but it did not elicit a behavioral response.

Regardless of the significance of the acetophenone response, it may lend itself to improved traps and ultimately better control procedures. There are no efficient synthetic lures and traps available to monitor opiine parasitoid populations in the field (Messing 1992). At present, most monitoring and delineation of fruit fly parasitoids is accomplished through laborious and time-consuming fruit sampling (Montoya et al. 2000; Sivinski et al. 2000). An improved trapping system would prove valuable in several ways. It could be used to determine the presence of

parasitoids in a given area as well as help understand their population dynamics. Traps could provide important wasp dispersal and patch retention information that might improve the application of parasitoids for inundative biological control. Knowing when and where to release parasitoids, as determined by previous trapping in various locations, could be crucial to their application in new sites, whether the goal is permanent establishment or mass releases.

Field testing of acetophenone is needed to examine its usefulness. Although only female wasps were attracted, females are the agents of mortality, and their distribution in time and space is generally of primary interest. It may be that acetophenone by itself will not be sufficiently attractive, but it could prove to have a role in a more efficacious mixture of compounds that could include other floral volatiles, fruit odors (Eben et al. 2000), fungal/bacterial odors (Greany et al. 1977), and pheromones.

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References

ALDRICH, J. R., ROSI, M. C., and BIN, F. 1995. Behavioral correlates for minor volatile compounds from stink bugs (Heteroptera: Pentatomidae). *J. Chem. Ecol.* 21:1907–1920.

ALTUZAR, A., MONTOYA, P., and ROJAS, J. C. 2004. Response of *Fopius arisanus* (Hymenoptera: Braconidae) to fruit volatiles in a windtunnel. *Fla. Entomol.* 87:616–618.

ANDERSEN, J. F. 1987. Composition of the floral odor of *Cucurbita maxima* Duchesne (Cucurbitaceae). *J. Agric. Food Chem.* 35:60–62.

ANDERSSON, S., NILSSON, L. A., GROTH, I., and BERGSTROM, G. 2002. Floral scents in butterfly-pollinated plants: possible convergence in chemical composition. *Bot. J. Linn. Soc.* 140:129–153.

AWANO, K., ICHIKAWA, Y., TOKUDA, K., and KURAOKA, M. 1997. Volatile components of the flowers of two *Calanthe* species. *Flavour Fragr. J.* 12:327–330.

BARANOWSKI, R., GLENN, H., and SIVINSKI, J. 1993. Biological control of the Caribbean fruit fly, *Anastrepha suspensa* (Loew). *Fla. Entomol.* 76:245–250.

BEGUM, M. 2004. Habitat manipulation to enhance biological control of lightbrown apple moth (*Epiphyas postvittana*). MS Thesis, University of Sydney.

BEGUM, M., GURR, G. M., and WRATTEN, S. D. 2004. Flower colour affects tri-trophic biocontrol interactions. *Biol. Control* 30:584–590.

BERGSTROM, G., DOBSON, H., and GROTH, I. 1995. Spatial fragrance patterns within the flowers of *Ranunculus acris* (Ranunculaceae). *Plant Syst. Evol.* 195:221–242.

BERNDT, L. A., and WRATTEN, S. 2005. Effects of alyssum flowers on the longevity, fecundity, and the sex ratio of the leafroller parasitoid *Dolichogenidea tasmanica*. *Biol. Control* 32:65–69.

BESS, H. A., BOSCH, R. V. D., and HARAMOTO, F. 1961. Fruit fly parasites and their activities in Hawaii. *Proc. Hawaii. Entomol. Soc.* 17:367–373.

BIRKETT, M. A., BRUCE, T. J. A., MARTIN, J. L., SMART, L. E., OAKLEY, J., and WADHAMS, L. J. 2004. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana*, to wheat panicle volatiles. *J. Chem. Ecol.* 30:1319–1328.

CHANEY, W. E. 1998. Biological control of aphids in lettuce using in-field insectaries, pp. 73–83, in C. H. Pickett, and R. L. Bugg (eds.). Enhancing Biological Control-Habitat Management to Promote Natural Enemies of Agricultural PestsUniversity of California Press, Berkeley, CA.

CLANCY, D., and DRESSNER, E. 1952. Importation of natural enemies to control oriental fruit fly in Hawaii. *J. Econ. Entomol.* 45:85–90.

EBEN, A., BENREY, B., SIVINSKI, J., and ALUJA, M. 2000. Host species and host plant effects on preference and performance of *Diachasmimorpha longicaudata* (Hymenoptera: Braconidae). *Environ. Entomol.* 29:87–94.

FOSTER, S. P., and HARRIS, M. O. 1997. Behavioral manipulation methods for insect pest- management. *Annu. Rev. Entomol.* 42:123–46.

FRANK, J. H., and PARKMAN, J. P. 1999. Integrated pest management of pest mole crickets with emphasis on the southeastern USA. *Integr. Pest Manag. Rev.* 4:39–52.

GREANY, P. D., TUMLINSON, J. H., CHAMBERS, D. L., and BOUSCH, G. M. 1977. Chemically mediated host finding by *Biosteres* (Opius) *longicaudatus*, a parasitoid of tephritid fruit fly larvae. *J. Chem. Ecol.* 3:189–195.

HEATH, R. R., and MANUKIAN, A. 1992. Development and evaluation of systems to collect volatile semiochemicals from insects and plants using charcoal-infused medium for air purification. *J. Chem. Ecol.* 14:441–453.

HERNANDEZ, M. M., SANZ, I., ALDELANTO, M., BALLACH, S., and PRIMO, E. 1996. Electroantennogram activity from antennae of *Ceratitis capitata* (Wied.) to fresh orange airborne volatiles. *J. Chem. Ecol.* 22:1607–1617.

IDSTEIN, H., and SCHERIER, P. 1985. Volatile constituents from guava (*Psidium guajava*, L.) fruit. *J. Agric. Food Chem.* 33:138–143.

JERVIS, M. A., KIDD, N. A. C., FITTON, M. G., HUDDLESTON, T., and DAWAH, H. A. 1993. Flower-visiting by hymenopteran parasitoids. *J. Nat. Hist.* 27:67–105.

JEWETT, D. K., and CARPENTER, J. E. 2001. Seasonal abundance of a pupal parasitoid, *Diapetimorpha introita* (Hymenoptera: Ichneumonidae). *Fla. Entomol.* 84:50.

JOHANOWICZ, D., and MITCHELL, E. 2000. Effects of sweet alyssum flowers on the longevity of the parasitoid wasps *Cotesia marginiventris* (Hymenoptera: Braconidae) and *Diadegma insulare* (Hymenoptera: Ichneumonidae). *Fla. Entomol.* 83:41–47.

KAINOH, Y. 1999. Parasitoids, pp. 383–404, in J. Hardie, and A. Minks (eds.). Pheromones of Non-Lepidopterous Insects Associated with Agricultural PlantsCABI Publishing, Wallingford, UK.

KOGAN, M. 1998. Integrated pest management: Historical perspectives and contemporary developments. *Annu. Rev. Entomol.* 43:243–270.

KOHNLE, U., USSONG, M. M., DUBBEL, V., and FRANCKE, W. 1987. Acetophenone in the aggregation of the beech bark beetle, *Taphrorychus bicolor* (Coleoptera: Scolytidae). *J. Appl. Entomol.* 103:249–252.

KOPTUR, S. 2005. Nectar as fuel for plant protectors, pp. 75–108, in F. Wackers, P. van Rijn, and J. Bruin (eds.). Plant-Provided Food for Carnivorous Insects: A Protective Mutualism and its ApplicationsCambridge University Press, Cambridge UK.

LANDOLT, P. J., ADAMS, T., REED, H. C., and ZACK, R. S. 2001. Trapping alfalfa looper moths (Lepidoptera: Noctuidae) with

single and double component floral chemical lures. *Environ. Entomol.* 30:667–672.

LEVINSON, H., LEVINSON, A., and OSTERRIED, E. 2003. Orange-derived stimuli regulating oviposition in the Mediterranean fruit fly. *J. Appl. Entomol.* 127:269–275.

LOPEZ, M., ALUJA, M., and SIVINSKI, J. 1999. Hymenopterous larval-pupal and pupal parasitoids of *Anastrepha* flies (Diptera: Tephritidae) in Mexico. *Biol. Control* 15:119–129.

MAINI, S., and BURGIO, G. 1999. *Ostrinia nubilalis* (Hb.) (Lepidoptera: Pyralidae) on sweet corn: relationship between adults caught in multibaited traps and ear damages. *J. Appl. Entomol.* 123:179–185.

MARTIN, W., NORDLAND, D., and NETTLES, W. 1990. Response of the parasitoid, *Eucelatoria bryani* to selected plant materials in an olfactometer. *J. Chem. Ecol.* 16:499–508.

MESSING, R. H. 1992. An effective trapping method for field studies of opiine braconid parasitoids of tephritid fruit flies. *Entomophaga* 37:391–396.

MESSING, R. H., and JANG, E. B. 1992. Response of the fruit fly parasitoid *Diachasmimorpha longicaudata* to host-fruit stimuli. *Environ. Entomol.* 21:1189–1195.

MESSING, R. H., KLUNGNESS, L. M., and JANG, E. B. 1997. Effects of wind on movement of *Diachasmimorpha longicaudata*, a parasitoid of tephritid fruit flies, in a laboratory flight tunnel. *Entomol. Exp. Appl.* 82:147–152.

MESSING, R. H., KLUNGNESS, L. M., JANG, E. B., and NISHIJIMA, K. 1996. Response of the melon fly parasitoid *Pysttalia fletcheri* (Hymenoptera: Braconid) to host-habitat stimuli. *J. Insect Behav.* 9:933–945.

MONTOYA, P., LIEDO, P., BENREY, B., CANCINO, J., BARRERA, J., SIVINSKI, J., and ALUJA, M. 2000. Biological control of *Anastrepha* spp. (Diptera: Tephritidae) in mango orchards through augmentative releases of *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae). *Biol. Control* 18:216–224.

NISHIDA, T. 1956. An experimental study of the ovipositional behavior of *Opius fletcheri*. *Proc. Hawaii. Entomol. Soc.* 16:126–134.

ONG, K., PETER, C., ACREE, T. E., and LEVIN, E. H. 1998. Characterization of volatiles in Rambutan fruit (*Nephelium lappaceum* L.). *J. Agric. Food Chem.* 46:611–615.

OVRUSKI, S., ALUJA, M., SIVINSKI, J., and WHARTON, R. 2000. Hymenopteran parasitoids of fruit-infesting Tephritidae (Diptera) in Latin America and the southern United States: Diversity, distribution, taxonomic status and their use in fruit fly biological control. *Integr. Pest Manag. Rev.* 5:81–107.

PENG, H., and YANG, X. 2005. Volatile constituents in the flowers of *Elsholtzia argyi* and their variation: a possible utilization of plant resources after phytoremediation. *Journal of Zhejiang University Natural Sciences Ed.* 6B:91–95.

PROKOPY, R. J. 1985. A low-spray apple pest-management program for small orchards. *Can. Entomol.* 117:581–585.

PURCELL, M. F. 1998. Contribution of biological control to integrated pest management of tephritid fruit flies in the tropics and subtropics. *Integr. Pest Manag. Rev.* 3:63–83.

RICE, R. E., and JONES, R. A. 1982. Collections of *Prospaltella perniviosi* Tower (Hymenoptera: Aphelinidae) in San Jose scale (Homoptera: Diaspididae) pheromone traps. *Environ. Entomol.* 11:876–880.

ROHRIG, E. 2006. A floral-derived compound attractive to the tephritid fruit fly parasitoid *Diachasmimorpha longicaudata.* MS Thesis, Department of Entomology and Nematology, University of Florida, Gainesville, FL.

SAS INSTITUTE INC. 2001.SAS System for Windows, Version 8.2.SAS Institute Inc., Cary, NC.

SCHULZ, S., BOPPRE, M., and VANE-WRIGHT, R. I. 1993. Specific mixtures of secretions from male scent organs of African milkweed butterflies (Danainae). *Philos. Trans. R. Soc. Lond. B.* 342:161–181.

SIVINSKI, J., and PETERSSON, E. 1997. Mate choice and species isolation in swarming insects, pp. 294–309, in J. Choe, and B. Crespi (eds.). Mating Systems in Insects and Arachnids Cambridge University Press, Cambridge UK.

SIVINSKI, J., ALUJA, M., and HOLLER, T. 2006. Food sources for adult *Diachasmimorpha longicaudata* (Ashmead) (Hymenoptera: Braconidae), a parasitoid of tephritid fruit flies (Diptera): Effects on longevity and fecundity. *Entomol. Exp. Appl.* 118:193–202.

SIVINSKI, J. M., CALKINS, C. O., BARANOWSKI, R., HARRIS, D., BRAMBILA, J., DIAZ, J., BURNS, R. E., HOLLER, T., and DODSON, G. 1996. Suppression of Caribbean fruit fly population through augmentative releases of the parasite *Diachasmimorpha longicaudata*. *Biol. Control* 6:177–185.

SIVINSKI, J., PINERO, J., and ALUJA, M. 2000. The distributions of parasitoids (Hymenoptera) of *Anastrepha* fruit flies (Diptera: Tephritidae) along an altitudinal gradient in Veracruz, Mexico. *Biol. Control* 18:258–269.

SUCKLING, D. M., GIBB, A. R., BURNIP, G. M., and DELURY, N. C. 2002. Can parasitoid sex pheromones help in insect biocontrol? A case study of the codling moth (Lepidoptera: Tortricidae) and its parasitoid *Ascogaster quadridentata* (Hymenoptera: Braconidae). *Environ. Ecol.* 31:947–952.

U.S. ENVIRONMENTAL PROTECTION AGENCY. 1987. Health and Environmental Effects Document for Acetophenone. ECAO-CIN-G001. Environmental Criteria and Assessment Office, Office of Health and Environmental Assessment, Office of Research and Development, Cincinnati, OH.

VENTURA, M. U., MARTINS, M. C., and PASINI, A. 2000. Responses of *Diabrotica speciosa* and *Cerotoma arcuata tingomariana* (Coleoptera: Chrysomelidae) to volatile attractants. *Fla. Entomol.* 83:403–410.

WHARTON, R. A. 1989. Classical biological control of fruit infesting Tephritidae, pp. 303–314, in A. S. Robinson, and G. Hooper (eds.). Fruit Flies: Their Biology, Natural Enemies, and Control. Vol3B. Elsevier, Amsterdam.

WHARTON, R. A. 1999. A review of the old world genus *Fopius* Wharton (Hymenoptera: Braconidae: Opiinae), with description of two new species reared from fruit infesting Tephritidae (Diptera). *J. Hymenopt. Res.* 8:48–64.

WHARTON, R. A., and MARSH, P. 1978. New world Opiinae (Hymenoptera: Braconidae) parasitic on Tephritidae (Diptera). *J. Wash. Acad. Sci.* 68:147–167.

WHITMAN, D. W., and ELLER, F. J. 1990. Parasitic wasps orient to green leaf volatiles. *Chemoecology* 1:69–75.

WONG, T. T. Y., RAMADAN, M. M., MCINNIS, D. O., MOCHIZUKI, N., NISHIMOTO, J. I., and HERR, J. C. 1991. Augmentative releases of *Dichasmimorpha tryoni* (Hymenoptera: Braconidae) to suppress a Mediterranean fruit fly population in Kula, Maui, Hawaii. *Biol. Control* 1:2–7.

ZAR, J. H. 1974. Biostatistical analysis. Prentice-Hall, Englewood Cliffs, NJ.

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Inter- and Intraspecific Comparisons of Antiherbivore Defenses in Three Species of Rainforest Understory Shrubs

R. M. Fincher · L. A. Dyer · C. D. Dodson · J. L. Richards · M. A. Tobler · J. Searcy · J. E. Mather · A. J. Reid · J. S. Rolig · W. Pidcock

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Abstract Plants defend themselves against herbivores and pathogens with a suite of morphological, phenological, biochemical, and biotic defenses, each of which is presumably costly. The best studied are allocation costs that involve trade-offs in investment of resources to defense versus other plant functions. Decreases in growth or reproductive effort are the costs most often associated with antiherbivore defenses, but trade-offs among different defenses may also occur within a single plant species. We examined trade-offs among defenses in closely related tropical rain forest shrubs (*Piper cenocladum*, *P. imperiale*, and *P. melanocladum*) that possess different combinations of three types of defense: ant mutualists, secondary compounds, and leaf toughness. We also examined the effectiveness of different defenses and suites of defenses against the most abundant generalist and specialist *Piper* herbivores. For all species examined, leaf toughness was the most effective defense, with the toughest species, *P. melanocladum*, receiving the lowest incidence of total herbivory, and the least tough species, *P. imperiale*, receiving the highest incidence. Although variation in toughness within each species was substantial, there were no intraspecific relationships between toughness and herbivory. In other *Piper* studies, chemical and biotic defenses had strong intraspecific negative correlations with herbivory. A wide variety of defensive mechanisms was quantified in the three *Piper* species studied, ranging from low concentrations of chemical defenses in *P. imperiale* to a complex suite of defenses in *P. cenocladum* that includes ant mutualists, secondary metabolites, and moderate toughness. Ecological costs were evident for the array of defensive mechanisms within these *Piper* species, and the differences in defensive strategies among species may represent evolutionary trade-offs between costly defenses.

Keywords *Piper cenocladum* · *Piper imperiale* · *Piper melanocladum* · Piperaceae · Amides · Secondary metabolites · Chemical defense · Plant-herbivore interactions · Costa Rica · Tropical rain forest

R. M. Fincher · L. A. Dyer (✉) · M. A. Tobler
Department of Ecology and Evolutionary Biology,
Tulane University,
400 Boggs,
New Orleans, LA 70118, USA
e-mail: ldyer@tulane.edu

C. D. Dodson · J. L. Richards · J. Searcy · J. E. Mather · A. J. Reid · J. S. Rolig · W. Pidcock
Department of Physical and Environmental Sciences,
Mesa State College,
1100 North Ave.,
Grand Junction, CO 81501, USA

Introduction

In response to strong selective forces exerted by a broad array of enemies, which include insect herbivores, vertebrate herbivores, and pathogens (Coley et al. 1985), plants employ a variety of morphological, biochemical, and biotic defenses (Maxwell et al. 1972; Johnson 1975; Coley 1983; Kursar and Coley 1992; Harborne 2001). Differences in plant palatability that result from these defenses contribute to substantial variation in the extent of herbivore damage to plants (Coley 1983). However, each kind of defense is potentially associated with allocation costs (Levin 1976; Herms and Mattson 1992; Gershenzon 1994; Bergelson and Purrington 1996; Koricheva 2002; Strauss et al. 2002).

Many studies have addressed intraspecific trade-offs between defense and other plant metabolic functions (Zangerl et al. 1997). Because most plants produce several

different defenses (reviewed in Romeo et al. 1996), intraspecific trade-offs may occur between defenses (e.g., between chemical and biotic defenses or between two different chemical defenses; Mattson et al. 1988; Steward and Keeler 1988; but see Koricheva et al. 2004). In this case, rather than reducing plant allocation of resources to reproduction or growth, plants may express the costs of heightened defense production by diverting resources from one defense to another. These trade-offs among different defenses are referred to as "ecological costs" (Heil 2002) since reducing a given defense only incurs costs under the appropriate ecological conditions.

Evolutionary trade-offs among defenses may also be evident between closely related species because sympatric plant species that differ in the type or quantity of their defenses may be at a selective advantage when defending themselves against specialist herbivores that have evolved resistance (Cates and Rhoades 1977). Producing multiple defenses may be selectively advantageous either because of synergistic action, where the effect of a combination of defenses exceeds the sum of the effect of all individual defenses (e.g., Berenbaum and Neal 1985; Berenbaum et al. 1991; Calcagno et al. 2002; Dyer et al. 2003), or because multiple defenses may protect a plant against a diverse suite of enemies (Hay et al. 1994; Lindroth and Hwang 1996; Nelson and Kursar 1999; Dyer et al. 2003; Koricheva et al. 2004).

Ant–plant associations ranging from obligate mutualisms to loose facultative relationships are common (Heil and McKey 2003) and have long been used as model systems in the study of plant defense (e.g., Janzen 1966; Rehr et al. 1973; Dyer et al. 2001). Allocation of resources to production of domatia, food bodies, nectar, or other ant rewards in these systems presumably incurs some costs (Folgarait and Davidson 1995; Dyer et al. 2001; Heil et al. 2002). Leaf toughness and production of secondary metabolites are two widely distributed defenses in tropical plants (Coley and Barone 1996). Several studies have shown that toughness is an effective defense (Feeny 1970; Coley 1983; Sagers and Coley 1995; Coley and Barone 1996), but relatively few studies include toughness in examinations of ecological costs. Plant secondary metabolites are often effective in reducing herbivory (reviewed by Harborne 2001) but can be metabolically costly to produce (Gershenzon 1994; Zangerl et al. 1997; Dyer et al. 2001; reviewed by Bergelson and Purrington 1996, and Strauss et al. 2002), potentially necessitating trade-offs in resource allocation. For example, allocation of limited plant resources may necessitate trade-offs between allocation of carbon to ant rewards, construction of structural carbohydrates to enhance toughness, and/or chemical defense. Similarly, nitrogen may be allocated to ant rewards, nitrogen-based chemical defenses, or the enzymes and physiological machinery necessary to produce tougher leaves or higher levels of chemical defense. Plant species or individuals that invest in ant mutualisms often show reduced chemical defense when compared to closely related species or individual plants within a species that lack ant defenders (Rehr et al. 1973; Dyer et al. 2001; Heil et al. 2002).

By using field experiments and surveys, we examined relative efficacies of and trade-offs among three ecologically important tropical plant defenses: ant mutualists, secondary metabolites, and leaf toughness. Three closely related species in the genus *Piper* [*P. imperiale* (Miq.) C. DC., *P. cenocladum* C. DC., and *P. melanocladum* C. DC.) served as a model system. The phytochemistry of the diverse tropical genus *Piper* is variable and well documented (reviewed by Parmar et al. 1997; Dyer et al. 2004a). All species investigated to date (more than 112) produce mixtures of secondary metabolites, and the compounds discovered include alkaloids/amides/imides, lignans, neolignans, terpenes, propenylphenols, steroids, kavapyrones, chalcones, flavones, flavanones, and piperolides (Dyer et al. 2004a). The three *Piper* species utilized are closely related (as part of the sect. *Macrostachys* clade, Tepe et al. 2004), and share many species of generalist and specialist herbivores (Marquis 1991; L. Dyer, personal observations). They display a unique combination of ant mutualism, chemical defense, and leaf toughness. *Piper cenocladum* is defended by ant mutualists and nitrogen-containing chemicals, *P. imperiale* has a facultative relationship with ants, but its chemistry has not previously been investigated, and *P. melanocladum* has no relationship with ants, and its chemistry was previously unknown. The relative leaf toughness of all three has not previously been measured.

We tested for inter- and intra-specific trade-offs between multiple putative plant defenses and their effects on herbivory in order to address these two general questions: How is intra- and interspecific variation in plant defense associated with herbivory by specialists and generalists? Are ecological costs of different types of defense evident within and among these three *Piper* species?

We predicted that generalist herbivores would respond more to chemical defenses, while small, specialists would be deterred by ant defenders or leaf toughness (Dyer et al. 2004b). We expected to find trade-offs in allocation of resources to toughness, ant associations, and chemical defense within and among the three *Piper* species.

Materials and Methods

Study System *Piper cenocladum* is a tall (to 5 m) understory plant with large, long-lived leaves (Letourneau and Dyer 1998) found in the lowland wet forest of Costa Rica (Burger 1971). This species has defenses in the form of

amide/imide secondary metabolites (Dodson et al. 2000) and resident obligate ant mutualists (Risch and Rickson 1981; Risch 1982). *Piper cenocladum* leaves contain two imides and one amide [Appendix, structures 1–3: piplartine (1), 4′-desmethylpiplartine (2), and cenocladamide (3); Dodson et al. 2000] in relatively high concentrations of up to 3.8% dry weight (Dyer et al. 2004b). Piplartine is cytotoxic in vitro (Duh et al. 1990), and all three compounds in combination act synergistically to directly and indirectly affect the fitness and feeding preferences of generalist and specialist herbivores from a wide range of taxa (Dyer et al. 2003). *Pheidole bicornis* Forel (Hymenoptera: Formicidae: Myrmicinae) ant colonies are housed inside of *P. cenocladum* sheathing leaf bases (petioles), and the plants produce amino acid and lipid rich opalescent food bodies on the adaxial sides of the petioles (Risch and Rickson 1981). These food bodies comprise the majority of the ants' diet (Fischer et al. 2002). In return, the ants remove insect eggs, vines, and small particles from the surface of the leaves, and kill small lepidopteran larvae and stem boring weevils (Risch et al. 1977; Letourneau 1983; Letourneau and Dyer 1998). When plants are not inhabited by ants, the production of these food bodies declines, and amide production increases threefold or greater (Dodson et al. 2000; Dyer et al. 2001).

Piper imperiale is a large shrub or small tree to 6 m tall found in moist, shaded forest areas (Burger 1971). This species is characterized by large leaves and tubercles on stems and leaves but in many ways is morphologically and ecologically similar to *P. cenocladum* (Burger 1971). Several species of ants facultatively inhabit the sheathing leaf bases, but the plants produce no food bodies inside of the domatia. Whether the ants benefit the plants in any way is unknown.

Piper melanocladum is a small understory shrub to 1.6 m tall, with thick, glabrous, shiny lanceolate leaves (Burger 1971). It has small, partially open leaf bases, and has no demonstrated relationship with ants. We describe the first characterization of the nitrogen-based secondary metabolites of *P. imperiale* and *P. melanocladum*.

Field Survey This study took place in two lowland tropical wet forests at Tirimbina Rainforest Center and Estacion Biologica La Selva, Heredia Province, Costa Rica. La Selva is located at 10°25′N 84°05′W at circa 100 m elevation on the Caribbean slope. Tirimbina is located nearby at circa 210 m elevation, 10°24′4″ N, 84°6′29″ W.

We sampled *P. imperiale* (N=81), *P. cenocladum* (N=84), and *P. melanocladum* (N=94) plants along trails at Tirimbina and La Selva. Because *P. imperiale* and *P. cenocladum* reproduce vegetatively through fragmentation (Dyer et al. 2004c), individuals that we collected were separated by at least 5 m. On each plant, we chose the first fully mature leaf from the top of the plant for our measurements to standardize leaf age. We measured leaf toughness by using a modified penetrometer described by Feeny (1970). This device measures leaf toughness as the force, measured in grams of sand, needed to punch a 5-mm steel rod through a taut leaf. Immediately after removing the leaf from the plant, we took three toughness measurements, two on one side of the midrib and one on the other side. These measurements were made near the leaf tip, thus avoiding all major veins. The mean of these three toughness values was used in all analyses.

We used a translucent grid to measure the percent area removed from each leaf by each of the following major *Piper* herbivores: *Atta cephalotes* (leaf-cutter ants; Hymenoptera: Formicidae: Myrmicinae), katydids (Orthoptera: Tettigoniidae), beetles (Coleoptera: Chrysomelidae and Curculionidae), *Quadrus cerealis* caterpillars (Lepidoptera: Hesperiidae), and at least two species of *Eois* caterpillars (Lepidoptera: Geometridae), which were grouped together. Each type of herbivore damage is easily discernible based upon the pattern and shape of damage. Total percent damage was calculated for each leaf as the sum of damage by all herbivore types.

Imide Isolation and Quantification Imides were isolated from *P. imperiale* and *P. melanocladum*, and their structures were determined with the methods for *P. cenocladum* described by Dodson et al. (2000). All imide structures were confirmed by synthesis (Appendix). A randomly selected subset of *P. imperiale* (N=30), *P. melanocladum* (N=44), and *P. cenocladum* (N=34) leaves were air-dried and analyzed to determine secondary chemical content. Each leaf was extracted ×2 with ethanol overnight. The resultant extract was resuspended in a 3:1 water/ethanol mixture and exhaustively extracted with chloroform in a separatory funnel. Combined extracts were dried, resuspended in chloroform, and analyzed by gas chromatography. Standards for analysis were synthesized at Mesa State College (Appendix). For a more detailed description of extraction and GC methods, see Dodson et al. (2000) and Dyer et al. (2001).

Atta cephalotes Bioassays To determine the effectiveness of the newly identified *P.* imides as defenses, we performed a feeding choice experiment with *Atta cephalotes* colonies. We modeled our experiment on those performed by Folgarait et al. (1996). We presented foraging *A. cephalotes* colonies with a selection of leaf fragments coated with different compounds. Treatments applied to experimental leaf fragments included piplaroxide (**5**), the newly discovered compounds (**6**) and (**4**), and a control that contained

only methanol (all imides were in methanol solution). Treatments were applied to *Hyeronima alchorneoides* Allemao (Euphorbiaceae) leaves (a species shown to be palatable to *A. cephalotes*; Folgarait et al. 1996) and allowed to dry. Randomly distributed *A. cephalotes* colonies were simultaneously presented with five fragments of each of the four treatments (for a total of 20 leaf fragments per trial) in a cafeteria-style display. These trials continued for 15 min or until all 5 fragments of one of the treatments had been removed by the ants. It was repeated with 22 different colonies (N=22).

We modified this *A. cephalotes* feeding trial to use *P. imperiale* leaf ethanol extracts, which would contain most secondary metabolites and other ethanol-soluble compounds produced by the plant, and to force the ants to cut fragments from a large leaf section, so that their mouthparts would be exposed to any secondary metabolites during the cutting process. We applied an extract of *P. imperiale* to leaves of *Hieronyma alchorneoides* and compared the amount of leaf material removed from leaves treated with extract to control leaves. Fresh and dry mass measurements from 144 strips of *H. alchorneoides* were used to create a conversion factor for estimation of the dry mass of fresh leaf strips used in feeding choice trials (dry mass=0.2512 (fresh mass) +0.0054, R^2=0.83). Leaves were cut into paired strips (two from each leaf) weighing 0.25 g fresh weight, avoiding all major veins, and the leaf area for each strip was measured with a LICOR leaf area meter (leaf area mean ± SE=12.27±0.15 cm^2). We cut a pair of treatment and control leaf strips from the same leaf, applied *P. imperiale* extract at high or low concentrations of 0.0095% leaf dry weight compound 4 (Appendix) and 0.0051% leaf dry weight compound 5 (Appendix) (N=42), or 0.0024% leaf dry weight compound 4 (Appendix) and 0.0013% leaf dry weight compound 5 (Appendix) (N=36; dissolved in 0.24 and 0.06 ml ethanol, respectively) to the treatment leaf strip and a corresponding amount of ethanol to the control leaf strip and allowed the extract to dry. These concentrations are an order of magnitude lower than those found in *P. imperiale* leaves, and so should measure the lower limits of ant abilities to detect and respond to secondary metabolites. Leaves were placed in pairs (one control leaf and one leaf treated with *P. imperiale* extract) in an actively foraging column of leaf-cutter ants (modeled after the methods of Folgarait et al. 1996). Each trial was allowed to continue for 2 hr or until the ants had removed all of one leaf strip. Leaf strips were large enough that ants cut small (approximately 1 cm^2) sections to carry away. After each trial, final leaf weight and leaf area were measured.

Statistical Analyses Because we were not able to transform the percent herbivory data to meet requirements of normality for parametric statistics due to the low frequency of occurrence of herbivory, we used two methods of analyses to test for differences among *Piper* species in herbivory by different herbivores. First, to examine qualitatively host choice by herbivores, we categorized leaves as experiencing herbivory or escaping from herbivory, and we performed chi-square tests for contingency tables to test for differences among plant species in the frequency of escape from herbivory. We then quantitatively examined the extent of feeding by herbivores once feeding was initiated (i.e., excluding all plants lacking herbivory) by performing an analysis of covariance (ANCOVA) with plant species as the independent variable, herbivore damage as the dependent variable, and toughness as a covariate, followed by Tukey's multiple range tests. For this analysis, we removed all zero herbivory values from the data set and log transformed damage and toughness values to meet assumptions of residual normality and homogeneity of variance. The covariate and the interactive term were insignificant in all tests. In combination, these two analyses compare both the frequency of herbivory and the amount of herbivore damage among plant species. Both of these analyses were repeated for all damage types (total herbivory, *A. cephalotes*, tettigoniid, beetle, *Eois*, and *Q. cerealis*). To test for interspecific differences in toughness and concentration of amides/imides, we performed an analysis of variance (ANOVA) followed by Tukey's multiple range test.

We used logistic regression to examine intraspecific relationships among chemical defense (with chemical defense content used as a predictor variable), toughness, and herbivory by different herbivores. One test was performed for each type of herbivore (with the exception of *Q. cerealis* and *A. cephalotes*, which were excluded because of rarity) and for total percent herbivory. Damage by each type of herbivore was classified as either present or absent. We tested for evidence of intraspecific trade-offs among different defenses by examining correlations between average leaf toughness and amide content for each *Piper* species.

Due to intrinsic difficulties in analyzing cafeteria-style feeding choice data (Lockwood 1998, and works cited therein) and nonnormality of the data, we analyzed the first *A. cephalotes* bioassay feeding preference data by using log-linear models (Floyd 2001). Each leaf fragment treatment was used as a variable in specified models. We used the maximum likelihood method for parameter estimation of linear models and Chi-square statistics for hypothesis testing (Folgarait et al. 1996). Because we were interested in testing specific hypotheses, we used nonhierarchical models to test the significance of leaf treatments as predictors of *A. cephalotes* preference. For the second *A. cephalotes* bioassay feeding preference experiment that

used leaf extracts, two paired *t* tests were used to test for differences in the leaf area and mass removed from control and *P. imperiale* leaf extract treated leaf strips, for each concentration of extract.

Results

Secondary Metabolites *Piper imperiale* contains two imides (Appendix, structures 4, 5). The first imide, compound 4, is an analog of piplartine, a compound we previously found in *P. cenocladum*. Compound 4 (5′-desmethoxydihydropiplartine) has not been isolated previously from a species of *Piper*, and is accompanied by its epoxide deviative, compound 5, which has been isolated previously from *P. tuberculatum* by Capron and Wiemer (1996) and is named piplaroxide. Total imide content ranged from 0.0044% to 0.029% dry weight. *P. imperiale* did not have alkaloids but contained at least five different sesquiterpenes (Appendix, Experimental).

We isolated compounds **4** and **5** (piplaroxide; Appendix) as well as the 4′-desmethyl analog of piplaroxide from *P. melanocladum*. The latter compound is also new to the genus *Piper* and has structure **6** (Appendix). These imides are present at high levels (ranging from 0.016% to 0.40% dry weight) in the leaves. No other defensive compounds were detected in *P. melanocladum* (Appendix, Experimental).

Piper cenocladum total amide/imide content ranged from 0.17% to 1.068% leaf dry weight. This range of concentrations is consistent with ranges reported in other studies with this species where synergy (Dyer et al. 2003) and trade-offs (Dodson et al. 2000) have been demonstrated. No other defensive compounds were detected in *P. cenocladum* (Appendix, Experimental).

Interspecific Differences The three *Piper* species were different in their leaf toughness ($F_{2,\ 256}$=168.2, P<0.001; Fig. 1a). *Piper melanocladum* is the toughest species with a penetrometer value of 559.3±13.9 g, nearly twice as tough as the least tough *P. imperiale* (296.6±6.7 g). The toughness of *P. cenocladum* was intermediate (389.9±13.9 g). The concentration of amides/imides in *P. cenocladum* was higher than that in *P. melanocladum*, which in turn was higher than that in *P. imperiale* ($F_{2,\ 100}$=107.18, P<0.001; Fig. 1b).

When leaves were categorized as either experiencing or escaping herbivory, *Piper* species had significant differences in frequency of leaves escaping damage from beetles, *Eois*, and all herbivores combined (Fig. 2a; beetle: X^2=17.3, df=2, P<0.001; *Eois*: X^2=13.1, df=2, P=0.001; total: X^2=21.4, df=2, P<0.001). The number of individuals escaping total herbivory and beetle herbivory was lowest for *P. imperiale*, while the number of individuals escaping *Eois* herbivory was lowest for *P. cenocladum*. *Piper melanocladum* individuals escaped all three types of herbivory more frequently than the other two *Piper* species. Damage by *Atta cephalotes* and *Q. cerealis* was rare (N=4 for each herbivore), indicating that these herbivores avoid all three *Piper* species. Tettigoniids showed no preference for one *Piper* species over another (X^2=4.0, df=2, P=0.1).

Once a plant is attacked by an herbivore, the extent of damage differs among *Piper* species and among herbivores. When individuals with no herbivore damage were omitted from the analysis, beetle and total herbivore damage were significantly different between *Piper* species (Table 1, Fig. 2b). Herbivores fed more on *Piper imperiale* than on *P. cenocladum* or *P. melanocladum* (Fig. 2b). Beetles

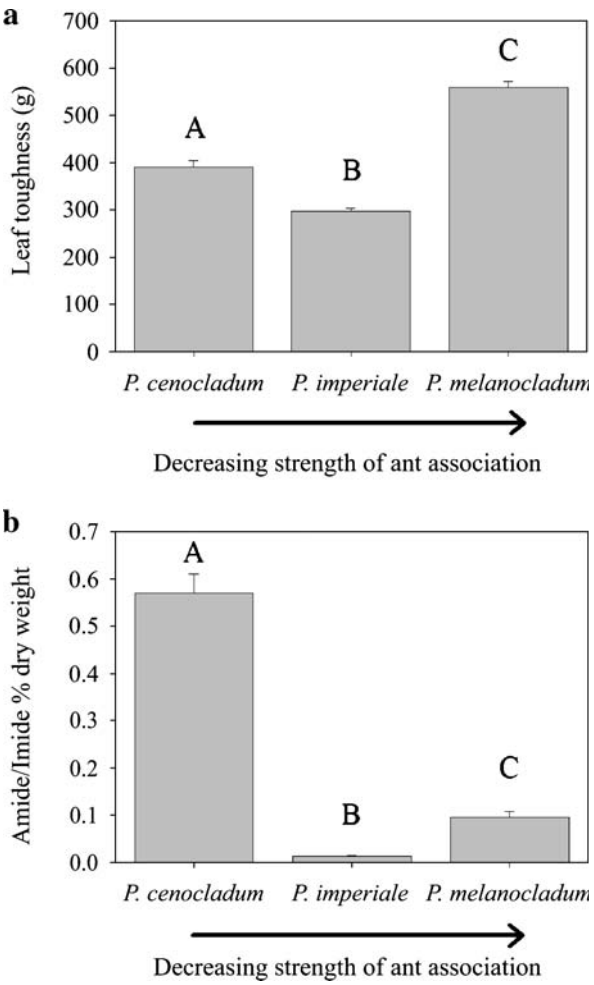

Fig. 1 Differences in: **a** mean leaf toughness (±1 SE) and **b** mean amide/imide content (±1 SE) among three species of *Piper* shrubs (*P. cenocladum*: N=84, *P. imperiale*: N=84, *P. melanocladum*: N=94). Data were analyzed using analysis of variance followed by Tukey's multiple range test; *different letters* above *Piper* species indicate significant differences

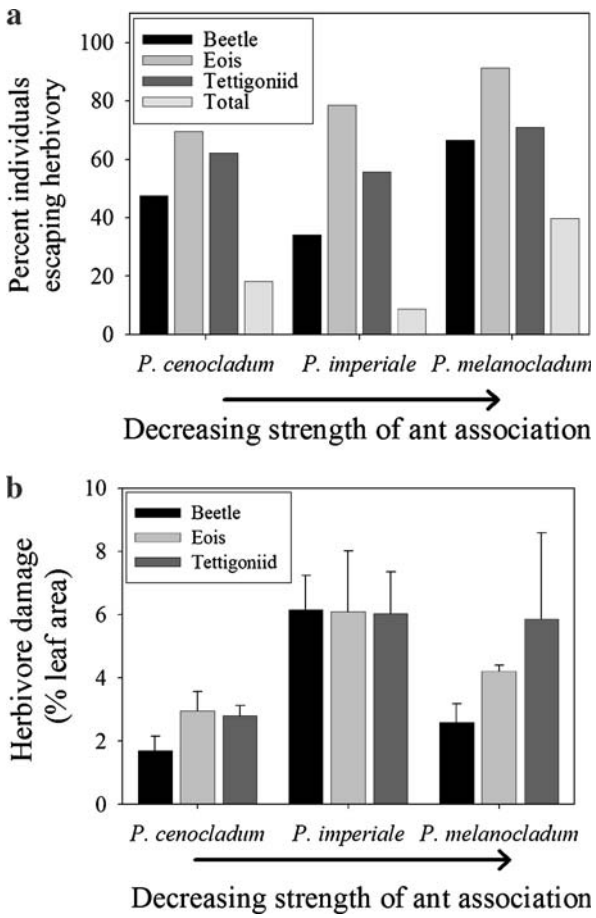

Fig. 2 Naturally occurring interspecific variation in herbivory for three closely related species of tropical shrubs, *Piper* spp. **a** Percentage of individuals completely escaping herbivory. Chi-square tests for contingency tables were utilized to test for differences between plant species in the frequency of escape from herbivory. Beetle, *Eois* and total herbivory differed significantly between *Piper* species (*P. cenocladum*: N=84, *P. imperiale*: N=84, *P. melanocladum*: N=94). A value of 100% indicates all individuals of that species escaped herbivory from a particular herbivore taxon. Total herbivory includes escape from all categories of herbivory, thus, it is always has a lower percentage than other categories. **b** Percent herbivory (±1 SE) on individual plants that did not escape herbivores. Analysis of Covariance (with toughness as a covariate) was utilized to test for differences in herbivory between species. *P. cenocladum*: N=72, *P. imperiale*: N=67, *P. melanocladum*: N=56)

showed a significant preference for *P. imperiale* over *P. cenocladum* but did not differentiate between *P. melanocladum* and the other two *Piper* species (Table 1). *Piper* damage by *Atta cephalotes* and *Q. cerealis* was excluded from the analysis due to small sample size. Levels of damage by *Eois* and tettigoniids were not significantly different among *Piper* species (Table 1).

Intraspecific Relationships Among Secondary Metabolites, Toughness, and Herbivory Secondary metabolite content in *P. melanocladum* was negatively correlated with levels of tettigoniid herbivory on leaves that experienced herbivory (R=−0.63, P=0.05). No other correlations between herbivory and secondary metabolites were found. We found no intraspecific relationships between toughness and herbivory (Table 2), but intraspecific variance was low compared to interspecific variance (Fig. 1a). We also found no evidence for trade-offs between toughness and secondary metabolites in the three species (*P. cenocladum* N=34, R=−0.22, P>0.05; *P. imperiale* N=30, R=0.033, P>0.05; *P. melanocladdum* N=44, R=0.10, P>0.05).

Atta cephalotes Feeding Trial In *P. imperiale* and *P. melanocladum*, compound **4**, piplaroxide (**5**), and compound **6** are clearly deterrent to *A. cephalotes* (Table 3, Fig. 3). The loglinear model revealed significant associations in removal of different leaf disks based on this deterrence. The (compound **4**×compound **6**) and (compound **4**×piplaroxide) interactions are significant since the frequencies of removal of both disk types are low in each case. In addition, associations between removal of control and treatment disks reflect the fact that the likelihood of the control disk being taken while the treatment disk was not taken was high, and the likelihood of the control disk being left while the treatment disk was taken was low. Whole leaf extract of *P. imperiale* was also deterrent to *A. cephalotes* (Fig. 4). Ants removed less leaf area and mass of leaves treated with leaf extracts in high (T=−2.38, df=41, P=0.02; T=−2.86, df=41, P=0.007) and low (T=−2.60, df=35, P=.01; T=−2.10, df=35, P=0.04) concentrations.

Discussion

We found evidence for ecological costs of the different defense mechanisms in the three *Piper* species studied. Secondary metabolites and their concentrations differed among species, along with toughness and the strength of the association of the plants with ants. *Piper melanocladum*, which has no ant association and low levels of chemical defense, has tough leaves. *Piper cenocladum* has the strongest association with ants and the highest levels of chemical defenses, but the leaves of this species are not likely to use toughness as a deterrent. *Piper imperiale* has tender leaves, low levels of defenses, and a loose association with ants. In addition to these contrasting suites of defenses, it is possible that there are trade-offs between the traits measured here and chemical defenses that are unknown or were not quantified, such as the sesquiterpenes that are present in *P. imperiale*. A comparison of toughness for the non-ant plant, *P. melanocladum*, with the ant-plants, *P. cenocladum* (obligate) and *P. imperiale* (facultative), provides support for the hypothesis that ant plants invest less in other defenses (Fig. 1a; Dyer et al. 2001; Heil et al.

Table 1 Qualitative differences in frequency of herbivory

Damage Type	Mean±SE (*N*)			*F* (*df*)
Plant Species (Total *N*)	*P. imperiale* (81)	*P. cenocladum* (84)	*P. melanocladum* (94)	
Total percent damage	9.6±1.21 (72)	4.5±1.1 (67)	5.0±1.4 (56)	9.6** (2)
Beetle percent damage	6.1±1.1% (52)	1.7±0.4% (43)	2.6±0.6% (31)	10.7** (2)
Tettigoniid percent damage	6.0±1.3 (35)	2.8±0.8 (31)	5.9±2.7 (27)	3.0* (2)
Eois percent damage	6.1±1.9 (17)	3.0 0.6 (25)	4.2 1.7 (8)	1.4 (2)
A. cephalotes percent damage	(0)	22.9±20.8 (3)	1.0 (1)	–
Q. cerealis percent damage	26.9±23.9 (2)	2.4 (1)	6.6 (1)	–

[a] Analysis of covariance
[b] Only herbivores experiencing herbivory were included in analyses, thus *N* varies by damage type and different types of herbivory do not add up to total percent damage.
*$P<.06$
**$P<.0001$

2002). On the other hand, the hypothesis that ant plants invest less than non-ant plants in chemical defenses, such as amides/imides, is only supported when comparing concentrations of these compounds in *P. imperiale* and *P. melanocladum* (Fig. 1b).

The effectiveness of different defensive mechanisms in these three species varies widely and differs according to the taxon of the attacking herbivore. The strong negative relationship between herbivory and toughness across the three species supports the findings of previous studies that demonstrate that toughness is an important defense (Coley 1983; Sagers and Coley 1995; Coley and Barone 1996). When choosing among multiple host species, generalist and oligophagous herbivores may avoid extremely tough species. In an examination of different types of anti-herbivore defenses and other plant characteristics across 46 tropical tree species, Coley (1983) found that leaf toughness was the plant characteristic most correlated with reduced herbivory. However, all patterns of herbivory cannot be accounted for with differences in leaf toughness. Specialist *Eois* caterpillar damage is higher on *P. cenocladum* vs. the other two species, although *P. cenocladum* is of intermediate toughness. *Eois* feeding may be deterred by both toughness (in *P. melanocladum*) and secondary metabolite content (in *P. imperiale*). Alternatively, as relatively specialized feeders, *Eois* species, some of which sequester imides/amides (Dyer et al. 2003), may be adapted to the defensive compounds in *P. cenocladum*. Tettigoniids, which are relatively generalized feeders, do not appear to have a host preference possibly because they feed on the young, expanding leaves of these species, which may have low toughness and secondary metabolite content (Kursar and Coley 1992).

Intraspecific variation in leaf toughness is not associated with changes in total herbivory, suggesting that feeding preferences within a single plant species may be motivated by other factors, such as nutrient content or secondary metabolism. Indeed, individual *Piper* species with lower levels of chemical defenses in their tissues may be preferred by insect enemies, such as leaf-cutter ants. We have shown here that piplaroxide (**5**) and compounds **4** and **6** (found in *P. imperiale* and *P. melanocladum*) are strongly deterrent to

Table 2 Logistic regression: toughness as predictor of herbivore damage

Species	Total Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	Beetle Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	Tettigoniid Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2	*Eois* Damage Likelihood Ratio χ^2, Standardized Estimate, Estimate χ^2
Piper cenocladum (*N*=84)	0.40, −0.54, 0.31	0.78, −0.73, 0.63	1.86, −1.13, 1.33	0.19, 0.42, 0.18
Piper imperiale (*N*=81)	2.80, 1.67, 2.71	0.42, 0.65, 0.42	0.28, 0.53, 0.28	0.29, 0.53, 0.28
Piper melanocladum (N=94)	2.11, 1.44, 2.04	2.86, 1.72, 2.75	0.30, 0.56, 0.30	0.15, 0.38, 0.15

[a] Data analyzed with logistic regression; degrees of freedom=1.
[b] $P>0.05$ for all likelihood ratios, indicating that models are a good fit.
[c] $P>0.05$ for all standardized estimates, indicating no association between toughness and herbivore damage.

Table 3 Effects of *Piper* imides on *Atta cephalotes* feeding preference

Source	Degrees of Freedom	Chi-Square	*P* Value	Standardized Estimate
Compound 5×Compound 6	1	30.58	<0.0001	5.5303
Compound 5×Compound 4	1	23.29	<0.0001	4.8243
Compound 6	1	7.67	0.0281	2.7695
Control×Compound 6	1	4.41	0.0499	2.1002
Control×Compound 6	1	4.38	0.0250	2.0924
Control	1	3.79	0.0486	−1.9470
Control×Compound 5	1	0.49	0.5792	0.6967
Compound 6[a]	1	0.91	0.3396	0.9549
Compound 5[a]	1	0.06	0.8001	−0.2230
Likelihood ratio	4	5.13	0.2738	

Data analyzed utilizing logit models

[a] Nonsignificant variables included in former models.

Compound 4=1-[(2E)-3-(3′,4′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one; Compound 5=piplaroxide; Compound 6=3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one

A. cephalotes (see also Capron and Wiemer 1996). In previous studies, we demonstrated also that the three imides/amides found in *P. cenocladum* deter feeding by *A. cephalotes* and act synergistically against a variety of herbivores (Dyer et al. 2003). Clearly, the imide/amide nitrogen-based defenses are effective deterrents to leaf-cutter ants, and the presence of these compounds is a likely explanation for why these *Piper* species are avoided by *A. cephalotes*.

The two sister species, *P. cenocladum* and *P. imperiale* exhibit different patterns of defense, tolerance, and trade-offs among defenses, despite the fact that these two species are morphologically similar, unresolved in molecular phylogenies (Tepe et al. 2004), and share almost identical herbivore fauna. In *P. imperiale*, herbivory is common, as the species appears to be investing few resources in defense and relying on tolerance. Dyer et al. (2004c) found that asexual reproductive success of *P. imperiale* was not affected by herbivory, which is consistent with a tolerance hypothesis. *Piper imperiale* fragments placed on the surface of lowland rainforest soils to simulate asexual reproduction through natural fragmentation appeared tolerant of high

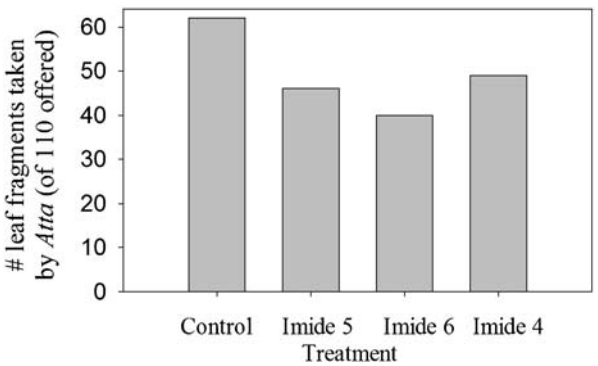

Fig. 3 The frequency of experimental leaf fragment removal by leafcutting ants, *Atta cephalotes* (Hymenoptera). Experimental fragments were treated with imides from two species of tropical shrubs, *P. imperiale* and *P. melanocladum*, and control fragments were treated only with solvent. A loglinear model uncovered significant associations between removal of pairs of leaf fragments with different solutions applied (e.g., when high numbers of control fragments were removed, low numbers of piplaroxide fragments were removed). Based on significant associations in this model, all defensive imides were deterrent to *A. cephalotes*. Imide 4=1-[(2E)-3-(3′,4′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one; Imide 5=piplaroxide; Imide 6=3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one

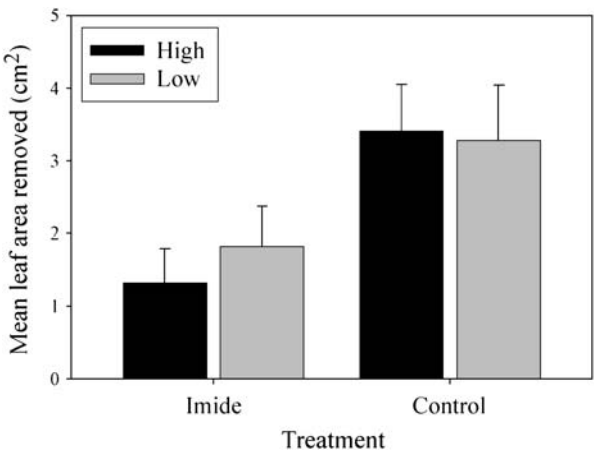

Fig. 4 Mean leaf area (±1 SE) removed from leaves of the tropical shrub, *P. imperiale*, by foraging leaf-cutter (*Atta cephalotes*; Hymenoptera) workers. Large leaf fragments were treated with *P. imperiale* extracts at high ($T=-2.38$, d.f.=41, $P=0.02$) and low ($T=-2.60$, d.f.=35, $P=0.01$) concentrations and ants were presented with a control leaf and a treatment leaf during a 2 h trial

levels of herbivory present on fragments before planting. In contrast, *P. cenocladum* exhibits high allocation of resources to defense, which may reflect low tolerance of herbivory in this species. *Piper cenocladum* relies primarily on vegetative fragmentation for reproduction (Greig 1993), and Dyer et al. (2004c) showed that *P. cenocladum* asexual reproductive success is reduced by natural herbivory on and artificial damage of the leaves of fragments. Prevention of herbivory by chemical and ant defense is associated with increased asexual reproductive fitness. One might predict that such high investment in defense would lead to trade-offs among different defenses. In *P. cenocladum*, a trade-off between secondary metabolite content and ant defense is indicated by a threefold increase in amide concentrations when ants are excluded from *P. cenocladum* plants (Dodson et al. 2000). In the current study, this increase in secondary metabolite content was not correlated with *Eois* herbivory, which is consistent with other studies that demonstrate that amides/imides do not protect *P. cenocladum* plants from feeding by specialized herbivores (Dyer et al. 2004b). There is evidence that the *Ph. bicornis* ant mutualists effectively protect plants against specialized herbivores, but they have little effect on large, generalist herbivores such as katydids or other caterpillars, which may be deterred by increased amide/imide concentrations (Dyer and Letourneau 1999; Dyer et al. 2001, 2004b). Although we found that *P. cenocladum* exhibits moderate investment in leaf toughness, we were unable to detect any correlations between toughness and herbivory in this species. Nevertheless, investment in leaf toughness could make it difficult for rare herbivores such as hesperiids (Lepidoptera) and *Atta* (Hymenoptera) from adding this plant species as a consistent food resource.

In summary, we have demonstrated a wide variety of defensive mechanisms among three closely related species of tropical shrubs. It is clear that redundancy in defense provides the plant with protection against a variety of herbivores. Previous work demonstrated intraspecific trade-offs between chemical and ant-mediated defense in *P. cenocladum*, but our interspecific results here were consistent with both the trade-off hypothesis as well as the alternative hypothesis that there are no trade-offs between indirect biotic and direct chemical defenses in plants (Heil et al. 2002). We were also unable to measure trade-offs between defense and other functions, but tough leaves may come at the expense of lower rates of growth or reproduction. Studies of chemical redundancy, ecological costs of antiherbivore defense, and interspecific variation in defensive attributes should consider the complex interaction between forces that select for optimum levels of defense against a variety of different herbivores and trade-offs between defenses, growth, and reproduction that allow plants to minimize the cost of defense.

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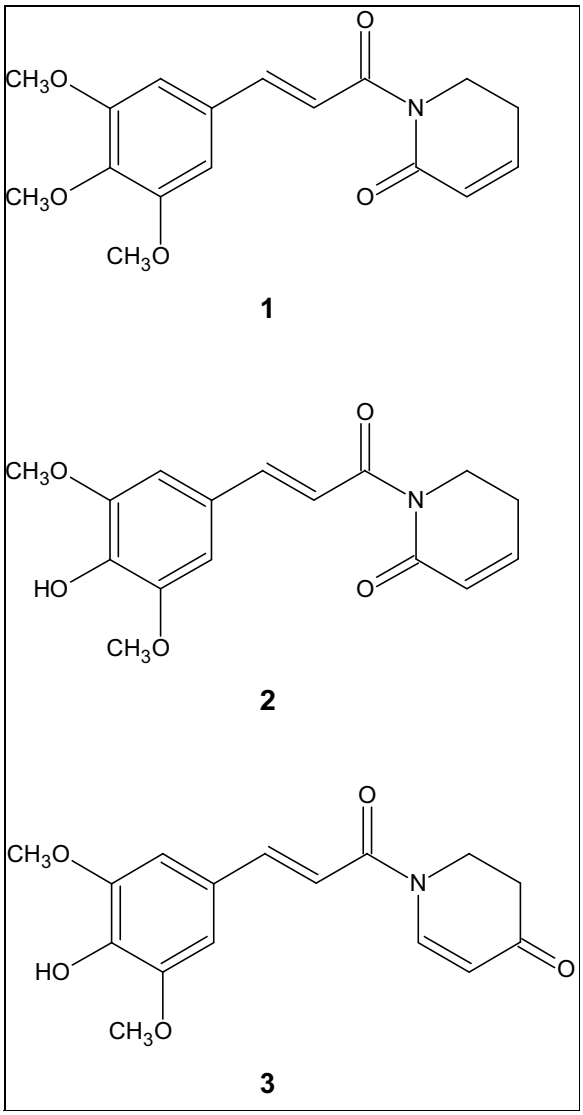
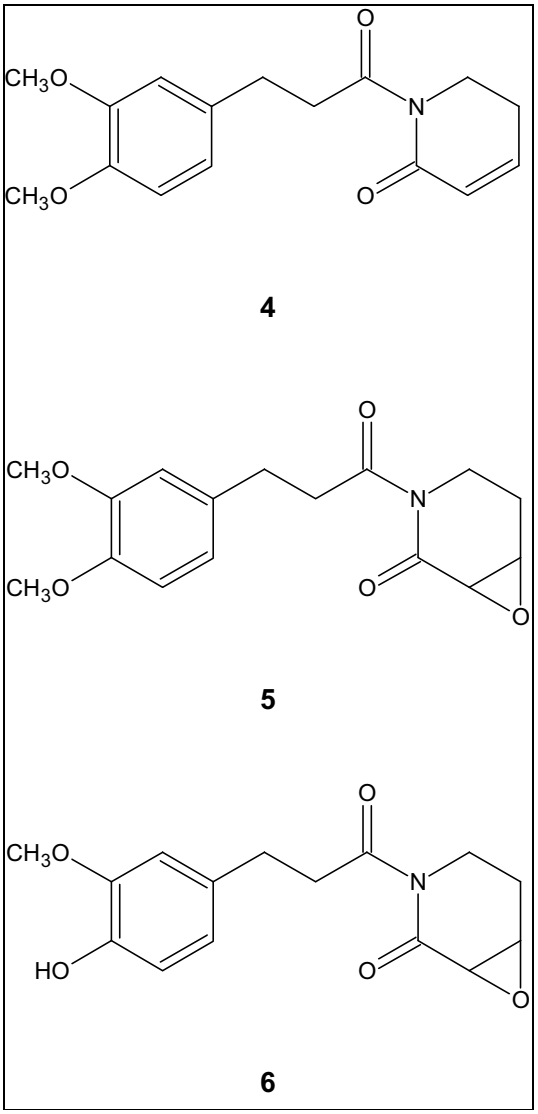
Appendix

Structures 1–3. Defensive metabolites isolated from *Piper cenocladum*



Structure 4–6. Defensive metabolites isolated from *Piper melanocladum*



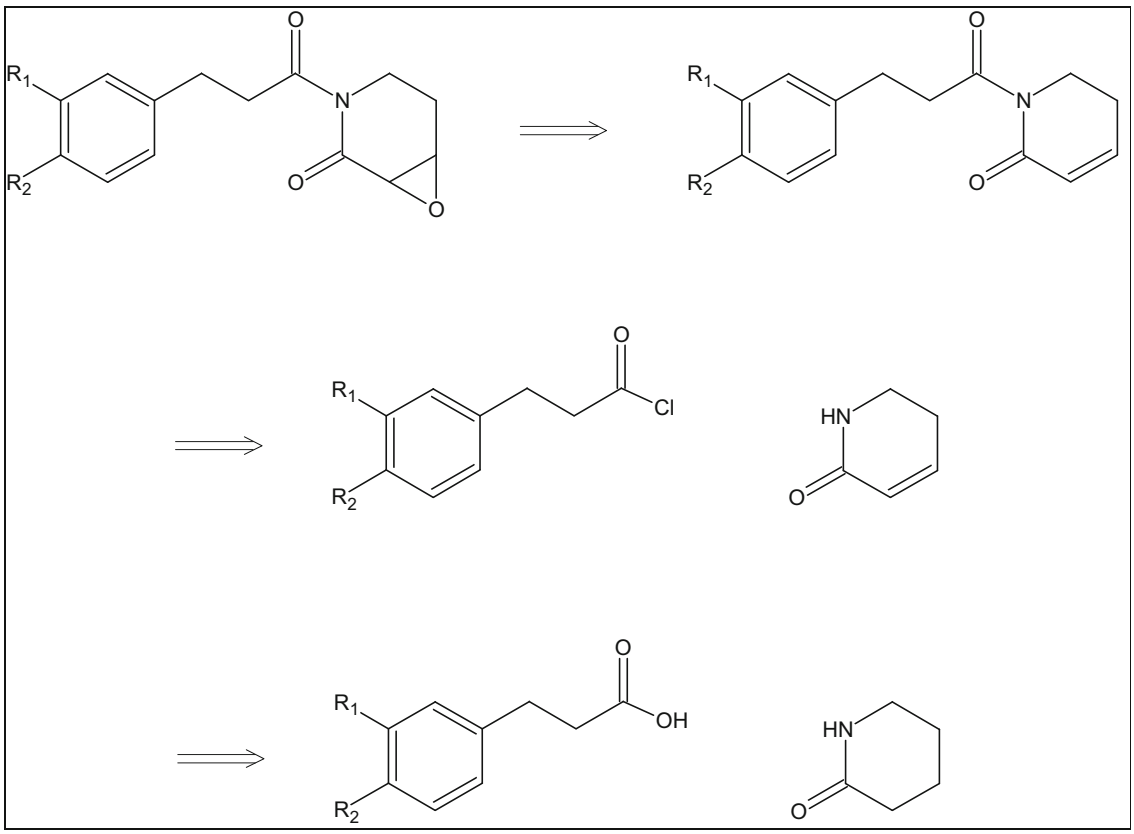
Synthesis of **4, 5, and 6** (Schemes 1, 2, 3, 4, 5, 6 and 7): The syntheses of 1-[(2E)-3-(3′′,4′′-dimethoxyphenyl)prop-2-enoyl]-5,6-dihydropridin-2(1H)-one (**4**), 3-[(2*E*)-3-(3,4-dimethylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo-[4.1.0] heptan-2-one (piplaroxide, **5**) and 3-[(2*E*)-3-(4-hydroxy-3-methylphenyl)prop-2-enoyl]-7-oxa-3-azabicyclo[4.1.0]-heptan-2-one (**6**) were accomplished via similar convergent approaches. These involved the synthesis of an appropriate functionalized pyridinone piece and its coupling with an appropriately activated acid derivative.

Experimental

General Information All ^{1}H NMR and ^{13}C NMR spectra were recorded at 300 MHz. All flash chromatography was performed on Aldrich silica gel (200–400 mesh, 60 Å). Tetrahydrofuran (THF) was redistilled from sodium/benzophenone. Dichloromethane (CH_2Cl_2) was redistilled from CaH_2. All other reagents and solvents were used as received unless otherwise noted.

1-(Methoxymethyl)piperidin-2-one (13) A 500-ml round bottom flask was flushed under N_2 while δ-valerolactam was melted on the oven. δ-Valerolactam (15.147 g, 153 mmol) and distilled THF (160 ml) were added to the flask, and the mixture was cooled on an ice bath for 10 min. After cooling, 2.25 M n-BuLi (50 ml) was added to the mixture slowly via syringe. CH_3OCH_2Cl (11.8 g, 157 mmol) was added to the flask, and the mixture stirred at room temperature for 2 h. The reaction mixture was transferred to a separatory funnel with hexane (50 ml), and washed with water (2×100 ml) and brine (1×50 ml). The combined aqueous layers were extracted with CH_2Cl_2 (5× 50 ml). The combined organic layers were washed with brine (1×60 ml), dried over anhydrous magnesium sulfate, and filtered. The solvent was removed under reduced pressure to yield a yellow liquid (15.2 g), which was purified via vacuum distillation (92–94°C @ 4 mm Hg) using a Vigeroux fractionating column to yield **13** as a colorless liquid [10.3 g, 49% yield; ^{1}H NMR ($CDCl_3$): δ 4.77 (2H, s), δ 3.32 (2H, t, *J*=5 Hz), δ 3.26 (3H, s), δ 2.39 (2H, t, *J*=5 Hz), δ 1.78 (4H, m). ^{13}C NMR ($CDCl_3$): δ 171.1, δ 77.1, δ 56.0, δ 46.2, δ 32.4, δ 23.1, δ 21.3].

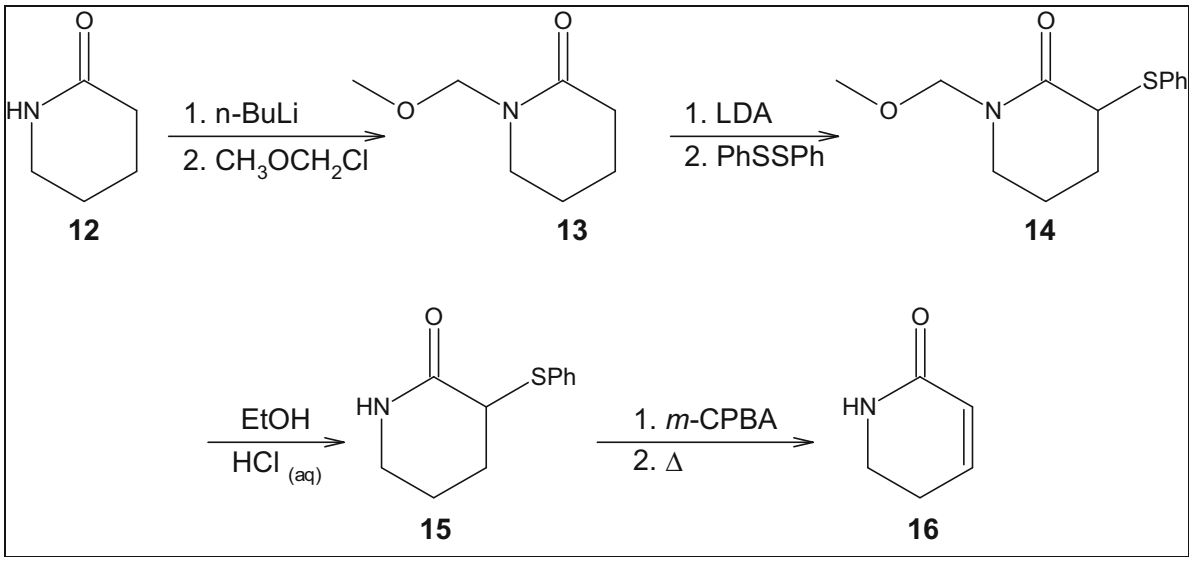
1-(Methoxymethyl)-3-(phenylthio)piperidin-2-one (14) A 250-ml three-necked round bottom was oven dried, fitted with an addition funnel and a septum, and flushed under N_2. Distilled THF (30 ml) and diisopropylamine (8 ml) were added to the flask and the solution stirred on ice for 10 min. 2.25 M n-BuLi (24 ml) was added through the septum via syringe and the mixture stirred on ice for 10 min. The mixture was cooled to −78°C in a dry ice/isopropanol bath. Compound **13** (4 g, 27.9 mmol) was dissolved in distilled THF (15 ml) and added to the reaction through the dropping funnel over 10 min. The mixture stirred at −78°C for an additional 45 min. A mixture of phenyldisulfide (6.01 g) and HMPA (4.8 ml) in distilled THF (15 ml) was added to the reaction mixture over 20 min. The reaction stirred at −78°C for an additional 40 min and was then allowed to warm to room temperature. The reaction mixture was transferred to a separatory funnel with water (60 ml) and extracted with diethyl ether (3× 60 ml). The combined ether layers were washed with 3 M NaOH (1×40 ml), water (1×40 ml), 3 M HCl (1×40 ml), water (1×40 ml), and brine (1×40 ml). The solution was dried over magnesium sulfate, filtered, and evaporated to yield **14** as an orange oil [6.25 g, 89% yield; ^{1}H NMR ($CDCl_3$): δ 7.54 (2H, m), δ 7.29 (3H, m), δ 4.83 (2H, q, *J*=



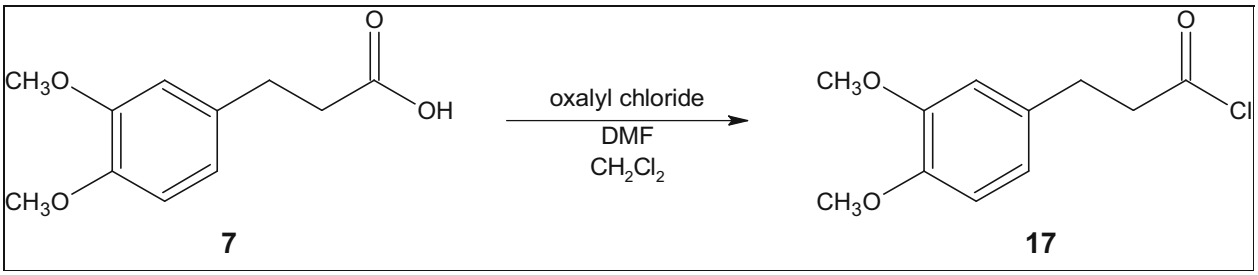
Scheme 1 Convergent retrosynthesis of "*Piper* amides" isolated from *P. melanocladum*

10 Hz), δ 3.89 (1H, t, *J*=5.5 Hz), δ 3.39 (2H, m), δ 3.30 (3H, s), δ 2.06 (3H, m), δ 1.7 (1H, m). ^{13}C NMR ($CDCl_3$): δ 169.8, δ 134.4, δ 132.7, δ 129.1, δ 127.7, δ 77.5, δ 56.2, δ 49.0, δ 45.9, δ 28.4, δ 20.4].

3-(Phenylthio)piperidin-2-one (15) Compound **14** (10 g) was placed in a 1-l round bottom flask with 95% ethanol (270 ml) and conc. HCl (54 ml). The reaction mixture was heated at reflux for 6 h. The volume was reduced to 100 ml



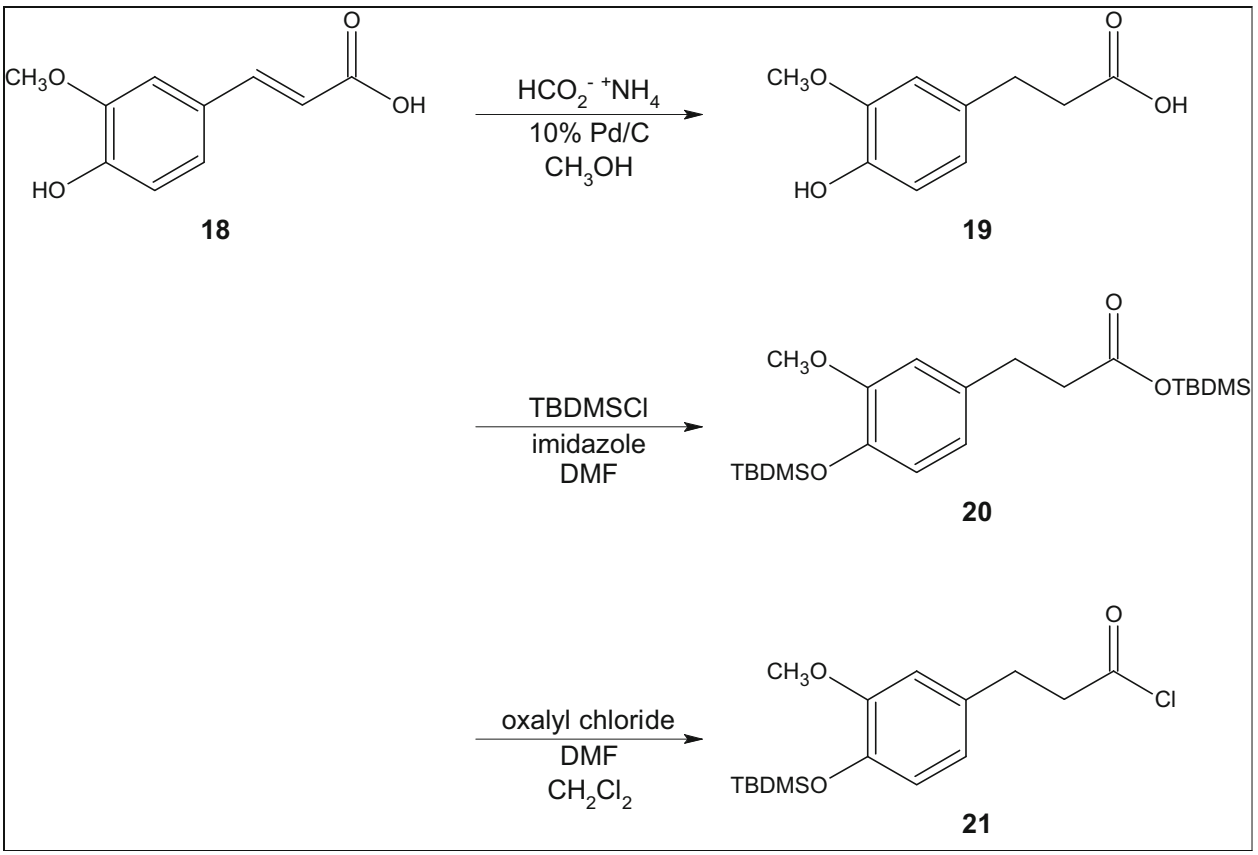
Scheme 2 The dihydropyridinone moiety was prepared from δ-valerolactam (**12**). The lactam nitrogen was protected (**13**) by treating the lithium salt of the lactam with chloromethyl methyl ether (MOMCl). The enolate of compound **13** was generated by treating the protected lactam with two equivalents of LDA in THF at −78°C and sulfenylated in the α-position with phenyl disulfide (PhSSPh). After deprotection of the sulfenylated lactam (**14**), the sulfide was oxidized to the sulfoxide and subjected to thermal elimination to produce 5,6-dihydropyridin-2(1*H*)-one (**16**)



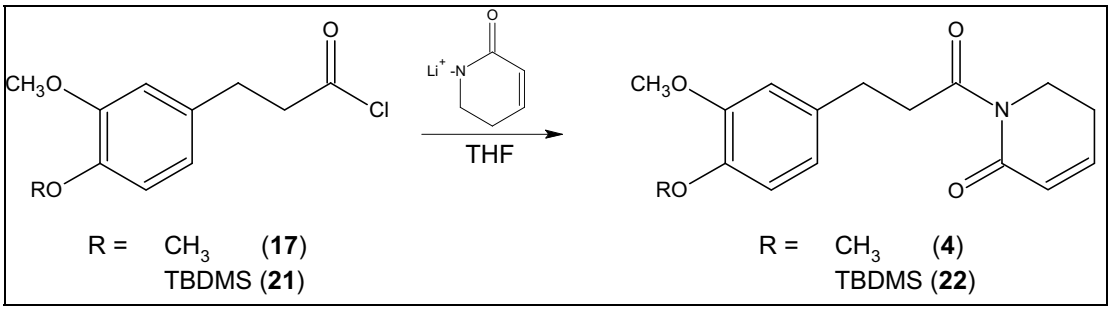
Scheme 3 The hydrocinnamoyl derivatives were prepared from appropriate carboxylic acid precursors. Consequently, commercially available 3,4-dimethoxyhydrocinnamic acid (**7**) was converted to 3,4-dimethoxyhydrocinnamoyl chloride (**17**) by treating the acid with oxalyl chloride in dichloromethane using catalytic amounts of dry DMF

under reduced pressure, and the mixture was extracted with CH_2Cl_2 (2×250 ml). The acid layer was neutralized with 6 M NaOH and extracted with CH_2Cl_2 (2×250 ml). Each CH_2Cl_2 layer was washed with saturated $NaHCO_3$ (2× 80 ml) and brine (1×80 ml). The combined organic layers were dried over sodium sulfate, filtered, and evaporated to yield a brown residue (7.4 g). The residue was recrystallized from ethyl acetate/hexane to yield **15** as an off-white solid [5.3 g, 64% yield; ^{1}H NMR ($CDCl_3$): δ 7.54 (2H, m), δ 7.31 (3H, m), δ 6.05 (1H, broad s), δ 3.83 (1H, t, *J*=6 Hz), δ 3.32 (2H, t, *J*=4 Hz), δ 2.03 (3H, m), δ 1.74 (1H, m)]. ^{13}C NMR ($CDCl_3$): δ 170.4, δ 134.1, δ 132.9, δ 129.1, δ 127.8, δ 48.4, δ 42.5, δ 28.3, δ 20.2.

5,6-Dihydropyridin-2(1H)-one (16) Compound **15** (3.0 g) was weighed into a 250-ml round bottom flask. CH_2Cl_2 (100 ml) and saturated $NaHCO_3$ (20 ml) were added to the flask, which was then cooled on an ice bath for 10 min.



Scheme 4 In the case of the 4-hydroxy-3-methoxy derivative, the appropriate acid (**19**) was first obtained via catalytic transfer hydrogenation of commercially available ferulic acid (**18**) with ammonium formate using 10% Pd/C in methanol. The free phenolic hydroxyl group was then protected as the TBDMS ether (**20**) using *t*-butyldimethylsilyl chloride and imidazole in dry DMF. Protection was necessary to avoid polymerization during and after the formation of the acyl halide. While this step also introduced a TBDMS group at the carboxyl oxygen, this group was directly converted to the acyl chloride (**21**) by treating the silyl ester with oxalyl chloride in dichloromethane using catalytic amounts of DMF



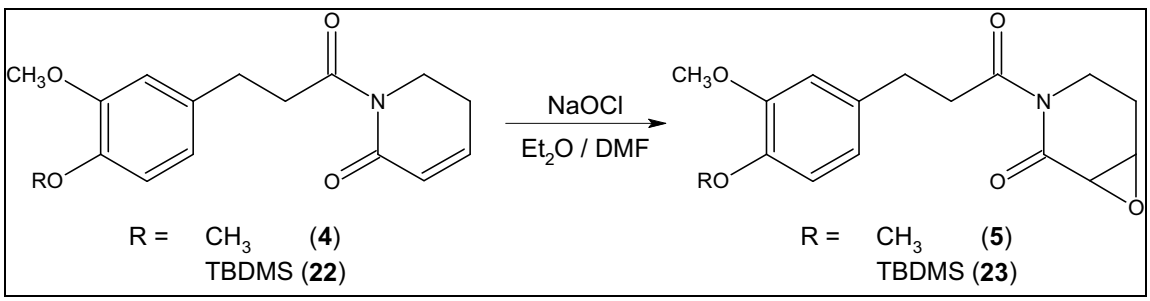
Scheme 5 In all cases, the next step involved the coupling of compound **16** with the appropriate acyl halide. Thus, the lithium salt of compound **16** was generated by treatment with KHMDS in dry THF and condensed with ether **17** or **21** to yield **4** or **22**, respectively

Seventy-seven percent 3-chloroperbenzoic acid (3.1 g) was added in five portions over 10 min. The mixture was vigorously stirred on an ice bath 1 h. The reaction mixture was transferred to a separatory funnel with CH_2Cl_2 (200 ml) and washed with saturated $NaHCO_3$ (2×30 ml). The solution was dried over sodium sulfate, filtered, and evaporated to yield a brown residue (3.2 g). Toluene (50 ml) was added to the brown residue and the solution was heated at reflux for 1 h. The solvent was removed under reduced pressure, and the brown oil was purified via flash chromatography (EtOAc) to yield compound **16** [1.25 g, 90% yield; ^{1}H NMR ($CDCl_3$): δ 6.64 (1H, m) δ 6.45 (1H, broad s), δ 5.89 (1H, m), δ 3.42 (2H, m), δ 2.34 (2H, m)]. ^{13}C NMR ($CDCl_3$): δ 166.6, δ 141.9, δ 129.2, δ 39.7, δ 23.9.

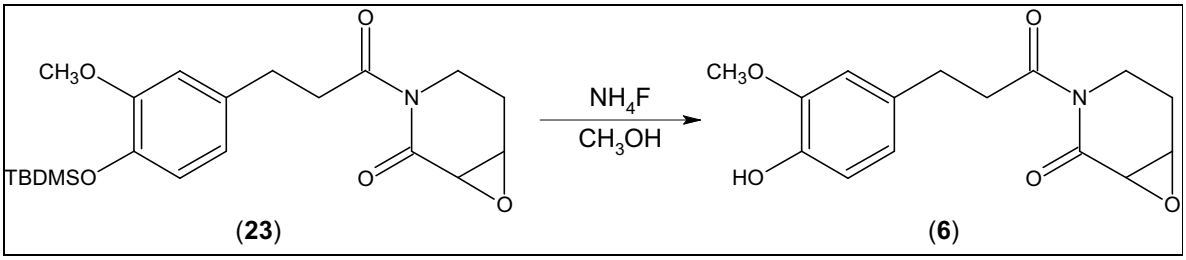
3-(3,4-Dimethoxyphenyl)propanoyl chloride (17) A 100-ml round-bottomed flask was flushed with N_2 and charged with **7** (2.1 g, 10 mmol), freshly distilled dichloromethane (40 ml), and dry DMF (four drops). Oxalyl chloride (1.75 ml, 20 mmol) was added slowly via pipet. The flask was fitted with a drying tube ($CaCl_2$), and the mixture was stirred at RT for 22 h. The volatiles were removed under reduced pressure to yield **17** as a pale yellow oil sufficiently pure for use [2.3 g, 100%; ^{1}H NMR ($CDCl_3$): δ 6.82–6.69 (3H, m, ArH), δ 3.87 (3H, s, -OCH_3), δ 3.86 (3H, s, -OCH_3), δ 3.19 (2H, t, J=7.4 Hz, -CH_2-), δ 2.96 (2H, t, J= 7.4 Hz, -CH_2-). ^{13}C NMR ($CDCl_3$): δ 173.22, δ 149.09, δ 147.94, δ 131.22, δ 120.30, δ 111.61, δ 111.43, δ 55.99, δ 55.94, δ 48.92, δ 30.75].

3-(4-Hydroxy-3-methylphenyl)propanoic acid (19) A mixture of **18** (2.0 g, 10 mmol), ammonium formate (2.0 g, 32 mmol), and 10% Pd/C (0.1 g) in methanol (50 ml) was stirred under N_2 at RT for 24 h. Celite filter aid (0.5 g) was added, and the mixture was vacuum filtered. The volatiles were removed under reduced pressure. The residue was transferred to a separatory funnel with the aid of 1 M HCl (50 ml) and dichloromethane (50 ml). The organic layer was isolated, and the aqueous solution was extracted with more dichloromethane (1×50 ml). The combined organic layers were washed with brine, dried over anhydrous $MgSO_4$, and filtered. The volatiles were removed under reduced pressure to yield **19** as a pure white solid [1.9 g, 95%; ^{1}H NMR ($CDCl_3$): δ 11.17 (1H, br s), δ 6.84 (1H, d), δ 6.71 (2H, m), δ 5.54 (1H, br s), δ 3.87 (3H,s), δ 2.89 (2H, t), δ 2.65 (2H, d). ^{13}C NMR ($CDCl_3$): δ 179.11, δ 146.50, δ 144.14, δ 132.14, δ 120.91, δ 114.47, δ 110.97, δ 55.94, δ 36.07, δ 30.41].

tert-Butyldimethylsilyl 3-(4-t-butyldimethylsilyloxy-3-methyoxyphenyl)propanoate (20) A mixture of **19** (2.7 g, 14 mmol), TBDMSCl (4.52 g, 30 mmol) and imidazole (4.1 g, 60 mmol) in dry DMF (12 ml) was stirred under N_2 at RT for 5 days. The mixture was poured into a separatory funnel with diethyl ether (75 ml) and hexanes (25 ml). The mixture was washed with water (1×50 ml), saturated $NaHCO_3$ (1×50 ml), and water (1×50 ml), and brine (1× 50 ml). The organic solution was dried over anhydrous $MgSO_4$ and filtered. Solvents were removed under reduced



Scheme 6 The corresponding epoxides (**5** and **23**) were prepared via epoxidation of the respective alkenes by treatment with NaOCl in a mixture of diethyl ether and DMF



Scheme 7 Finally, compound **6** was prepared after deprotection of **23** with methanolic NH_4F

pressure to yield a colorless oil. The remaining volatiles were removed under high vacuum to yield **20** as a pure, pale yellow oil [5.77 g, 99%; ^{1}H NMR ($CDCl_3$): δ 6.74 (1H, d), δ 6.68 (1 H, d), δ 6.63 (1H, d of d), δ 3.78 (3H, s), δ 2.85 (2H, t), δ 2.62 (2H, t), δ 0.98 (9 H, s), δ 0.90 (9H, s), δ 0.23 (6H, s), δ 0.13 (6H, s). ^{13}C NMR ($CDCl_3$): δ 173.57, δ 150.81, δ 143.36, δ 134.22, δ 120.84, δ 120.35, δ 112.38, δ 55.53, δ 37.78, δ 30.88, δ 25.82, δ 25.63, δ 18.52, δ 17.67, δ −4.58, δ −4.74].

3-(4-t-butyldimethylsilyloxy-3-methyoxyphenyl)propanoyl chloride (21) A 250-ml round-bottomed flask was flushed with N_2 and charged with **20** (5.768 g, 13.6 mmol), freshly distilled dichloromethane (25 ml), and dry DMF (five drops). Oxalyl chloride (1.80 ml, 20.6 mmol) was added slowly via pipet. The flask was fitted with a drying tube ($CaCl_2$), and the mixture was stirred at RT for 20 h. The volatiles were removed under reduced pressure to yield **21** as a pale yellow oil sufficiently pure for further use [2.3 g, 100%; ^{1}H NMR ($CDCl_3$): δ 6.77 (1H, d), δ 6.66 (1H,d), δ 6.62 (1H,d), δ 3.79 (3H, s), δ 3.18 (2H, t), δ 2.93 (2H, t), δ 0.98 (9H, s), δ 0.13 (6H, s). ^{13}C NMR ($CDCl_3$): δ 173.25, δ 151.03, δ 143.91, δ 132.09, δ 121.09, δ 120.45, δ 112.38, δ 55.58, δ 48.95, δ 30.84, δ 25.80, δ 18.52, δ −4.56].

1-[3-(3,4-Dimethoxyphenyl)propanoyl]-5,6-dihydropyridin-2(1H)-one (4) A 100-ml three-necked, round-bottomed flask was oven dried and flushed with N_2 while cooling. Freshly distilled THF (15.6 ml) and a solution of KHMDS (8.2 ml, 0.5 M in toluene) were added to the flask and cooled on ice for 10 min. Compound **16** (0.4 g, 4 mmol) was dissolved in THF (4.5 ml) and added dropwise to the reaction mixture, which was then stirred on ice for 10 min. Compound **17** (1.0 g, 4.4 mmol) was added to the reaction mixture, which was then stirred on ice for 10 min. The reaction mixture was transferred to a separatory funnel with water (20 ml), ethyl acetate (30 ml), and hexane (10 ml). The organic layer was isolated and washed with saturated $NaHCO_3$ (2×20 ml), 0.1 M HCl (1×20 ml), and brine (1× 20 ml). The organic solution was dried over sodium sulfate, filtered, and the solvent was evaporated to yield an orange oil (800 mg). The oil was purified via flash chromatography (4:1 EtOAc/hexane) to yield compound **4** as a white solid [470 mg, 40% yield; ^{1}H NMR ($CDCl_3$): δ 6.86 (1H, d of t), δ 6.78 (3H, s), δ 5.97 (1H, d of t), δ 3.96 (2H, t), δ 3.86 (3H, s), δ 3.84 (3H, s), δ 3.24 (2H, t), δ 2.93 (2H, t), δ 2.37 (2H, m); ^{13}C NMR ($CDCl_3$): δ 175.7 (s), δ 165.5 (s), δ 149.0 (s), δ 147.5 (s), δ 145.3 (d), δ 133.8 (s), δ 126.0 (d), δ 120.4 (d), δ 111.9 (d), δ 111.2 (d), δ 56.0 (q), δ 55.9 (q), δ 41.2 (t), δ 41.1 (t), δ 30.8 (t), δ 24.7 (t). LREIMS *m/z* (rel. int.): 289 (37), 192 (51), 164 (100), 151 (50). HRMS: Found *m/z* 289.1315, calculated for $C_{16}H_{19}O_4N$ 289.1314. Anal. Calcd. for $C_{16}H_{19}NO_4$: C, 66.42; H, 6.62; N, 4.84. Found: C, 66.52; H, 6.62; N, 4.65.

3-[3-(3,4-dimethoxyphenyl)propanoyl]-7-oxa-3-azabicyclo [4.1.0]heptan-2-one (5) A 250-ml round-bottomed flask was charged with **4** (3.47 g, 12 mmol), DMF (100 ml) and Et_2O (100 ml). The mixture was cooled to 5°C on an ice bath. Cold, 6% $NaOCl_{(aq)}$ (Clorox, 20 ml, 24 mmol) was added, and the resultant mixture was vigorously stirred for 10 min. The reaction mixture was then transferred to a separatory funnel with 5% $Na_2S_2O_3$ (100 ml) and Et_2O (50 ml). The layers were mixed and separated. The aqueous layer was further extracted with Et_2O (2×100 ml). The combined organic layers were then washed with brine (1× 100 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield **5** as a pale solid that was sufficiently pure for further use (1.23 g, 34%). Further purification can be accomplished via recrystallization from 95% EtOH [^{1}H NMR ($CDCl_3$): δ 6.77 (3H, br s), δ 4.32 (3H, dddd), δ 3.87 (3H, s), δ 3.85 (3H, s), δ 3.68 (1H, dd), δ 3.56 (1H, d), δ 3.21 (2H, m), δ 3.15 (1H, dd), δ 2.90 (2H, t), δ 2.41 (1H, dm), δ 1.98 (1H, dddd). ^{13}C NMR ($CDCl_3$): δ 174.8 (s), δ 169.7 (s), δ 148.8 (s), δ 147.4 (s), δ 133.5 (s), δ 120.4 (d), δ 111.9 (d), δ 111.2 (d), δ 56.0 (q), δ 55.9 (q), δ 53.6 (d), δ 52.5 (d), δ 41.5 (t), δ 35.8 (t), δ 30.6 (t), δ 24.0 (t). LREIMS *m/z* (rel. int.): 305 (24), 192 (35), 164 (100), 151 (62). Anal. Calcd. for $C_{16}H_{19}NO_5$: C, 62.94; H, 6.27; N, 4.59. Found: C, 63.05; H, 6.08; N, 4.54.

1-[3-(4-t-Butyldimethylsilyloxy-3-methyoxyphenyl)-propanoyl]-5,6-dihydropyridin-2(1H)-one (22) A 250-ml three neck, oven dried, round bottom was cooled while flushing with N_2. KHMDS (0.5 M in toluene, 28.4 ml) was

added and the flask cooled on an ice bath for 10 min. Compound **16** (1.38 g, 14 mmol) was dissolved in THF (30 ml) and added dropwise to the reaction mixture which then stirred for 10 min. Compound **21** (4.5 g, 14 mmol) was added to the reaction which then stirred for 10 min. The reaction mixture was transferred to a separatory funnel with water (50 ml), ethyl acetate (75 ml), and hexanes (15 ml). The water layer was removed and the organic layer was washed with saturated $NaHCO_3$ (2×50 ml), 0.1M HCl (1× 50 ml), and brine (1×50 ml). The solution was dried over sodium sulfate, filtered, and evaporated to yield a brown oil (5.2 g). The oil was purified via flash chromatography (1:1 EtOAc/hexanes) to yield compound **22** [2.2 g, 42% yield; ^{1}H NMR ($CDCl_3$): δ 6.86 (1H, d of t), δ 6.70 (3H, m), δ 5.97 (1H, d of t), δ 3.95 (2H, t), δ 3.77 (3H, s), δ 3.21 (2H, t), δ 2.90 (2H, t), δ 2.36 (2H, m), δ 0.97 (9H, s), δ 0.12 (6H, s). ^{13}C NMR ($CDCl_3$): δ 175.81, δ 165.44, δ 150.73, δ 145.26, δ 143.26, δ 134.64, δ 125.97, δ 120.72, δ 120.66, δ 112.68, δ 55.55, δ 41.14, δ 41.08, δ 30.94, δ 25.82, δ 24.72, δ 18.51, δ 14.28, δ -4.55].

3-[3-(4-t-Butyldimethylsilyloxy-3-methoxyphenyl)-propanoyl]-7-oxa-3-azabicyclo[4.1.0]heptan-2-one (23) A 250-ml round-bottomed flask was charged with **22** (1.34 g, 3.44 mmol), DMF (36 ml) and Et_2O (36 ml). The mixture was cooled to 5°C on an ice bath. Cold, 6% $NaOCl_{(aq)}$ (Clorox, 20 ml, 17.2 mmol) was added, and the resultant mixture was vigorously stirred for 1 h. The reaction mixture was then transferred to a separatory funnel with 5% $Na_2S_2O_3$ (180 ml) and Et_2O (90 ml). The layers were mixed and separated. The aqueous layer was further extracted with Et_2O (2×90 ml). The combined organic layers were then washed with brine (1×180 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield **23** as a pale orange oil that was sufficiently pure for further use (1.27 g, 91%). Further purification can be accomplished via flash chromatography on silica [1:1 EtOAc/hexanes; ^{1}H NMR ($CDCl_3$): δ 6.75–6.64 (3H, m), δ 4.32 (1H, dddd), 3.78 (3H, s), 3.68 (1H, dd), 3.55 (1H, d), 3.18 (2H, m), 3.13 (1H, dd), 2.34 (1H, dm), δ 1.98 (1H, dddd), 0.98 (9H, s), 0.13 (6H, s)].

3-[3-(4-hydroxy-3-methoxyphenyl)propanoyl]-7-oxa-3-aza-bicyclo[4.1.0]heptan-2-one (6) A mixture of **23** (1.27 g, 3.12 mmol), ammonium fluoride (0.579 g, 15.6 mmol), and methanol (75 ml) was heated at reflux for 5 min. The reaction mixture was transferred to a separatory funnel with the aid of water (90 ml). The aqueous layer was extracted with CH_2Cl_2 (2×90 ml). The combined organic extracts were washed with brine (1×90 ml) and dried over anhydrous $MgSO_4$. The volatiles were evaporated under reduced pressure to yield a tan solid. This was recrystallized from EtOAc-hexanes to yield **6** as an off-white solid [0.41 g, 48%; ^{1}H NMR ($CDCl_3$): δ 6.83–6.70 (3H,m), δ 5.47 (1H,s), δ 4.35 (1H, ddt), δ 3.87 (3 H, m), δ 3.68 (1 H, br t), δ 3.56 (1H, d), δ 3.21 (2H, t), δ 3.21 (1H, m), δ 2.90 (2 H, t), δ 2.40 (1H, m), δ 1.98 (1H, ddd). ^{13}C NMR ($CDCl_3$): δ 175.1 (s), δ 169.9 (s), δ 146.6 (s), δ 144.2 (s), δ 133.0 (s), δ 121.2 (d), δ 114.3 (d), δ 111.2 (d), δ 56.0 (q), δ 53.5 (d), δ 52.4 (d), δ 41.5 (t), δ 35.7 (t), δ 30.6 (t), δ 23.9 (t). LREIMS *m/z* (rel. int.): 291 (13), 178 (23), 150 (100), 137 (50). ES(+)MS *m/z* 292.1 [M+H], 314.1 [M+Na]. Anal. Calcd. for $C_{15}H_{17}NO_5$: C, 61.85; H, 5.88; N, 4.81. Found: C, 62.16; H, 6.11; N, 4.81.

Other potential compounds All three species were screened for basic alkaloids using a standard acid/base partitioning technique followed by TLC with visualization using the alkaloid specific spray reagent, iodoplatinic acid, and found to be negative. Each species was examined by GC-MS using the same conditions described above for imide/amide quantitation. *Piper cenocladum* and *P. melanocladum* were found to be devoid of compounds other than the imides/amides described (other peaks in the chromatograms had areas at least 1,000 times less than the imides/amides). *Piper imperiale* was found to contain a series of seven peaks with areas similar to or greater than the imides described. The mass spectra of all of these compounds are consistent with sesquiterpenes or mono-oxygenated sesquiterpenes. Five peaks had mass spectra that were excellent matches to the library spectra of copaene, cyclopropylazulene, (-) spathulenol, ledrol, and caryophyllene oxide. The peak for caryophyllene oxide was the largest in the chromatogram. Lack of standards for these compounds precluded confirmation of their identities and quantification. Other classes of natural products were not actively pursued.

References

BERENBAUM, M., and NEAL, J. J. 1985. Synergism between myristicin and xanthotoxin, a naturally co-occurring plant toxicant. *J. Chem. Ecol.* 11:1349–1349.

BERENBAUM, M. R., NITAO, J. K., and ZANGERL, A. R. 1991. Adaptive significance of furanocoumarin diversity in *Pastinaca sativa* (Apicaceae). *J. Chem. Ecol.* 17:207–207.

BERGELSON, J., and PURRINGTON, C. B. 1996. Surveying patterns in the cost of resistance in plants. *Am. Nat.* 148:536–558.

BURGER, W. 1971. Flora costaricensis. *Fieldiana Bot.* 35:1–1.

CALCAGNO, M. P., COLL, J., LLORIA, J., FAINI, F., and ALONSO-AMELOT, M. E. 2002. Evaluation of synergism in the feeding deterrence of some furanocoumarins on *Spodoptera littoralis*. *J. Chem. Ecol.* 28:175–175.

CAPRON, M. A., and WIEMER, D. F. 1996. Piplaroxide an ant-repellent piperidine epoxide from *Piper tuburculatum*. *J. Nat. Prod.* 59:794–794.

CATES, R. G., and RHOADES, D. 1977. Patterns in the production of antiherbivore chemical defenses in plant communities. *Biochem. Syst. Ecol.* 5:185–185.

COLEY, P. D. 1983. Herbivory and defensive characteristics of tree species in a lowland tropical forest. *Ecol. Monogr.* 34:209–234.

COLEY, P. D., and BARONE, J. A. 1996. Herbivory and plant defenses in tropical forests. *Annu. Rev. Ecol. Syst.* 27:305–305.

COLEY, P. D., BRYANT, J. P., and CHAPIN, F. S. III 1985. Resource availability and plant anti-herbivore defense. *Science* 230:895–899.

DODSON, C. D., DYER, L. A., SEARCY, J., WRIGHT, Z., and LETOURNEAU, D. K. 2000. Cenocladamide, a dihydropyridone alkaloid from *Piper cenocladum*. *Phytochemistry* 53:51–51.

DUH, C. Y., WU, Y. C., and WANG, S. K. 1990. Cytotoxic pyridine alkaloids from *Piper aborescens*. *Phytochemistry* 29:2689–2689.

DYER, L. A., and LETOURNEAU, D. K. 1999. Trophic cascades in a complex terrestrial community. *Proc. Nat. Acad. Sci. USA* 96:5072–5072.

DYER, L. A., DODSON, C. D., BEIHOFFER, J., and LETOURNEAU, D. K. 2001. Trade-offs in antiherbivore defenses in *Piper cenocladum*: Ant mutualists versus plant secondary metabolites. *J. Chem. Ecol.* 27:581–581.

DYER, L. A., DODSON, C. D., STIREMAN, J. O., TOBLER, M. A., SMILANICH, A. M., FINCHER, R. M., and LETOURNEAU, D. K. 2003. Synergistic effects of three *Piper* amides on generalist and specialist herbivores. *J. Chem. Ecol.* 29:2499–2499.

DYER, L. A., DODSON, C. D., and RICHARDS, J. 2004a. Isolation, synthesis, and evolutionary ecology of Piper amides, pp. 117–139, in L. A. Dyer, and A. N. Palmer (eds.). A Model Genus for Studies of Evolution, Chemical Ecology, and Trophic InteractionsKluwer Academic Publishers, Boston.

DYER, L. A., DODSON, C. D., LETOURNEAU, D. K., TOBLER, M. A., HSU, A., and STIREMAN, J. O. I. 2004b. Ecological causes and consequences of variation in defensive chemistry of a neotropical shrub. *Ecology* 85:2795–2803.

DYER, L. A., GENTRY, G., and TOBLER, M. A. 2004c. Fitness consequences of herbivory: Impacts on asexual reproduction of tropical rain forest understory plants. *Biotropica* 36:68–68.

FEENY, P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–565.

FISCHER, R. C., RICHTER, A., WANEK, W., and MAYER, V. 2002. Plants feed ants: food bodies of myrmecophytic *Piper* and their significance for the interaction with *Pheidole bicornis* ants. *Oecologia* 133:186–186.

FLOYD, T. 2001. Causal categorical data analysis, pp. 197–216, in S. M. Scheiner, and J. Gurevitch (eds.). Design and Analysis of Ecological ExperimentsOxford University Press, Oxford.

FOLGARAIT, P. J., and DAVIDSON, D. W. 1995. Myrmecophytic *Cecropia*: Antiherbivore defences under different nutrient treatments. *Oecologia* 104:189–189.

FOLGARAIT, P. J., DYER, L. A., MARQUIS, R. J., and BRAKER, H. E. 1996. Leaf-cutting ant (*Atta cephalotes*) preferences for five native tropical plantation tree species growing under different light conditions. *Entomol. Exp. App.* 80:521–521.

GERSHENZON, J. 1994. The cost of plant chemical defense against herbivory: A biochemical perspective, pp. 105–173, in E. Bernays (ed.). Insect-plant interactions, Vol VCRC Press, Boca Raton, IL.

GREIG, N. 1993. Regeneration mode in Neotropical *Piper*: Habitat and species comparisons. *Ecology* 74:2125–2135.

HARBORNE, J. B. 2001. Twenty-five years of chemical ecology. *Nat. Prod. Rep.* 18:361–379.

HAY, M. E., KAPPEL, Q. E., and FENICAL, W. 1994. Synergisms in plant defenses against herbivores-interactions of chemistry, calcification, and plant-quality. *Ecology* 75:1714–1726.

HEIL, M. 2002. Ecological costs of induced resistance. *Curr. Opin. Plant Biol.* 5:345–350.

HEIL, M., and MCKEY, D. 2003. Protective ant-plant interactions as model systems in ecological and evolutionary research. *Annu. Rev. Ecol. Syst.* 34:425–453.

HEIL, M., DELSINNE, T., HILPERT, A., SCHURKENS, S., ANDARY, C., LINSENMAIR, K. E., SOUSA, M. S., and MCKEY, D. 2002. Reduced chemical defence in ant-plants? A critical re- evaluation of a widely accepted hypothesis. *Oikos* 99:457–468.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: to grow or defend. *Q. Rev. Biol.* 67:283–283.

JANZEN, D. H. 1966. Coevolution of mutualism between ants and Acacias in Central America. *Evolution* 20:249–249.

JOHNSON, H. B. 1975. Plant pubescence: An ecological perspective. *Bot. Rev.* 41:233–258.

KORICHEVA, J. 2002. Meta-analysis of sources of variation in fitness costs of plant antiherbivore defenses. *Ecology* 83:176–176.

KORICHEVA, J., NYKANEN, H., and GIANOLI, E. 2004. Meta-analysis of trade-offs among plant antiherbivore defenses: are plants jacks-of-all-trades, masters of all?. *Am. Nat.* 163:E64–E75.

KURSAR, T. A., and COLEY, P. D. 1992. Delayed greening in tropical leaves: An antiherbivore defense?. *Biotropica* 24:256–256.

LETOURNEAU, D. K. 1983. Passive aggression: An alternative hypothesis for the *Piper-Pheidole* association. *Oecologia* 60:122–122.

LETOURNEAU, D. K., and DYER, L. A. 1998. Experimental manipulations in lowland tropical forest demonstrate top-down cascades through four trophic levels. *Ecology* 79:1678–1678.

LEVIN, D. A. 1976. The chemical defenses of plants to pathogens and herbivores. *Annu. Rev. Ecol. Syst.* 7:121–121.

LINDROTH, R. L., and HWANG, S. Y. 1996. Diversity, redundancy, and multiplicity in chemical defense systems of aspen, pp. 25–56, in J. T. Romeo, J. A. Saunders, and P. Barbosa (eds.). Phytochemical Diversity and Redundancy in Ecological InteractionsPlenum Press, New York.

LOCKWOOD, J. R. 1998. On the statistical analysis of multiple-choice feeding preference experiments. *Oecologia* 116:475–475.

MARQUIS, R. J. 1991. Herbivore fauna of Piper (Piperaceae) in a Costa Rican wet forest: diversity, specificity, and impact, pp. 179–208, in P. W. Price, T. M. Lewinsohn, G. W. Fernandes, and W. W. Benson (eds.). Plant-Animal Interactions: Evolutionary Ecology in Tropical and Temperate RegionsJohn Wiley & Sons, New York.

MATTSON, W. J., LAWRENCE, R. K., HAACK, R. A., HERMS, D. A., and CHARLES, P. J. 1988. Defensive strategies of woody plants against different insect-feeding guilds in relation to plant ecological strategies and intimacy of association with insects, pp. 3–38, in W. J. Mattson, J. Levieux, and C. Bernard-Dagan (eds.). Mechanisms of Woody Plant Defenses Against InsectsSpringer, New York.

MAXWELL, F. G., JENKINS, J. N., and PARROTT, W. L. 1972. Resistance of plants to insects. *Adv. Agron.* 24:187–265.

NELSON, A. C., and KURSAR, T. A. 1999. Interactions among plant defense compounds: a method for analysis. *Chemoecology* 9:81–92.

PARMAR, V. S., JAIN, S. C., BISCT, K. S., JAIN, R., TANEJA, P., JHA, A., TYAGI, O. D., PRASAD, A. K., WENGEL, J., OLSEN, C. E., and BOLL, P. M. 1997. Phytochemistry of the genus *Piper*. *Phytochemistry* 46:597–597.

REHR, S. S., FEENY, P. P., and JANZEN, D. H. 1973. Chemical defence in Central American non-ant-acacias. *J. An. Ecol.* 42:405–405.

RISCH, S. 1982. How Pheidole ants help *Piper* plants. *Brenesia* 19/20:545–548.

RISCH, S. J., and RICKSON, F. R. 1981. Mutualism in which ants must be present before plants produce food bodies. *Nature* 291:149–150.

RISCH, S., MCCLURE, M., VANDERMEER, J., and WALTZ, S. 1977. Multualism between three species of tropical *Piper* (Piperaceae) and their ant inhabitants. *Am. Midl. Nat.* 98:433–433.

ROMEO, J. T., SAUNDERS, J. A., and BARBOSA, P. 1996. Phytochemical Diversity and Redundancy in Ecological Interactions. Plenum Press, New York.

SAGERS, C. L., and COLEY, P. D. 1995. Benefits and costs of defense in a neotropical shrub. *Ecology* 76:1835–1835.

STEWARD, J. L., and KEELER, K. H. 1988. Are there trade-offs among antiherbivore defenses in *Ipomoea* (Convolvulaceae)?. *Oikos* 53:79–79.

STRAUSS, S. Y., RUDGERS, J. A., LAU, J. A., and IRWIN, R. E. 2002. Direct and ecological costs of resistance to herbivory. *Trends Ecol. Evol.* 17:278–278.

TEPE, E., VINCENT, M., and WATSON, L. 2004. Phylogenetic patterns, evolutionary trends, and the origin of ant-plant associations in *Piper* section Macrostachys: Burger's hypothesis revisited, pp. 156–178, in L. A. Dyer, and A. D. N. Palmer (eds.). *Piper*: A Model Genus for Studies of Phytochemistry, Ecology, and EvolutionKluwer Academic Publishing, New York.

ZANGERL, A. R., ARNTZ, A. M., and BERENBAUM, M. R. 1997. Physiological price of an induced chemical defense: photosynthesis, respiration, biosynthesis and growth. *Oecologia* 109:433–433.

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The Chemistry of the Postpharyngeal Gland of Female European Beewolves

Erhard Strohm · Gudrun Herzner · Martin Kaltenpoth · Wilhelm Boland · Peter Schreier · Sven Geiselhardt · Klaus Peschke · Thomas Schmitt

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Abstract Females of the European beewolf, *Philanthus triangulum*, possess a large glove-shaped gland in the head, the postpharyngeal gland (PPG). They apply the content of the PPG to their prey, paralyzed honeybees, where it delays fungal infestation. Here, we describe the chemical composition of the gland by using combined GC-MS, GC-FTIR, and derivatization. The PPG of beewolves contains mainly long-chain unsaturated hydrocarbons (C23–C33), lower amounts of saturated hydrocarbons (C14–C33), and minor amounts of methyl-branched hydrocarbons (C17–C31). Additionally, the hexane-soluble gland content is comprised of small amounts of an unsaturated C25 alcohol, an unknown sesquiterpene, an octadecenylmethylester, and several long-chain saturated (C25, C27) and unsaturated (C23–C27) ketones, some of which have not yet been reported as natural products. Surprisingly, we found a dimorphism with regard to the major component of the PPG with some females having (*Z*)-9-pentacosene, whereas others have (*Z*)-9-heptacosene as their predominant component. The biological relevance of the compounds for the prevention of fungal growth on the prey and the significance of the chemical dimorphism are discussed.

Keywords Antifungal · Crabronidae · GC-FTIR · Hymenoptera · *Philanthus triangulum* · Postpharyngeal gland · PPG · Sphecidae

E. Strohm (✉) · G. Herzner · M. Kaltenpoth
Department of Zoology, University of Regensburg,
93040 Regensburg, Germany
e-mail: erhard.strohm@biologie.uni-regensburg.de

E. Strohm · G. Herzner · M. Kaltenpoth · T. Schmitt
Department of Animal Ecology and Tropical Biology,
Theodor-Boveri-Institute, University of Würzburg,
97074 Würzburg, Germany

W. Boland
Max-Planck-Institute for Chemical Ecology,
Jena, Germany

P. Schreier
Department of Food Chemistry, University of Würzburg,
97074 Würzburg, Germany

S. Geiselhardt · K. Peschke · T. Schmitt
Department of Evolutionary Biology and Animal Ecology,
Faculty of Biology, University of Freiburg,
Freiburg, Germany

Introduction

Hymenoptera possess a huge variety of exocrine glands (e.g., Hölldobler and Wilson 1990). The chemistry and function of different types of these have been studied for a number of social species, whereas comparatively little is known from solitary bees and wasps. Recently, a postpharyngeal gland (PPG) has been described from a species of digger wasp (Strohm et al. 2007), the European beewolf, *Philanthus triangulum* Fabricius 1775 (Hymenoptera: Crabronidae, formerly Sphecidae, Melo 1999). The occurrence of this gland is surprising since the PPG was assumed to be restricted to ants (Hölldobler and Wilson 1990; Schoeters and Billen 1997; Lenoir et al. 1999) where it functions in generating the colony odor (e.g., Hefetz et al. 1992, 1996; Soroker et al. 1994, 1995, 1998; Vienne et al. 1995; Dahbi et al. 1998; Lenoir et al. 1999, 2001; Oldham et al. 1999; Soroker and Hefetz 2000; for a review of other proposed functions, see Eelen et al. 2006).

In beewolves, the PPG has a unique function in protecting the larval provisions from microbial attack (Strohm and Linsenmair 2001; Herzner and Strohm 2007; Herzner et al. 2007a). Female European beewolves hunt

and paralyze honeybees, bring them to their nest burrow, and provision one to six bees in a brood cell as larval food for one progeny. Due to the humid and warm conditions in the brood cell, the highly nutritive provisions are prone to detrimental microbial attack (Strohm and Linsenmair 2001). Early fungus infestation inevitably destroys the food resources, and larvae are killed by fungal toxins or starve to death. Observations in special cages (Strohm and Linsenmair 1994–1995) revealed that beewolf females intensively lick the bodies of the paralyzed bees and apply the secretion from the PPG to the bees' surface (Strohm and Linsenmair 2001; Herzner et al. 2007a). This treatment has the effect of delaying fungus growth for 2 to 3 days, which is a highly relevant effect given the short larval feeding period of only 8 to 11 days. The primary mechanism of this delay is not a direct chemical effect of the secretion on fungi, but the prevention of water condensation on the bees that in turn impairs the germination and growth of fungal spores (Herzner and Strohm 2007). Male European beewolves also have a PPG that is even larger than in females (Herzner et al. 2007b). However, it serves as a reservoir for the scent marking pheromone that males apply to their territories to attract females (Kroiss et al. 2006).

Despite the advanced understanding of the function of the secretion of the PPG of female European beewolves, there has been no detailed investigation of its chemistry. Therefore, we analyzed the chemical composition by using combined gas chromatography-mass spectrometry (GC-MS), gas chromatography-Fourier transform infrared spectroscopy (GC-FTIR), fractionation, and derivatizations.

Materials and Methods

Sampling Beewolf females were taken either from a field population close to the Biocenter of the University of Würzburg, Germany, or from a laboratory population kept at the University of Würzburg (daughters of field caught females). They were all mated with actively provisioned nests with honeybees and were between 1 and 4 weeks old. To identify the chemicals of the PPG, females that were freshly killed by CO_2 were decapitated, and their PPGs were removed from the heads by grasping the hypopharynx with tweezers and gently pulling the attached gland out through the mouth (Strohm et al. 2007). The glands were immersed immediately in 0.25 ml *n*-hexane (Fluka Chemie GmbH, Buchs, Switzerland) that had been distilled prior to use. For the identification of the components in the PPG, the glands of four females were pooled. This enabled us to identify minor components that were not reliably detectable in the extracts of individual PPGs. In order to have an easier method to obtain the content of the PPG, we investigated whether extracts of whole heads differed in composition from dissected glands. There was a large amount of hydrocarbons in the PPG, and extracts of entire heads were identical to extracts of the dissected glands. There were no additional peaks detectable and the proportions were the same (see Herzner et al. 2007b for an analogous procedure for male PPGs). Thus, as an easier alternative to the dissection of the PPG, entire heads were extracted. To obtain data on PPG content variability, we analyzed individual extracts made from the heads of 37 females. Heads of freshly killed females were cut off and extracted in distilled hexane for 4 h. An internal standard (octadecane, Sigma, St Louis, MO, USA) was added to assess the absolute amount of compounds in the PPG (although octadecane could be detected in pooled samples, it was not detectable in individual samples and was therefore employed as an internal standard).

Identification Identification of the chemicals was accomplished by GC-MS. Head extracts were fractionated by solid phase extraction (SPE, Chromabond, unmodified silica, 3 ml, 500 mg, Macherey-Nagel, Düren, Germany) with hexane as the first and dichloromethane as the second eluent to separate nonpolar and polar fractions. Alkanes were characterized by comparison of mass spectra and retention indices with those of purchased standard alkanes (Aldrich, Deisenhofen, Germany). Corresponding alkenes were tentatively identified by their typical mass spectra, their retention indices, and (depending on availability) with commercially available (Aldrich) and synthesized standards. Dimethyl disulfide (DMDS) derivatization was carried out to determine the position of double bonds according to the method of Dunkelblum et al. (1985). The configurations of the double bonds were determined by using GC-FTIR (Attygalle 1994). Methyl-branched alkanes were identified by using MS databases and diagnostic ions, and by determining retention indices (Carlson et al. 1998). Details on the identification of polar compounds are given in the results section.

For the identification of the hydrocarbons, we used a Hewlett Packard HP 6890 Series GC System coupled to a Hewlett Packard HP 5973 Mass Selective Detector (Agilent Technologies, Böblingen, Germany). The GC was equipped with a DB-1 fused silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness; J & W, Folsom, CA, USA). Temperature was programmed from 100°C to 300°C with a 6°C/min heating rate, held for 20 min at 300°C. Helium was used as carrier gas with a constant flow of 1 ml/min. Injection was carried out at 300°C in the splitless mode for 2 min. The electron impact mass spectra (EI-MS) were recorded with an ionization voltage of 70 eV and a source temperature of 230°C. The software ChemStation (Agilent Technologies) for windows was used for data acquisition.

GC-FTIR spectra were obtained by using an HP 5890 GC (Agilent Technologies) coupled to an FTS 575C Tracersystem (BioRad, Hercules, CA, USA). The GC was equipped with the same column as described above. Temperature was programmed from 80 to 270°C with a 4°C/min heating rate. Helium was used as carrier gas with a constant flow of 1–2 ml/min. Injections were carried out by using a split/splitless injector at 250°C in the splitless mode for 60 s. Injection volume was 0.1 µl. IR spectra were recorded by scanning 256 times in a frequency range from 4,000 to 700 cm^{-1} with a resolution of 1 cm^{-1}. Data system was a Dell Optiplex GX110-PC with BioRad WinIR Pro (Version 2.7) Tracer Software and Sadtler IRSearchMaster.

Data Handling and Statistical Analysis Because the relative amounts of compounds constitute compositional data, they were transformed to logcontrasts according to Aitchison (1986): $Z_{ij}=\log_{10}(Y_{ij}/g(Y_j))$ where Z_{ij} is the standardized peak area i for individual j, Y_{ij} is the peak area i for individual j, and $g(Y_j)$ is the geometric mean of all peaks for individual j, prior to statistical analysis. Several peaks had to be combined for the quantitative analysis of individuals because they were not always clearly separated in the chromatograms. To test for differences in the proportions of components between groups, we subjected the transformed data to exact tests for two independent groups (SPSS 13.0, SPSS Inc. 2004).

Results

Identification and Analysis of Hydrocarbons in the PPG The chromatograms of the crude hexane extracts of the PPG showed a total of 53 peaks that represented 62 different compounds (Fig. 1, Table 1). The mean amount of all compounds was 337±292 µg (minimum, 36.7 µg; maximum, 1,410 µg, N=37). All alkanes as well as (*Z*)-9-tricosene, (*Z*)-9-pentacosene, and (*Z*)-9-heptacosene were identified by comparison of mass spectra and retention indices with those of available standards. The location and configuration of double bonds of other alkenes were determined with DMDS derivatives and GC-FTIR data (band at 721 cm^{-1}: *cis* configuration of RCH=CHR′; Attygalle 1994). Some components could, however, not be completely characterized (location of methyl groups or double bond, configuration of double bond) due to the small amounts in the extracts. One component could not be identified at all. There were more unsaturated (81.5%) than saturated compounds in the nonpolar fraction of the PPG extract from beewolf females.

Identification of the Constituents in the Polar Fraction The polar fraction contained a group of uncommon constituents.

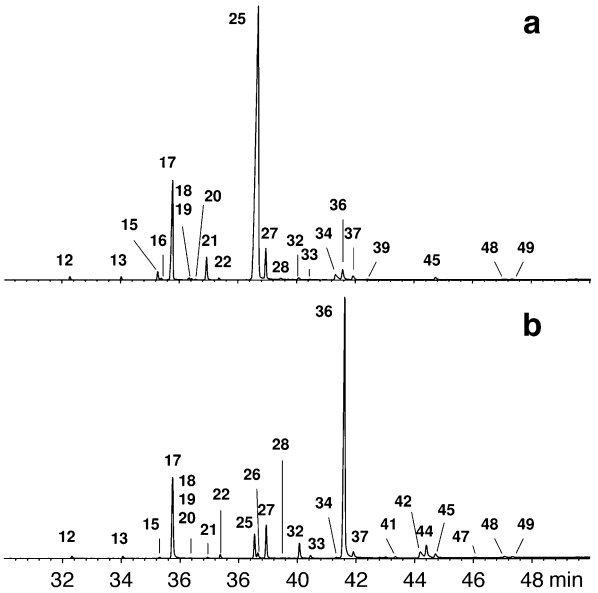

Fig. 1 Total ion chromatograms of the hexane extract of the PPG from individual female European beewolves, *Philanthus triangulum*, with **a** (*Z*)-9-pentacosene (C25-type) and **b** (*Z*)-9-heptacosene (C27-type) as the predominant hydrocarbon peak. Numbers correspond to the numbers in the peak list (Table 1). Some compounds listed in Table 1 were present in quantities too low to be visible in these chromatograms

Hydrogenation of double bonds with H_2 and palladium on carbon (Attygalle 1998) as well as DMDS adducts were used to obtain sufficient information by mass spectrometry to identify these compounds. 18-Heptacosen-10-one was identified by comparison with an already published mass spectrum (Yasui et al. 2003). The mass spectrum of this compound showed a molecular ion of *m/z* 392 and two diagnostic fragment ions at *m/z* 155 for $[C_9H_{19}–CO]^+$ and at *m/z* 265 for $[C_{17}H_{33}–CO]^+$ indicating the carbonyl position at C10. After hydrogenation of this unsaturated ketone, the resulting 10-heptacosanone as well as the 10-heptacosanone of the untreated extract showed identical mass spectra (*m/z*: 155 $[C_9H_{19}\text{-}CO]^+$, 267 $[C_{17}H_{35}\text{-}CO]^+$, 394 M^+) and identical retention indices (2,868) with that already published by Yasui et al. (2003). 14-Tricosen-6-one was characterized by its diagnostic masses (*m/z*: 99 $[C_6H_{11}\text{-}CO]^+$, 265 $[C_{17}H_{33}\text{-}CO]^+$, 336 M^+). Also, hydrogenated 14-tricosen-6-one was tentatively identified as 6-tricosanone by comparison with a commercially available MS library (NIST 2.0, Stein et al. 2001). The double bond positions in these and additional unsaturated ketones were determined from mass spectra of DMDS adducts. Additionally, three unsaturated and one saturated ketone were tentatively identified as 15-tetracosen-7-one, 16-pentacosen-8-one, 17-hexacosen-9-one, and 8-pentacosanone. The corresponding alcohol 16-pentacosen-8-ol was tentatively identified by its diagnostic masses after hydrogenation of the polar fraction

Table 1 List of compounds in the postpharyngeal gland of females of the European beewolf, *Philanthus triangulum*

Peak No.	RI	Compound Name	Diagnostic MS Ions
1	1400	Tetradecane[a]	198
2	1500	Pentadecane[a]	212
3	1600	Hexadecane[a]	226
4	1700	Heptadecane[a]	240
5	1710	X-Methylheptadecane	–
6	1800	Octadecane[a]	254
7	1814	X-Methyloctadecane	–
8	1900	Nonadecane[a]	268
9	1906	Sesquiterpene	41, 69, 81, 93, 107, 120, 133, 222
10	2000	Eicosane[a]	282
11	2080	(*Z*)-9-Octadecenylmethylester[a]	222, 264, 296
12	2100	Heneicosane[a]	296
13	2200	Docosane[a]	310
14	2267	Δ-X-Tricosene	322
15	2273	(*Z*)-9-Tricosene[a]	322
16	2286	Δ-7-Tricosene	322
17	2300	Tricosane[a]	324
18	2338	11-; 9-Methyltricosane	168/169, 196/197; 140/141, 224/225
19	2343	7-Methyltricosane	112/113, 252/253
20	2352	5-Methyltricosane	84/85, 280/281
21	2371	Δ-X-Tetracosene	336
22	2400	Tetracosane[a]	338
23	2436	Δ-14-Tricosen-6-one	99, 115, 127, 181, 265, 336
24	2464	Δ,Δ-X,Y-Pentacosadiene	348
25	2471	(*Z*)-9-Pentacosene[a]	350
26	2492	Δ-Pentacosene	350
27	2500	Pentacosane[a]	352
28	2537	13-; 11-; 9-Methylpentacosane	196/197; 168/169, 224/225; 140/141, 252/253
29	2538	Δ-15-Tetracosen-7-one	94, 113, 141, 181, 195, 265, 350
30	2543	7- Methylpentacosane	112/113, 280/281
31	2554	Δ-16-Pentacosen-8-ol	67, 348
32	2572	(*Z*)-9-Hexacosene	364
33	2600	Hexacosane[a]	366
34	2639	Δ-16-Pentacosen-8-one	127, 155, 195, 209, 265, 364
35	2668	Pentacosan-8-one	127, 143, 155, 267, 283, 366
36	2672	(*Z*)-9-Heptacosene[a]	378
37	2700	Heptacosane[a]	380
38	2714	NI	–
39	2735	13-; 11-; 9-Methylheptacosane	196/197, 224/225; 168/169, 252/253; 140/141, 280/281
40	2740	Δ-17-Heptacosen-9-one	141, 169, 209, 223, 265, 378
41	2800	Octacosane[a]	394
42	2840	Δ-18-Heptacosen-10-one	155, 171, 183, 223, 237, 265, 392
43	2868	Heptacosan-10-one	127, 143, 155, 171, 267, 283, 295, 394
44	2872	(*Z*)-9-Nonacosene	406
45	2900	Nonacosane[a]	408
46	2933	15-; 13-; 11-Methylnonacosane	224/225; 196/197, 252/253; 168/169, 280/281
47	3000	Triacontane[a]	422
48	3076	(*Z*)-9-Hentriacontene	434
49	3100	Hentriacontane[a]	436
50	3134	15-; 13-Methylhentriacontane	224/225, 252/253; 196/197, 280/281
51	3200	Dotriacontane[a]	450
52	3275	(*Z*)-9-Tritriacontene	462
53	3300	Tritriacontane[a]	464

RI retention index; *X*, *Y* position of the methyl-group or double bond not known; *Δ* configuration of the double-bond not known; *NI* not identified
[a] Identification with available standards.

with lithium aluminum hydride (Attygalle 1998; Table 1). (*Z*)-9-Octadecenylmethylester was characterized by using a commercially available standard (Aldrich). Finally, a compound with a typical sesquiterpene mass spectrum was found in small amounts in the polar fraction of the extract. We could not determine the type of functional group in this sesquiterpene.

Chemical Dimorphism One alkene dominated the composition of the PPG, and surprisingly, this major compound differed among individuals (Fig. 2, Table 2). Among the 37 females whose PPG content was analyzed, 29 had (*Z*)-9-pentacosene and eight had (*Z*)-9-heptacosene as their major peak (from here on referred to as the C25- and C27-type, respectively). The frequency of the two types differed significantly from equality (χ^2=15.4, *df*=1, *P*<0.001). By using exact tests for independent samples to assess whether the proportions of other components were also affected by the major peak, we revealed that 14 of the 21 peaks differed significantly between the C25- and the C27-type (Table 2). Generally, the C27-type had larger proportions of components with longer chain lengths.

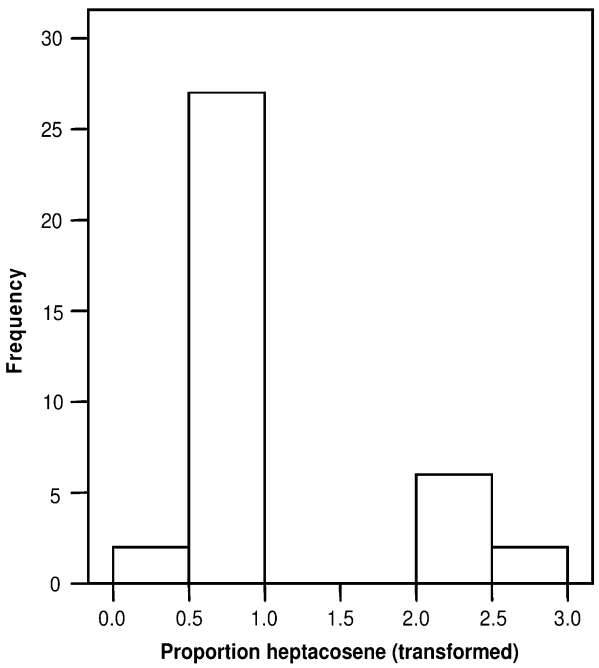

Fig. 2 Frequency distribution (population of *N*=37) of individual female European beewolves, *Philanthus triangulum*, based on the proportion of (*Z*)-9-heptacosene in their PPG secretion (values transformed to logcontrasts (Aitchison 1986): $Z_{ij}=\log_{10}(Y_{ij}/g(Y_j))$ where Z_{ij} is the standardized peak area *i* for individual *j*, Y_{ij} is the peak area *i* for individual *j*, and $g(Y_j)$ is the geometric mean of all peaks for individual *j*). There is a clear bimodal distribution, i.e., some individuals have a large proportion of (*Z*)-9-heptacosene, whereas some have a small proportion; there are no intermediate types

Discussion

The PPG secretion of beewolf females contains predominantly unbranched unsaturated long chain hydrocarbons (C23–C33, mainly either C25 or C27), smaller amounts of saturated hydrocarbons (C14–C33), and small or trace amounts of methylalkanes (C17–C31), unsaturated ketones (C23–C27), saturated ketones (C25, C27), a sesquiterpene, an unsaturated C18 methylester, and an unsaturated C25 alcohol.

The secretion of the PPG of beewolf females is involved in the preservation of their honeybee prey that serves as larval food (Herzner and Strohm 2007). Females apply large amounts of the PPG secretion to the prey prior to oviposition (Strohm and Linsenmair 2001; Herzner et al. 2007a). The prevalence of unsaturated hydrocarbons in the PPG of beewolf females is probably related to the preservation function. The preservation seems to be mainly accomplished by a physical mechanism (Herzner and Strohm 2007): the secretion prevents the condensation of water on the bees and in this way renders the microclimatic conditions unsuitable for the growth of fungi. Possibly, the PPG secretion covers structures on the paralyzed bee that would otherwise function as effective nuclei for the condensation of water. Scanning electron microscopy revealed that the PPG secretion forms a contiguous layer over the whole surface of the prey (Herzner and Strohm 2007). A hydrocarbon mixture of predominately alkenes might be an ideal means to build up such layers, because at the temperatures that prevail in beewolf brood cells, the unsaturated hydrocarbons might be in a more or less liquid state and can be spread easily over the bee surface.

The composition of hydrocarbons on the cuticle of paralyzed honeybees is dramatically modified due to the treatment by beewolf females (Herzner et al. 2007a, b). Untreated honeybees and most other insects bear predominantly saturated straight or branched hydrocarbons (e.g., Howard and Blomquist 1982, 2005; Schmitt et al. 2007). Less frequently, alkenes constitute large proportions of cuticular hydrocarbons (e.g., on a termite, Howard et al. 1978; on an aphid parasitoid, Liepert and Dettner 1996; on workers of European hornets, Ruther et al. 2002; on stingless bee foragers, Abdalla et al. 2003; on diapausing butterfly pupae, Kaneko and Katagiri 2004; or on nesting females of the burrowing bee, Simmons et al. 2003). The reasons for the prevalence of saturated hydrocarbons in some species and unsaturated hydrocarbons in other species are unknown. Possibly, the physicochemical properties of the surface can be adjusted to specific requirements by a particular mixture of saturated and unsaturated compounds (e.g., Gibbs and Pomonis 1995; Gibbs 1998).

In addition to the ubiquitous alkanes and alkenes that we found in the PPG, we also identified long chain unsaturated

Table 2 Mean proportions of selected hydrocarbons and ketones from the postpharyngeal glands of individual female European beewolves, *P. triangulum*, that had either (*Z*)-9-pentacosene (C25-type) or (*Z*)-9-heptacosene (C27-type) as the major component[a]

Compound name	C25-type		C27-type		Differences[b]	*P* values
(Peak no. in Table 1)	Mean	SD	Mean	SD	Pent-Hept	
Heneicosane (12)	0.20	0.14	0.18	0.13	0.02	0.086
Docosane (13)	0.16	0.09	0.13	0.08	0.04	**0.001**
Tricosenes (14, 15, 16)	0.36	0.27	0.08	0.07	0.28	**<0.001**
Tricosane (17)	14.06	3.74	12.93	3.02	1.13	**0.003**
Methyltricosanes (18, 19, 20)	0.10	0.10	0.05	0.04	0.05	**0.003**
Tetracosene (21)	1.19	0.65	0.14	0.08	1.06	**<0.001**
Tetracosane (22)	0.17	0.10	0.14	0.07	0.02	**0.003**
Pentacosenes (25, 26)	77.48	7.96	8.29	5.50	69.19	**<0.001**
Pentacosane (27)	2.05	0.92	2.82	1.20	−0.78	0.335
Methylpentacosanes (28)	0.06	0.03	0.08	0.03	−0.02	0.704
Hexacosene (32)	0.13	0.05	1.62	0.72	−1.50	**<0.001**
Hexacosane (33)	0.05	0.03	0.05	0.02	0.00	0.067
16-Pentacosen-8-one (34)	1.13	0.73	0.25	0.15	0.88	**<0.001**
Heptacosene (36)	1.00	0.39	69.80	8.86	−68.80	**<0.001**
Heptacosane (37)	0.38	0.22	0.31	0.12	0.07	**0.032**
Octacosane (41)	0.03	0.03	0.03	0.02	0.00	0.182
18-Heptacosen-10-one (42)	1.02	5.18	1.73	0.75	−0.70	**<0.001**
Nonacosene (44)	0.04	0.06	0.95	0.43	−0.90	**<0.001**
Nonacosane (45)	0.30	0.30	0.32	0.11	−0.02	0.550
Hentriacontene (48)	0.01	0.04	0.04	0.02	−0.03	**<0.001**
Hentriacontane (49)	0.07	0.08	0.06	0.02	0.01	0.062

[a] Table entries include mean and one standard deviation (SD) of the proportion (%) of the components for females of the C25 (*N*=29) and of the C27-type (*N*=8), as well as the difference (C25–C27) and the significance level for the difference according to an exact test (*P*, significant differences in bold).
[b] Differences were calculated from the original data, rounding of values produced some rounding error.

ketones, some of which had not yet been described as natural products. Only (*Z*)-18-heptacosen-10-one and 10-heptacosanone had previously been reported as components of a contact sex pheromone from females of the white-spotted longicorn beetle, *Anoplophora malasiaca* (Yasui et al. 2003). The function of these compounds in the secretion of beewolf females is unclear. Besides a not-completely identified sesquiterpene, (Z)-9-octadecenylmethylester, and 16-pentacosen-8-ol, these ketones are the only identified components in the PPG of beewolf females that have a functional group. They might be likely candidates to exhibit an antifungal effect. However, in bioassays, no direct antifungal effect of the PPG content could be detected (Herzner et al. 2007a). Male European beewolves have some of the same ketones and a slightly shorter unsaturated ketone in their marking secretion (Schmitt et al. 2003; Kroiss et al. 2006). Their exact function in males is also unknown (see below).

The estimated amount of hydrocarbons in the secretion of the PPG differed considerably among females. This might be due to differences in physiological status, size, and age. Interestingly, the mean (approximately 330 μg) and maximum (approximately 1,400 μg) amounts match the quantities found on honeybees that were embalmed with the PPG secretion by beewolf females. Bees are each embalmed with approximately 110 μg (Herzner et al. 2007a) and females provision on average three bees per day and a maximum of ten bees per day (Strohm and Linsenmair 1997). Thus, an average female has available the necessary amount of PPG secretion for the embalming of the average number of bees caught on 1 day. Likewise, the maximum amount found in some individuals would be sufficient to embalm the maximum number of bees that the most successful females hunt per day. Moreover, according to a three-dimensional reconstruction based on histological sections, the maximum volume of the gland was estimated to be 3–4 μl (Strohm et al. 2007). The comparatively large size of the gland is explained by the need to provide enough secretion for the treatment of several prey items per day. The supply with such large amounts of unsaturated hydrocarbons might be costly and might represent a considerable part of the cost of parental care in this species (Strohm and Linsenmair 1999, 2000; Strohm and Marliani 2002).

Remarkably, beewolf females show a striking dimorphism with regard to the chemical composition of the PPG

secretion. The major component is either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene, and there are no intermediate individuals. Both compounds are widespread among aculeate Hymenoptera and other insects (e.g., Ruther et al. 2002; Simmons et al. 2003). Most other components of the PPG secretion also differ between the two morphs, in that C27-types tend to have larger proportions of long-chain compounds. Possibly, the whole metabolism of hydrocarbons is adjusted to longer chain lengths in the C27-types. Why females have either (*Z*)-9-pentacosene or (*Z*)-9-heptacosene as the major compound is unclear. Preliminary analyses (E. Strohm, G. Herzner, M. Kaltenpoth, unpublished data) suggest that there is no effect of age or physiological status on the expression of the major component. One proximate explanation could be that conditions during development differ between the females and cause differential gene activation [for example, the synthesis of (*Z*)-9-heptacosene might be induced by high temperatures because of the presumably higher melting point, e.g., Gibbs et al. 1998; Rouault et al. 2000]. However, our study specimens were bred under identical temperature conditions in the same climate chamber with the same diet (honeybees from the same population) and, nevertheless, showed this dimorphism. Furthermore, an analysis of beewolf females from different populations ranging from northern Germany to the southern valleys of the Alps not only showed both types of females, but also revealed that they occurred in similar proportions (Strohm et al. 2008). Together with the lack of intermediate individuals, this might suggest that the dimorphism has a genetic basis. Such a dimorphism would have to be balanced because otherwise one morph would disappear at least from some populations either because it has a selective disadvantage or because of genetic drift. One possible explanation for a balanced dimorphism is a spatial difference in the suitability of the two alleles. Such spatial heterogeneity might either be generated by differences in abiotic or biotic conditions. There are specialized cuckoo wasps that enter the nests and oviposit on the bees (Strohm et al. 2001). These wasps seem to employ chemical mimicry in order not to leave traitorous signs in the nest. Most notably, the chemical mimicry only refers to the C27-type of females (Strohm et al. 2008). Thus, the C27-type might be disadvantaged with regard to the rate of parasitism, but this could be balanced by other advantages. Again, this hypothesis is weakened by the fact that the proportions of both types of individuals are similar over a wide geographical range despite probable differences in the abundance of the cuckoo wasps.

Compared to the content of the PPG of males of the European beewolf (Schmitt et al. 2003; Kroiss et al. 2006), females have fewer components with functional groups and lower proportions thereof, whereas the composition of the alkanes and alkenes is similar. The function of the secretion of the male PPG is also quite different. Males use this secretion to scent mark their territories and to attract receptive females (Simon-Thomas and Poorter 1972; Evans and O'Neill 1988; Strohm 1995; Strohm and Lechner 2000; Schmitt et al. 2003). The marking secretion is stored in and delivered from the PPG (Kroiss et al. 2006). Therefore, the compounds with functional groups may play a role in the attraction of females (Herzner et al. 2005; Kroiss et al. 2006) and may also convey some additional information on male quality and suitability as a mate (Herzner et al. 2006; Kaltenpoth and Strohm 2006).

In summary, females of the European beewolf, *P. triangulum*, have large amounts of mainly unsaturated hydrocarbons in their PPG. The composition of the secretion is probably shaped by its function as an antifungal coating of the prey, paralyzed honeybees. Thus, the function of the PPG of beewolf females differs entirely from this gland in ants. However, the general chemistry is consistent with that found in ants. This supports earlier arguments based on morphology, ultrastructure, and behavioral context (Strohm et al. 2007) that the PPGs of these two taxa are homologous. Comparison of physiological aspects of the PPGs of ants and beewolves, as well as the investigation of other aculeate Hymenoptera is necessary to obtain further insights into the evolution and function of the PPG in this group of insects.

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References

ABDALLA, F. C., JONES, G. R., MORGAN, E. D., and DA, CRUZ-LANDIM, C. 2003. Comparative study of the cuticular hydrocarbon composition of *Melipona bicolor* Lepeletier, 1836 (Hymenoptera, Meliponini) workers and queens. *Genet. Mol. Res.* 2:191–199.

AITCHISON, J. 1986. The Statistical Analysis of Compositional Data. Chapman and Hall, London.

ATTYGALLE, A. B. 1994. Gas phase infrared spectroscopy in characterization of unsaturated natural products. *Pure Appl. Chem.* 66:2323–2326.

ATTYGALLE, A. B. 1998. Microchemical techniques, pp. 207–294, in J. G. Millar, and K. F. Haynes (eds.). Methods in Chemical Ecology: Chemical Methods. Kluwer Academic Press, New York.

CARLSON, D. A., BERNIER, U. R., and SUTTON, B. D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol.* 24:1845–1865.

DAHBI, A., CERDA, X., and LENOIR, A. 1998. Ontogeny of colonial hydrocarbon label in callow workers of the ant *Cataglyphis iberica*. *C. R. Acad. Sci. Sér. 3 Sci. Vie* 321:395–402.

DUNKELBLUM, E., TAN, S. H., and SILK, P. J. 1985. Double-bond location in monosaturated fatty acids by dimethyl sulfide derivatization and mass spectrometry: Application to analysis of fatty acids in pheromone glands of four Lepidoptera. *J. Chem. Ecol.* 11:265–277.

EELEN, D., BØRGESEN, L., and BILLEN, J. 2006. Functional morphology of the postpharyngeal gland of queens and workers of the ant *Monomorium pharaonis* (L.). *Acta Zool.* 87:101–111.

EVANS, H., and O'NEILL, K. 1988. The Natural History of North American Beewolves. Cornell University Press, Ithaca, New York.

GIBBS, A. G. 1998. Water-proofing properties of cuticular lipids. *Am. Zool.* 38:471–482.

GIBBS, A., and POMONIS, J. G. 1995. Physical properties of insect cuticular hydrocarbons - the effects of chain-length, methyl-branching and unsaturation. *Comp. Biochem. Physiol. B-Biochem. Mol. Biol.* 112:243–249.

GIBBS, A. G., LOUIE, A. K., and AYALA, J. A. 1998. Effects of temperature on cuticular lipids and water balance in a desert *Drosophila*: is thermal acclimation beneficial? *J. Exp. Biol.* 201:71–80.

HEFETZ, A., ERRARD, C., and COJOCARU, M. 1992. Heterospecific substances in the postpharyngeal gland secretion of ants reared in mixed groups. *Naturwissenschaften* 79:417–420.

HEFETZ, A., ERRARD, C., CHAMBRIS, A., and LE NEGRATE, A. 1996. Postpharyngeal gland secretions as a modifier of aggressive behavior in the myrmicine ant *Manica rubida*. *J. Ins. Behav.* 9:709–717.

HERZNER, G., and STROHM, E. 2007. Fighting fungi with physics - prey embalming by a solitary wasp prevents water condensation. *Curr. Biol.* 17:R46–R47.

HERZNER, G., SCHMITT, T., LINSENMAIR, K. E., and STROHM, E. 2005. Prey recognition by females of the European beewolf and its potential for a sensory trap. *Anim. Behav.* 70:1411–1418.

HERZNER, G., SCHMITT, T., HECKEL, F., SCHREIER, P., and STROHM, E. 2006. Brothers smell similar: variation in the sex pheromone of male European beewolves *Philanthus triangulum* F. (Hymenoptera: Crabronidae) and its implications for inbreeding avoidance. *Biol. J. Linn. Soc.* 89:433–442.

HERZNER, G., SCHMITT, T., PESCHKE, K., HILPERT, A., and STROHM, E. 2007a. Food wrapping with the postpharyngeal gland secretion by females of the European beewolf *Philanthus triangulum*. *J. Chem. Ecol.* 33:849–859.

HERZNER, G., GOETTLER, W., KROISS, J., PUREA, A., WEBB, A. G., JAKOB, P. M., RÖSSLER, W., and STROHM, E. 2007b. Males of a solitary wasp possess a postpharyngeal gland. *Arth. Struc. Dev.* 36:123–133.

HÖLLDOBLER, B., and WILSON, E. O. 1990. The Ants. Springer, Heidelberg, Germany.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27:149–172.

HOWARD, R. W., and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–391.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

KALTENPOTH, M., and STROHM, E. 2006. The scent of senescence: Age-dependent changes in the composition of the cephalic gland secretion of the male European beewolf, *Philanthus triangulum*. *J. Ins. Sci.* 6:20.

KANEKO, J., and KATAGIRI, C. 2004. Epicuticular wax of large and small white butterflies, *Pieris brassicae* and *P. rapae crucivora*: qualitative and quantitative comparison between diapause and non-diapause pupae. *Naturwissenschaften* 91:320–323.

KROISS, J., SCHMITT, T., SCHREIER, P., STROHM, E., and HERZNER, G. 2006. A postpharyngeal gland functions as a sex pheromone reservoir in males of the solitary wasp *Philanthus triangulum*. *J. Chem. Ecol.* 32:2763–2776.

LENOIR, A., FRESNEAU, D., ERRARD, C., and HEFETZ, A. 1999. Individuality and colonial identity in ants: the emergence of the social representation concept, pp. 219–237, in C. Detrain, C. J. L. Deneubourg, and J. M. Pasteels (eds.). Information Processing in Social Insects. Birkhäuser Verlag, Basel.

LENOIR, A., HEFETZ, A., SIMON, T., and SOROKER, V. 2001. Comparative dynamics of gestalt odour formation in two ant species *Camponotus fellah* and *Aphaenogaster senilis* (Hymenoptera: Formicidae). *Physiol. Entomol.* 26:275–283.

LIEPERT, C., and DETTNER, K. 1996. Role of cuticular hydrocarbons of aphid parasitoids in their relationship to aphid-attending ants. *J. Chem. Ecol.* 22:695–707.

MELO, G. A. R. 1999. Phylogenetic relationships and classification of the major lineages of Apoidea (Hymenoptera), with emphasis on the crabronid wasps. *Scientific papers, Nat. Hist. Mus. Univ. Kans.* 14:1–55.

OLDHAM, N. J., MORGAN, E. D., AGOSTI, D., and WEHNER, R. 1999. Species recognition from postpharyngeal gland contents of ants of the *Cataglyphis bicolor* group. *J. Chem. Ecol.* 25:1383–1393.

ROUAULT, J., CAPY, P., and JALLON, J. -M. 2000. Variations of male cuticular hydrocarbons with geoclimatic variables: an adaptative mechanism in *Drosophila melanogaster*? *Genetica* 110:117–130.

RUTHER, J., SIEBEN, S., and SCHRICKER, B. 2002. Nestmate recognition in social wasps: manipulation of hydrocarbon profiles induces aggression in the European hornet. *Naturwissenschaften* 89:111–114.

SCHMITT, T., STROHM, E., HERZNER, G., BICCHI, C., KRAMMER, G., HECKEL, F., and SCHREIER, P. 2003. (*S*)-2,3-Dihydrofarnesoic acid, a new component in cephalic glands of male European beewolves *Philanthus triangulum*. *J. Chem. Ecol.* 29:2469–2479.

SCHMITT, T., HERZNER, G., SCHREIER, P., and STROHM, E. 2007. Volatiles of foraging honeybees *Apis mellifera* L. (Hymenoptera: Apidae) and their possible role as semiochemicals and kairomones. *Apidologie* 38:164–170.

SCHOETERS, E., and BILLEN, J. 1997. The post-pharyngeal gland in *Dinoponera* ants (Hymenoptera: Formicidae): unusual morphology and changes during the secretory process. *Int. J. Insect Morph. Embryol.* 26:443–447.

SIMMONS, L. W., ALCOCK, J., and EEDER, A. 2003. The role of cuticular hydrocarbons in male attraction and repulsion by female Dawson's burrowing bee, *Amegilla dawsoni*. *Anim. Behav.* 66:677–685.

SIMON-THOMAS, R. T., and POORTER, E. P. R. 1972. Notes on the behavior of males of *Philanthus triangulum* (F.) (Hymenoptera, Sphecidae). *Tijdschr. Entomol.* 115:141–151.

SOROKER, V., and HEFETZ, A. 2000. Hydrocarbon site of synthesis and circulation in the desert ant *Cataglyphis niger*. *J. Ins. Physiol.* 46:1097–1102.

SOROKER, V., VIENNE, C., and HEFETZ, A. 1994. The postpharyngeal gland as a "Gestalt" organ for nestmate recognition in the ant *Cataglyphis niger*. *Naturwissenschaften* 81:510–513.

SOROKER, V., HEFETZ, A., COJOCARU, M., BILLEN, J., FRANKE, S., and FRANCKE, W. 1995. Structural and chemical ontogeny of the postpharyngeal gland in the desert ant *Cataglyphis niger*. *Physiol. Entomol.* 20:323–329.

SOROKER, V., FRESNEAU, D., and HEFETZ, A. 1998. Formation of colony odor in ponerine ant *Pachycondyla apicalis*. *J. Chem. Ecol.* 24:1077–1090.

SPSS INC 2004. SPSS 13.0 for windows. SPSS Inc, Chicago, IL.

STEIN, S., MIROKHIN, Y., TCHEKHOVSKOI, D., and MALLARD, G. 2001. The NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library (Version 2.0). Gaithersburg, MD, USA.

STROHM, E. 1995. Allokation elterlicher Investitionen beim Europäischen Bienenwolf *Philanthus triangulum* Fabricius (Hymenoptera: Sphecidae). Verlag Dr. Köster, Berlin.

STROHM, E., and LECHNER, K. 2000. Male size does not affect territorial behaviour and life history traits in a sphecid wasp. *Anim. Behav.* 59:183–191.

STROHM, E., and LINSENMAIR, K. E. 1994/95. Leaving the cradle: how beewolves (*Philanthus triangulum* F.) obtain the necessary spatial information for emergence. *Zoology* 98:137–146.

STROHM, E., and LINSENMAIR, K. E. 1997. Female size affects provisioning and sex allocation in a digger wasp. *Anim. Behav.* 54:23–34.

STROHM, E., and LINSENMAIR, K. E. 1999. Measurement of parental investment and sex allocation in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Behav. Ecol. Sociobiol.* 47:76–88.

STROHM, E., and LINSENMAIR, K. E. 2000. Allocation of parental investment among individual offspring in the European beewolf *Philanthus triangulum* F. (Hymenoptera: Sphecidae). *Biol. J. Linn. Soc.* 69:173–192.

STROHM, E., and LINSENMAIR, K. E. 2001. Females of the European beewolf preserve their honeybee prey against competing fungi. *Ecol. Entomol.* 26:198–203.

STROHM, E., and MARLIANI, A. 2002. The cost of parental care: prey hunting in a digger wasp. *Behav. Ecol.* 13:52–58.

STROHM, E., LAURIEN-KEHNEN, C., and BORDON, S. 2001. Escape from parasitism: Spatial and temporal strategies of a sphecid wasp against a specialised cuckoo wasp. *Oecologia* 129:50–57.

STROHM, E., HERZNER, G., and GOETTLER, W. 2007. A 'social' gland in a solitary wasp? The postpharyngeal gland of female European beewolves (Hymenoptera, Crabronidae). *Arthr. Struct. Dev.* 36:113–122.

STROHM, E., KROISS, J., HERZNER, G., LAURIEN-KEHNEN, C., BOLAND, W., SCHREIER, P., and SCHMITT, T. 2008. A cuckoo in wolves' clothing? Chemical mimicry in a specialized cuckoo wasp of the European beewolf (Hymenoptera, Chrysididae and Crabronidae). *Front. Zool.* 5:2

VIENNE, C., SOROKER, V., and HEFETZ, A. 1995. Congruency of hydrocarbon patterns in heterospecific groups of ants: transfer and/or biosynthesis? *Ins. Soc.* 42:267–277.

YASUI, H., AKINO, T., YASUDA, T., FUKAYA, M., ONO, H., and WAKAMURA, S. 2003. Ketone components in the contact sex pheromone of the white-spotted longicorn beetle, *Anoplophora malasiaca*, and pheromonal activity of synthetic ketones. *Entomol. Exp. Appl.* 107:167–176.

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Developmental and Geographical Variation in the Chemical Defense of the Walkingstick Insect *Anisomorpha buprestoides*

Aaron T. Dossey · Spencer S. Walse · Arthur S. Edison

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Abstract *Anisomorpha buprestoides*, a walkingstick common in the southeastern United States, sprays chemicals that irritate and repel threatening insects, birds, or mammals. The active chemical in this substance was initially identified as a monoterpene dialdehyde. This compound can be present in several stereoisomeric forms, and subsequent studies have revealed that *A. buprestoides* produces at least three diastereomers: anisomorphal, dolichodial, and peruphasmal. However, no inquiry has been made to date into the geographical or developmental dependence of this variation. We report here that different populations of adult *A. buprestoides* spray either anisomorphal, or peruphasmal, or a mixture of the two stereoisomers. Additionally, offspring of a peruphasmal-producing population produced a variable mixture of anisomorphal and dolichodial but switched to peruphasmal upon reaching sexual maturity. This appears to be the first report of a developmentally regulated change in walkingstick insect chemical defense. Our results suggest a more complex role of these substances in the overall chemical ecology of walkingstick insects.

Keywords Insect · *Anisomorpha buprestoides* · Phasmatodea · Development · Chemical ecology · Monoterpene · Anisomorphal · Peruphasmal · Dolichodial

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A. T. Dossey · A. S. Edison (✉)
Department of Biochemistry & Molecular Biology,
University of Florida,
P.O. Box 100245, Gainesville, FL 32610-0245, USA
e-mail: art@mbi.ufl.edu

A. T. Dossey
e-mail: idpcwboy@ufl.edu

S. S. Walse
Center for Medical, Agricultural and Veterinary Entomology,
USDA-ARS,
Gainesville, FL 32604, USA

A. S. Edison
McKnight Brain Institute, University of Florida,
Gainesville, FL 32610, USA

A. S. Edison
National High Magnetic Field Laboratory, University of Florida,
Gainesville, USA

Present address:
S. S. Walse
Chemical Engineering, Yale University,
Mason Laboratory Room 307A, 9 Hillhouse Avenue,
New Haven, CT 06520-8286, USA

Introduction

Many living organisms rely on the use of chemical compounds for communication. Insects are particularly well-known for their chemical signaling and defense (Eisner et al. 2005). Many of the over 2,500 known species of walkingstick insects (Order Phasmatodea; Bedford 1978) produce noxious defensive secretions. However, the defensive chemistry of only a few species to date has been elucidated (Schneider 1934; Meinwald et al. 1962; Eisner 1965; Smith et al. 1979; Chow and Lin 1986; Ho and Chow 1993; Bouchard et al. 1997; Eisner et al. 1997; Schmeda-Hirschmann 2006; Dossey et al. 2006, 2007).

Defensive secretions from other types of insects are known to serve multiple functions (Blum 1996). For example, components of defensive secretions have been found to possess alarm pheromone activity in termites (Order Ispotera) and cockroaches (Order Blattodea) (Roisin

et al. 1990; Farine et al. 1997). Developmental changes in defensive secretion production have also been observed in insects such as true bugs (Order Hemiptera) (Blatt et al. 1998) and grasshoppers (Order Orthoptera) (Blum 1996). It has been postulated that walkingstick insect secretions could serve functions beyond warding off predators (Tilgner 2002), but so far, no studies have addressed this hypothesis.

We recently analyzed single defensive secretions from individual immature *Anisomorpha buprestoides* walkingsticks (Dossey et al. 2006) by using a novel 1-mm-high temperature superconducting NMR probe (Brey et al. 2006). This spray contained three monoterpene diastereomers: anisomorphal (Meinwald et al. 1962), dolichodial (Cavill and Hinterberger 1961; Cavill and Whitfield 1964; Cavill et al. 1976), and peruphasmal (Fig. 1a); glucose was also secreted with the monoterpenes (Dossey et al. 2006). Anisomorphal and dolichodial were produced in proportionally high concentrations compared with peruphasmal, which was present in only trace amounts. The ratios of anisomorphal and dolichodial varied from animal to animal and as a function of time (Dossey et al. 2006).

In two independent studies of *A. buprestoides* defensive spray, only anisomorphal was reported from *A. buprestoides* collected at Archbold Biological Research Station (Highlands Co., FL, USA) (Meinwald et al. 1962; Eisner et al. 1997). There were two major differences between the animals we worked with (Dossey et al. 2006) and those from the previous reports: (1) age/life stage and (2) geographical source of the animals from which spray was collected. Interestingly, *A. buprestoides* exists in various regionally specific color forms in Florida, USA (Hetrick

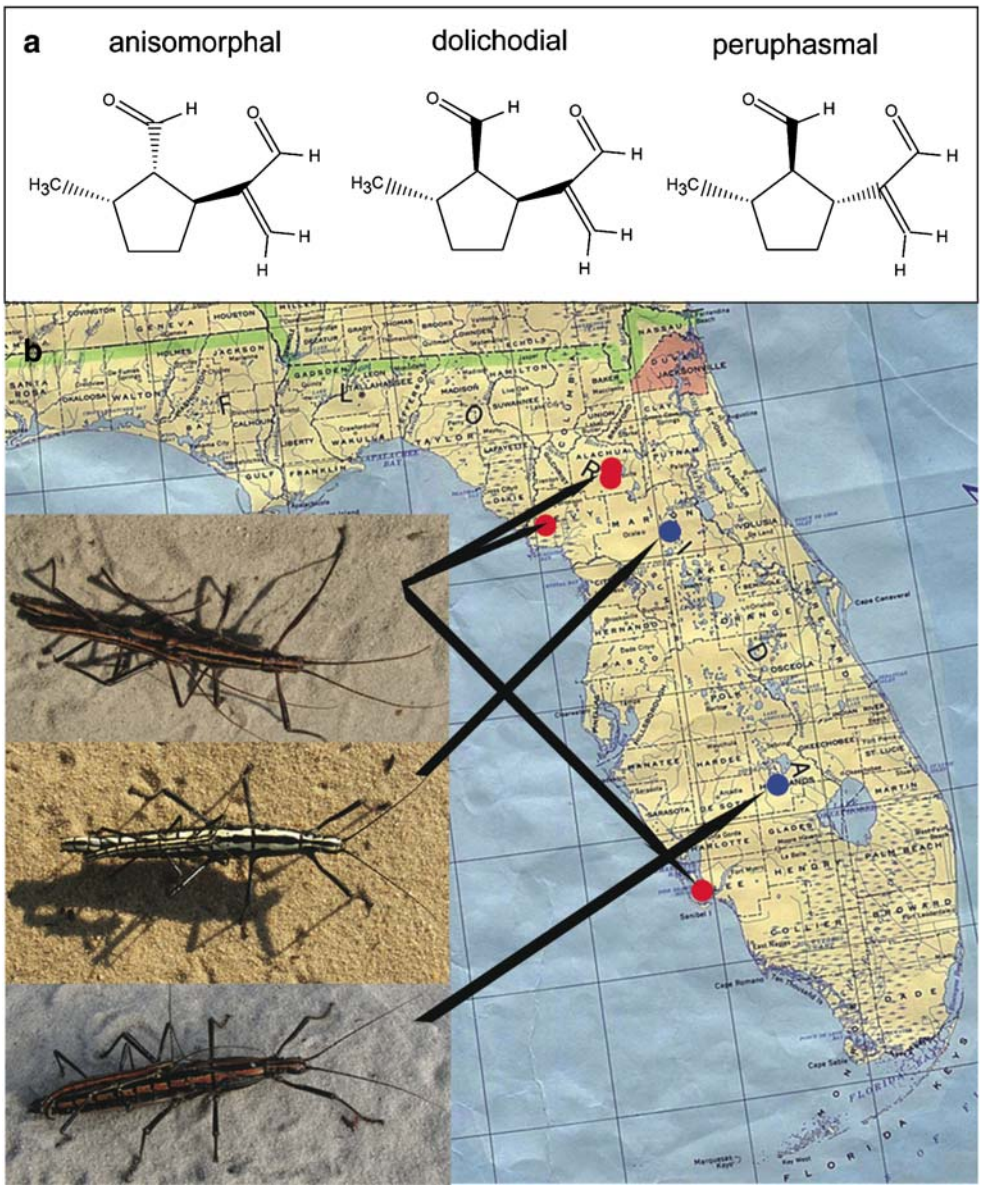

Fig. 1 Defensive spray variability in adult *A. buprestoides*. **a** The three monoterpene isomers produced by *A. buprestoides*. The relative configuration of dolichodial and anisomorphal were previously reported (Pagnoni et al. 1976). We have verified these studies and determined the relative configuration of peruphasmal (Wang et al., unpublished). **b** Geographic locations and photographs of color forms of *A. buprestoides* studied. The brown form (*top photo*) was found in two Gainesville locations (GNV and UFNA; 18 animals analyzed), GH (18 animals), and SI (three animals). The white-striped form (*middle photo*) was found in the ONF (15 animals). The orange-striped form (*bottom photo*) was found in AB (21 animals). The *colored dots* indicate collecting locations: *Blue dots* represent adult populations that only produced anisomorphal and *red dots* represent populations that produce peruphasmal or a mixture of peruphasmal and anisomorphal. One of the animals analyzed at Archbold produced about 25% dolichodial (Fig. 3), but that was the only significant amount of dolichodial found in the entire geographical study. No obvious dependence on gender of the animals was observed. Photographs by Aaron T. Dossey. The Florida map is courtesy of the University of Texas Libraries, The University of Texas at Austin

1949, Thomas 2001). The insects collected from Archbold for the current study all had a pair of orange dorsal stripes. Insects collected in Ocala National Forest (Florida, USA) were from a population with bright white dorsal stripes (Hetrick 1949). Aside from the stripes, the bodies of the Archbold and Ocala color forms are black. The bodies and dorsal stripes for *A. buprestoides* found at most other locations are usually brown with dark brown bodies and tan dorsal stripes. For simplicity, we refer to these color forms as the brown form (Fig. 1b, top photo), the white-striped form (Fig. 1b, middle photo), and the orange-striped form (Fig. 1b, bottom photo).

Here, we report both a developmental study and a geographical survey of the monoterpene dialdehydes used by *A. buprestoides* for defense. Additionally, we use ^{13}C labeling and ^{1}H NMR to demonstrate that *A. buprestoides* can synthesize its own defensive monoterpene dialdehyde from glucose. These results shed some light on the possible biological mechanisms behind the isomeric heterogeneity of defensive monoterpene dialdehyde production observed within this species.

Methods and Materials

Field Collections Individual secretions were collected in the field from sexually mature adult animals, as evidenced by the smaller males riding on the backs of larger females. The samples were frozen in the field and later analyzed by using NMR and gas chromatography-flame ionization detection (GC-FID). Each sample was an individual spray collected in a 1.5-ml glass vial held on top of the defensive gland while lightly agitating the insect. Samples were kept on ice and transported back to our lab for storage and analysis. The gender and location of each animal were recorded and are available in Supplementary Table S1. Global positioning satellite coordinates for collecting sites are as follows: Gainesville Airport "GNV": 29° 44′ 3″ N, 82° 16′ 27″ W; University of Florida Natural Area "UF-NA": 29° 38′ 1″ N, 82° 22′ 11″ W; Gulf Hammock "GH": 29° 14′ 46″ N, 82° 43′ 43″ W; Archbold Research Station "AB": 27° 10′ 50″ N, 81° 21′ 2″ W; Ocala National Forest "ONF": 29° 3′ 00″ N, 81° 38′ 50″ W; Sanibel Island "SI" two locations: 26° 27′ 5″ N, 82° 00′ 57″ W and 26° 27′ 37″ N, 82° 09′ 25″ W.

Developmental Study Eggs from the peruphasmal-producing GNV population of *A. buprestoides* were collected and reared in the laboratory. The hatchling animals were separated into their own plastic containers and reared as previously described (Dossey et al. 2006). Briefly, individuals were kept in separate containers, fed variegated privet (*Ligustrum sinense*), and raised from hatchling to adult. Defensive secretions were obtained as described for field collections of large animals or with a glass pipette placed directly over the defensive gland for very small animals. The secretions were extracted directly into methyl *tert*-butyl ether (MTBE) and analyzed by gas chromatography with mass spectrometric (GC-MS). The genders of the insects used in this study are available in Supplementary Table S2.

Isotopic Labeling An adult male *A. buprestoides* was fed 10–50 μl of 400 mM $^{13}C_6$ D-glucose (Cambridge Isotope Laboratories, Inc.) alternated every other day with variegated privet (*L. sinense*) for about 1 month. The glucose was given as a drop on the end of a blunt syringe needle and was readily accepted by the insect (Supplementary Video S1). Defensive secretions were collected periodically to monitor incorporation of ^{13}C into the defensive monoterpene. ^{13}C enrichment can easily be identified by large couplings between about 120 and 160 Hz (depending on the type of C–H bond) of the carbon-bound proton resonances in a standard ^{1}H 1D spectrum. This enrichment was quantified by using 1D ^{1}H NMR by dividing the sum of the integrals of the ^{13}C coupled "satellites" (e.g., pairs of coupled resonances) on each side of aldehyde ^{1}H resonances by the integral of the center ^{12}C-bound ^{1}H resonance.

Analytical Methods One-dimensional ^{1}H NMR spectra were collected as previously described (Dossey et al. 2006) on secretions dissolved directly with D_2O and subsequently analyzed with a custom built 1-mm HTS probe (Brey et al. 2006). GC-MS and flame ionization detection (GC-FID) were done as in (Dossey et al. 2006). Briefly, gas chromatography utilized He carrier gas (1.4 ml/min) and columns connected in series: a deactivated guard (L=8 cm, ID=0.53 mm), an HP-1MS retention-gap (L=2 m, ID=0.25 mm, df=0.25 mm), and a J&W DB-5 analytical (L=30 m, ID=0.25 mm, df=0.25 mm). Cool on-column or split-less injections (1 μl) were at 40°C and 200°C, respectively, and the oven program was isothermal at 40°C for 5 min, heated to 200°C at 11°C/min, and held 10 min, then heated to 250°C at 25°C/min and held 15 min. The FID was 260°C with N_2 make-up gas. Mass spectra were recorded with a Finnigan MAT Magnum® ion trap with 70-eV electron impact or isobutane chemical ionization over m/z 40–400; transfer-line and manifold temperatures were 240 and 220°C, respectively. Retention relative to tetradecane internal standard: peruphasmal, 0.81; dolichodial, 0.82; and anisomorphal, 0.83.

Results

Walkingstick Insects are Herbivores Thus, one explanation for chemical variability could be due to exogenous

synthesis of defensive compounds; if so, the different stereoisomers might be trivially explained as a consequence of diet. We note that dolichodial is also produced by ants (Cavill and Hinterberger 1961) and plants (Cavill and Hinterberger 1961; Pagnoni et al. 1976). To rule this out, we fed a few microliters of 400 mM $^{13}C_6$ D-glucose (see Supplementary Video S1) to a male walkingstick, alternating daily with food plant (privet—*Ligustum sinense*). We continued this feeding protocol for approximately 30 days and periodically analyzed the defensive secretions by NMR and mass spectrometry. Peruphasmal was enriched by 14%, 20%, and 25% in ^{13}C after 2, 3, and 4 weeks, respectively, clearly demonstrating that *A. buprestoides* can biosynthesize their own defensive monoterpene (Fig. 2).

Another explanation for chemical variability could be geographic location, so we sampled a total of seventy-five *A. buprestoides* from six different regions in Florida (Fig. 1b). As mentioned, *A. buprestoides* has at least three different color forms that are found in different geographical locations (Hetrick 1949; Thomas 2001). Figure 1b shows photographs of each color form, geographical collecting locations, and a summary of the defensive spray composition from insects in each location. Quantitative results are shown in Fig. 3. The white-striped (Hetrick 1949) and orange-striped forms collected in the Ocala National Forest and Archbold Research Station, respectively, secreted only anisomorphal, consistent with the original studies (Meinwald et al. 1962; Eisner et al. 1997). The brown forms, collected in Gainesville, Gulf Hammock and Sanibel Island, were more heterogeneous, producing only anisomorphal (28 animals), only peruphasmal (five animals), or a mixture of anisomorphal and peruphasmal (six animals). With one exception (Fig. 3), no adult *A. buprestoides* produced more than a trace amount of dolichodial.

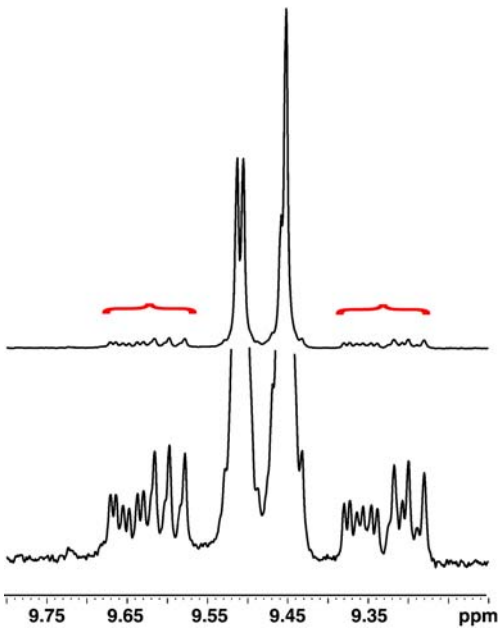

Fig. 2 Isotopic labeling of *A. buprestoides*. Animals were fed 10–50 µl of 400 mM uniformly labeled ^{13}C glucose alternated with plants for 15–30 days. This sample was collected on day 17, and 14% of the carbons in peruphasmal were labeled with ^{13}C, demonstrating that the insects can synthesize their own venom. The ^{13}C incorporation increased to 25% at 30 days (not shown). The spectrum shown is an expansion of the aldehyde region; the *top* shows the full intensity of the peaks, and the *bottom* is the same spectrum vertically expanded to show details of the ^{13}C satellites, indicated by *brackets*. The satellite resonances result from large one-bond ^{13}C–^{1}H scalar coupling, and the amount of ^{13}C incorporation can be easily determined from the ratio of the sum of the integrals of the satellite resonances to the integral of the central, ^{12}C-bound, resonances. A video showing the feeding is available as Supplementary Material, video S1

In our previous study, we found significant but variable amounts of dolichodial in immature *A. buprestoides* (Dossey et al. 2006). Because we found almost no dolichodial in the adult populations (Fig. 3), we investigated the role of development in the chemical composition of defensive secretions. Fourteen animals (ten males, three females, and one, which died before gender could be determined) were reared from hatchlings to adults as described in "Methods and Materials". These were offspring of adults collected from a peruphasmal-producing Gainesville population (GNV). Defensive secretions were collected and analyzed by GC-MS over a period of about 3 months (Fig. 4). Immature hatchlings (Fig. 4a) produced significant but variable amounts of dolichodial and anisomorphal but only trace amounts of peruphasmal, consistent with our previous findings (Dossey et al. 2006). We observed a consistent trend within each individual animal of increasing amounts of dolichodial after about 2 months of development (see yellow and orange circles in Fig. 4b and Supplementary Table S2 with a complete table of results). To our surprise, upon reaching sexual maturity, all animals stopped producing dolichodial and anisomorphal and started secreting only peruphasmal. Additionally, hatchlings reared in identical conditions but from another brown form population (Gulf Hammock, FL, USA) produced anisomorphal as adults (data not shown). This verified that peruphasmal was not the only isomer produced by adults reared under these conditions. Interestingly, these developmental changes in isomer production corresponded to the same sub-adult developmental stage when the males would normally mount the backs of females, the standard behavior for *A. buprestoides*.

Discussion

The results illustrate several aspects of the chemical defense system utilized by *A. buprestoides*. First, it is able to synthesize its own defensive monoterpene *de novo* from D-glucose. We hypothesize that the variability in the defensive

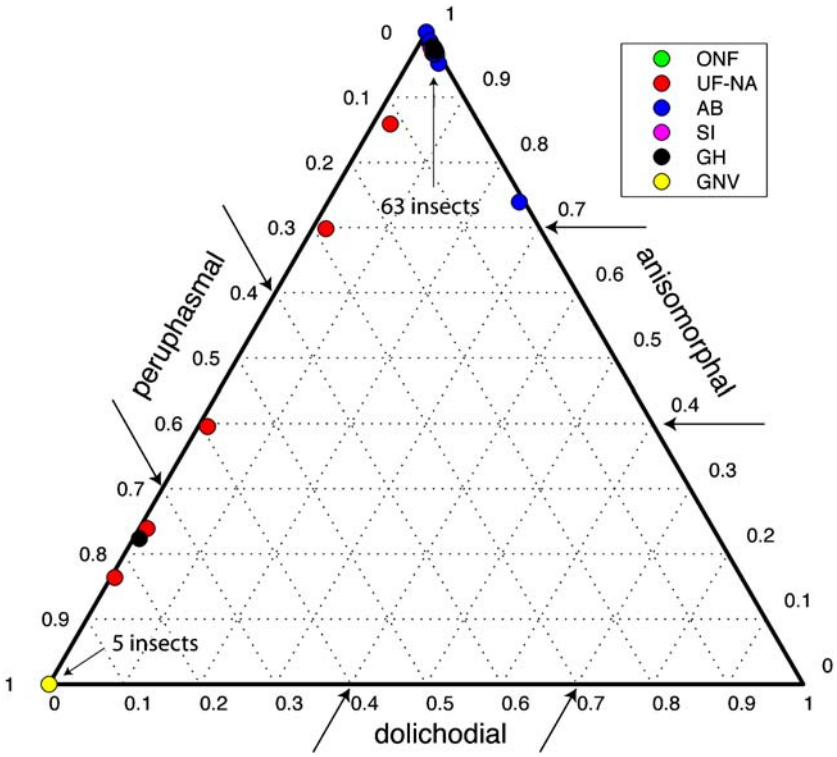

Fig. 3 Field milkings of individual adult *A. buprestoides*. Single defensive secretions were collected from animals in six different geographic locations in Florida (see Fig. 1b for abbreviations). The samples were frozen and analyzed by GC-FID and/or NMR as described in "Methods and Materials", and the total amount of anisomorphal, peruphasmal, and dolichodial for each animal was normalized to 1. The *arrows* on ternary plot axes indicate the direction that each component is plotted (e.g., anisomorphal is *horizontal*). With the exception of one sample with 25% dolichodial from AB, all other samples contained predominantly anisomorphal (63 insects), peruphasmal (five insects), or a mixture of anisomorphal and peruphasmal (six insects)

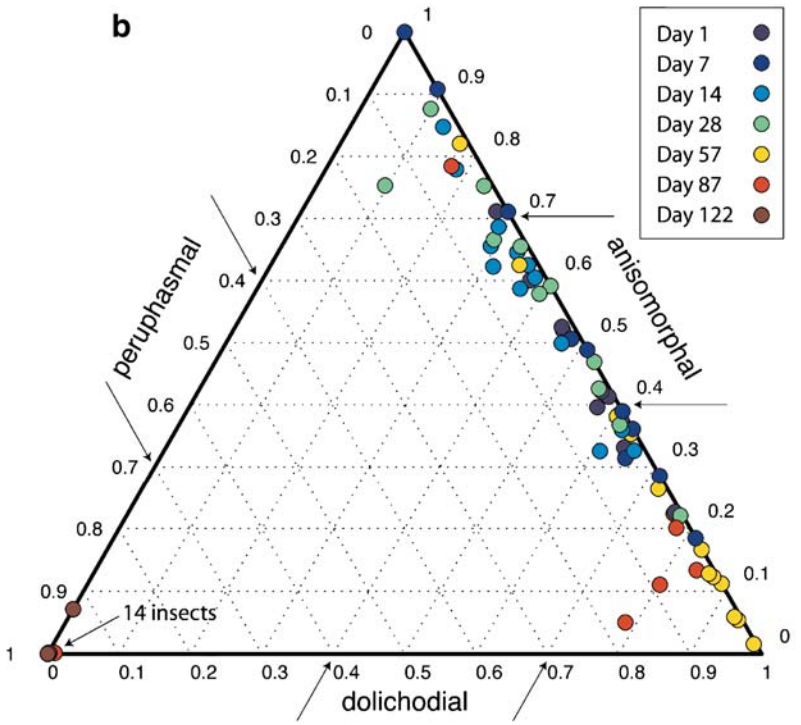

Fig. 4 *A. buprestoides* defensive secretions as a function of development. Fourteen animals (ten males, three females, and one undetermined) from a peruphasmal-producing GNV population (Figs. 1b and 3) were raised from hatchling to adult in individual containers. Individual secretions were collected from 1- to 3-day-old hatchlings (**a**) and at subsequent intervals ranging from 1 to 5 weeks, and secretions were analyzed by GC-MS. **b** Ternary plot of normalized anisomorphal, peruphasmal, and dolichodial concentrations, as described in Fig. 3. The data from *Day 1* were from 1- to 3-day-old hatchlings. By *Day 122*, all animals had reached sexual maturity. Photograph by Aaron T. Dossey

secretion composition likely results from variation in insect biosynthetic enzyme expression, primary sequence, or both. Such a system could evolve independently of food plant selection. However, further experiments are needed to determine whether enzymes or other non-protein mechanisms such as acid/base catalysis are responsible for the variability. Second, different populations of *A. buprestoides* produce different ratios of monoterpene dialdehydes in their defensive secretions, which also suggests an underlying genetic mechanism. Population dependent variability also could be the result of group isolation and, considering drastic differences between different color forms, the early stages of speciation. Since these insects do not fly, gene flow across their geographic range is probably slow, and certain populations are likely to become genetically isolated over time. However, a complete population genetic study of this species has not been pursued and is beyond the scope of the current study.

Finally, the composition of the monoterpene dialdehydes in *A. buprestoides* defensive spray is dependent on developmental life stage, showing both a trend to increased levels of dolichodial at about 2 months and an abrupt transition to ~100% peruphasmal by about 4 months (Fig. 4b). In the geographic study, we also found that adult *A. buprestoides* may sometimes produce anisomorphal or a combination of anisomorphal and peruphasmal, depending on which population they come from. In contrast, although dolichodial can be a major component of the spray before animals reach sexual maturity (Fig. 4b; Dossey et al. 2006), it is present only in trace amounts in adults (Fig. 3). One possible explanation for these observations is adaptive response to different predatory environments. For example, it is possible that young *A. buprestoides* encounter smaller invertebrate predators such as other insects or spiders. These may be more sensitive to dolichodial. Adult *A. buprestoides* are probably more vulnerable to attack by large vertebrate animals such as birds or mammals. It is possible that these animals are less sensitive to dolichodial and more sensitive to anisomorphal and peruphasmal. The developmental changes in *A. buprestoides* defensive spray chemistry also suggest their possible use as pheromones. *A. buprestoides* have an unusual behavior among insects: Males begin to copulate with the females before the females are fully grown, with one or two molts remaining. It is, therefore, possible that *A. buprestoides* defensive compounds also function as mating pheromones to regulate this behavior. In fact, many walkingstick species do not produce any secretion from their thoracic defense glands or produce secretions that are not irritating, at least to humans (Tilgner 2002). However, these hypotheses have yet to be tested. Overall, the findings of this study emphasize the value of extensively sampling natural products over both development and geographic range at individual organism resolution in order to more fully understand underlying chemical biodiversity and ecology.

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References

BEDFORD, G. O. 1978. Biology and ecology of the phasmatodea. *Annu. Rev. Entomol.* 23:125–149.

BLATT, S. E., BORDEN, J. H., PIERCE, H. D., GRIES, R., and GRIES, G. 1998. Alarm pheromone system of the western conifer seed bug, *Leptoglossus occidentalis*. *J. Chem. Ecol.* 24:1013–1031.

BLUM, M. S. 1996. Semiochemical parsimony in the arthropoda. *Annu. Rev. Entomol.* 41:353–374.

BOUCHARD, P., HSIUNG, C. C., and YAYLAYAN, V. A. 1997. Chemical analysis of defense secretions of sipyloidea sipylus and their potential use as repellents against rats. *J. Chem. Ecol.* 23:2049–2057.

BREY, W. W., EDISON, A. S., NAST, R. E., ROCCA, J. R., SAHA, S., and WITHERS, R. S. 2006. Design, construction, and validation of a 1-mm triple-resonance high-temperature-superconducting probe for nmr. *J Magn. Reson.* 179:290–3.

CAVILL, G. W. K., and HINTERBERGER, H. 1961. Chemistry of ants. 5. Structure and reactions of dolichodial. *Aust. J. Chem.* 14:143–149.

CAVILL, G. W. K., HOUGHTON, E., MCDONALD, F. J., and WILLIAMS, P. J. 1976. Isolation and characterization of dolichodial and related compounds from argentine ant, iridomyrmex-humilis. *Insect Biochem.* 6:483–490.

CAVILL, G. W. K., and WHITFIELD, F. B. 1964. Insect venoms, attractants & repellents .6. Synthesis of dolichodials. *Aust. J. Chem.* 17:1260–1269.

CHOW, Y. S., and LIN, Y. M. 1986. Actinidine, a defensive secretion of stick insect, megacrania alpheus westwood (orthoptera, phasmatidae). *J. Entomol. Sci.* 21:97–101.

DOSSEY, A. T., WALSE, S. S., CONLE, O. V., and EDISON, A. S. 2007. Parectadial, a monoterpenoid from the defensive spray of *parectatosoma mocquerysi*. *J. Nat. Prod.* 70(8):1335–1338.

DOSSEY, A. T., WALSE, S. S., ROCCA, J. R., and EDISON, A. S. 2006. Single insect nmr: a new tool to probe chemical biodiversity. *ACS Chem. Biol.* 1:511–4.

EISNER, T., MORGAN, R. C., ATTYGALLE, A. B., SMEDLEY, S. R., HERATH, K. B., and MEINWALD, J. 1997. Defensive production of quinoline by a phasmid insect (oreophoetes peruana). *J. Exp. Biol.* 200:2493–500.

EISNER, T. 1965. Defensive spray of a phasmid insect. *Sci.* 148:966–968.

EISNER, T., EISNER, M., and SIEGLER, M. 2005. Secret weapons: defenses of insects, spiders, scorpions, and other many-legged creatures. Belknap Press of Harvard University Press, Cambridge, Massachusetts.

FARINE, J. P., EVERAERTS, C., LEQUERE, J. L., SEMON, E., HENRY, R., and BROSSUT, R. 1997. The defensive secretion of eurycotis floridana (dictyoptera, blattidae, polyzosteriinae): Chemical iden-

tification and evidence of an alarm function. *Insect Biochem. Mol. Biol.* 27:577–586.

HETRICK, L. 1949. Field notes on a color variant of the two-striped walkingstick, *Anisomorpha buprestoides*, (stoll). *Fla. Entomol.* 32:74–77.

HO, H. Y., and CHOW, Y. S. 1993. Chemical-identification of defensive secretion of stick insect, megacrania tsudai shiraki. *J. Chem. Ecol.* 19:39–46.

MEINWALD, J., CHADHA, M. S., HURST, J. J., and EISNER, T. 1962. Defense mechanisms of arthropods .9. Anisomorphal, the secretion of a phasmid insect. *Tett. Letters.* 29–33.

PAGNONI, U. M., PINETTI, A., TRAVE, R., and GARANTI, L. 1976. Monoterpenes of teucrium-marum. *Aus. J. Chem.* 29:1375–1381.

ROISIN, Y., EVERAERTS, C., PASTEELS, J. M., and BONNARD, O. 1990. Castedependent reactions to soldier defensive secretion and chiral alarm recruitment pheromone in nasutitermes-princeps. *J. Chem. Ecol.* 16:2865–2875.

SCHMEDA-HIRSCHMANN, G. 2006. 4-methyl-1-hepten-3-one, the defensive compound from agathemera elegans (philippi) (phasmatidae) insecta. *Zeitschrift Fur Natur. C - J. Biosci.* 61:592–594.

SCHNEIDER, C. O. 1934. Las emanaciones del chinchemayo paradoxomorpha crassa. *Rev. Chil. Hist. Nat.* 38:44–46.

SMITH, R. M., BROPHY, J. J., CAVILL, G. W. K., and DAVIES, N. W. 1979. Iridodials and nepetalactone in the defensive secretion of the coconut stick insects, graeffea crouani. *J. Chem. Ecol.* 5:727–735.

THOMAS, M. C. 2001. The twostriped walkingstick, *Anisomorpha buprestoides* (stoll), (phasmatodea: Pseudophasmatidae). Fla. Dept. Agri. & Consumer Svcs. Division of Plant Industry. *Entomol. Circ.* 408:1–4.

TILGNER, E. H. 2002. Systematics of phasmida. Ph.D. Dissertation. University of Georgia, Athens, GA.

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The Iridoid Glucoside, Antirrhinoside, from *Antirrhinum majus* L. has Differential Effects on Two Generalist Insect Herbivores

Clifford W. Beninger · Renée R. Cloutier · Bernard Grodzinski

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Abstract The iridoid glucoside, antirrhinoside, is constitutively distributed throughout *Antirrhinum majus* L. in a manner consistent with its possible role as an allelochemical, but there is no evidence that it has a defensive function with respect to insect herbivory. To address this question, two generalist herbivores, *Lymantria dispar* L. (gypsy moth) and *Trichoplusia ni* Hübner (cabbage looper) were chosen for feeding trials on excised whole leaves of *A. majus* and in artificial diet assays. In leaf excision feeding trials, fourth instar gypsy moth rejected, without sampling, the leaves of *A. majus* regardless of what node the leaf was excised from. In contrast, fourth instar cabbage looper readily fed on the excised leaves, and antirrhinoside was not found in their bodies or feces (frass) as determined by thin layer and high-pressure liquid chromatography. In the leaf and diet assays, a second major leaf iridoid in *A. majus*, antirrhide, was found in both cabbage looper and gypsy moth frass. In diet feeding assays, the growth of gypsy moth and cabbage looper were not inhibited by methanol extracts, iridoid fractions, or pure antirrhinoside at concentrations of 0.6% in diet, but cabbage looper growth was enhanced. At an antirrhinoside concentration of 3.3% in diet, gypsy moth growth was reduced, whereas cabbage looper growth again increased significantly relative to the control. It is likely that antirrhinoside functions as defense against herbivory for one generalist insect herbivore but also, at low concentrations, enhances the growth of another.

C. W. Beninger (✉) · R. R. Cloutier · B. Grodzinski
Department of Plant Agriculture, University of Guelph, Guelph, ON, Canada N1G 2W1
e-mail: cbeninge@uoguelph.ca

Keywords *Antirrhinum majus* · Antirrhinoside · Iridoid glucoside · Generalist herbivores · *Lymantria dispar* · *Trichoplusia ni*

Introduction

Antirrhinoside is a monoterpene iridoid glucoside characteristic of species in the tribe Antirrhineae of the family Scrophulariaceae (Kooiman 1970) that includes *Antirrhinum majus* L. (Scarpati et al. 1968), various *Linaria* spp. (Ilieva et al. 1992; Bianco et al. 2004), *Cymbalaria* spp. (formerly placed in *Linaria*; Bianco et al. 1997; Serafini et al. 2004), *Asarina* spp. (Gowan et al. 1995; Voitsekhovskaja et al. 2007), *Kickxia* spp. (Handjieva et al. 1995; Al-Rehaily et al. 2006), and *Maurandya antirrhiniflora* Willd. (Boros et al. 1991). The taxonomy of genera within the Scrophulariaceae currently is the subject of debate. Recently, in a study based on molecular phylogenetics, *Antirrhinum* was placed in the Plantaginaceae family (Albach et al. 2005). This reassignment may indicate a closer relationship to *Plantago lanceolata* L., which has the iridoids catalpol and aucubin (Fig. 1). Catalpol is structurally similar to antirrhinoside, and it is found at high concentrations in new foliage, whereas aucubin predominates in older leaves (Klockars et al. 1993).

We have shown previously (Beninger et al. 2007) that for *A. majus* L., the iridoid glucoside, antirrhinoside, is likely phloem mobile and is allocated constitutively at much higher concentrations in young leaves, buds, and flowers, reaching 20% of the dry weight of those tissues, whereas the lowest concentrations are found in the root tissue. While antirrhinoside is present in all tissues of *A. majus*, a second major iridoid, antirrhide, is found only in the leaves. The relative concentrations of these two

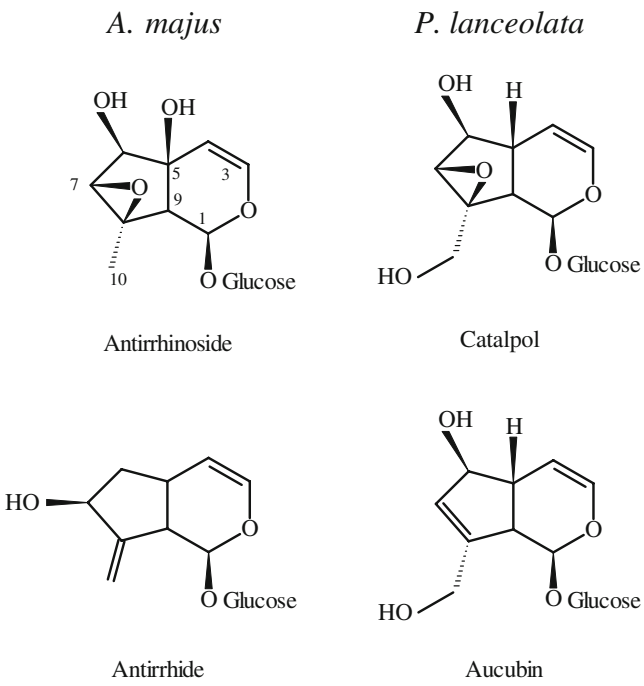

Fig. 1 Chemical structures of antirrhinoside and antirrhide found in *A. majus* and catalpol and aucubin found in *P. lanceolata*

iridoids change with time: as leaves age, antirrhinoside declines and the concentration of antirrhide increases (Beninger et al. 2007).

While much is known about the biosynthesis of antirrhinoside (Breinholt et al. 1992; Damtoft et al. 1993, 1995) there is, to our knowledge, little known of the function of this compound with regard to plant homeostasis or possible plant–plant and plant–animal interactions. The suggestion that it may be involved in homeostasis comes from a comparative study of two species within the Scrophulariaceae. Voitsekhovskaja et al. (2007) found that in *Asarina barclaiana* Pennell, antirrhinoside accounted for 39% of the total carbon transported in the phloem, whereas in *Alonsoa meridionalis* O. Kuntze, which does not have antirrhinoside, sugars, predominantly sucrose, were loaded into the phloem. From this, they concluded that antirrhinoside as well as sucrose in *A. barclaiana* may function to increase the osmotic pressure within the phloem. In another species within the Scrophulariaceae, *Asarina scandens* (Cav.) Penn., Gowan et al. (1995), by using ^{14}C labeling found that antirrhinoside accounted for up to 25% of the label relative to sucrose in lamina and petiole tissue. However, the potential of antirrhinoside as an allelochemical is not known. Other iridoids such as catalpol, catalposide, aucubin, loganin, and asperuloside negatively affect the growth of larval gypsy moth (Bowers and Puttick 1988, 1989). Indirect evidence for antirrhinoside acting as an allelochemical is the finding that it is sequestered by two species of moths that are aposematic as larvae and adapted to feeding on high antirrhinoside-containing plants (Boros et al. 1991). Antirrhinoside, however, was not found in the cryptic adults of the two species.

The purpose of the present study was to determine if antirrhinoside from *A. majus* would affect the feeding and growth of generalist insect herbivores. To accomplish this, two broadly polyphagus herbivores were chosen: gypsy moth (*Lymantria dispar* L.) and cabbage looper (*Trichoplusia ni* Hübner). Cabbage looper is a generalist that feeds on more than 160 species of plants in 36 families (Sutherland and Greene 1984) and may have encountered iridoid containing plants in its diet, as it has been known to be problematic in greenhouse production of *A. majus* (Naegele and Johnson 1962; Sutherland and Greene 1984). Gypsy moth is also considered a generalist that feeds on trees and shrubs in a number of families, but appears not to favor those that contain alkaloids (Barbosa and Krischik 1987).

Larval insects were presented with excised leaves from *A. majus* with different concentrations of antirrhinoside taken from different heights on the plant. Larvae were then tested in artificial diet experiments where methanol extract, iridoid fraction, and pure antirrhinoside isolated from *A. majus* were incorporated into diet. The fate of the two major iridoids in *A. majus*, antirrhinoside and antirrhide, was then determined by thin layer chromatography (TLC) and high-pressure liquid chromatography (HPLC) examination of the larval bodies and feces (frass) of the two species.

Methods and Materials

Plant Material The snapdragon cultivar used in this study was Potomac Ivory White. Seed were provided by PanAmerican Seed™ (Chicago, IL, USA) and sown and grown as described previously in Beninger et al. (2007). Briefly, seeds were sown in a soil-less growth medium (Pro-mix GPX®; Premier Horticulture Ltée/LTD Rivière du Loup, QC) in "128 cavity plug trays" (Landmark Co. Plastic, Akron, OH, USA). Germination took place on a greenhouse misting bench at 22/15°C day/night temperature regime under natural light conditions. After 3–4 wk, snapdragon seedlings were transplanted into a hydroponic basket with Leca as substrate and placed in 1.8-l containers filled with aerated nutrient solution as described by Johnstone et al. (2005). Hydroponic single-plant units were positioned in a growth chamber with a 12-hr photoperiod and provided 350±50 μmol m^{-2} sec^{-1} of photosynthetically active radiation at plant canopy height. Day/night temperatures were approximately 23.5/18°C. Plants were fertilized with a commercial hydroponic formulation (1.15 g of 6:11:35 N/P/K supplemented with 0.85 g of $CaNO_3$ per liter; Plant Products Ltd., Brampton, ON, Canada) with a pH of 6.0 and electrical

conductivity of 1.9 mS/cm. Each main stem was supported by a galvanized wire stake (gauge 12). Seedlings received a half strength nutrient solution for 5 d after transplant. Thereafter, seedlings received full strength nutrient solution, and the solution level was maintained during the experiment. Leaves for the insect bioassays and extraction of the iridoid fraction were excised at commercial maturity.

Insects Cabbage looper eggs and larvae were obtained from Agriculture and Agri-Food Canada, Southern Crop Protection & Food Research Centre, London, ON, Canada. Gypsy moth eggs were obtained from the Canadian Forest Service Insect Production Unit, Great Lakes Forest Research Centre, 1219 Queen St. E., Sault Ste. Marie, ON, Canada. Larvae were reared to the appropriate stage on BioServ® (Frenchtown, NJ, USA) gypsy moth and cabbage looper (general purpose Lepidoptera) artificial diets.

Isolation of the Iridoid Fraction from Leaf Tissue Freeze-dried, ground leaf tissue was loaded into a glass column (6.0×36.0 cm) and extracted sequentially with hexane, ethyl acetate, and methanol. The methanol fraction was found by HPLC to contain the iridoids. A 2.0×30.0-cm glass column was packed with a slurry of chlorophorm/methanol/water 6:3:1 and chromatographic silica (Fisher Scientific 100×200 mesh) and equilibrated with the same solvent system. One gram portions of powdered dry leaf crude methanol extract were dissolved in 2.0 ml methanol and loaded onto the column, which was then eluted with chloroform/methanol/water 6:3:1. Most of the chlorophyll and carotenoids were washed off the column, while the iridoids remained adhered to the silica. The mobile phase was changed to 100% methanol, which eluted the iridoids, and then this fraction was rotary evaporated until dry. It was taken up in 2.0 ml of methanol and loaded onto the top of a Sephadex LH-20 (Sigma-Aldrich®, Oakville, ON, Canada) 2.5×24.0-cm glass column, which had been equilibrated with 100% methanol and eluted with the same solvent. The column was monitored with a hand-held UV lamp at 366 nm (Model UVGL-58 Mineralight Lamp) and the blue band that contained the iridoids collected (Fig. 2a). This was rotary-evaporated to dryness, taken up in 2.0 ml of water, frozen at −80°C, and freeze-dried for use in the diet bioassays.

Isolation of Antirrhinoside Antirrhinoside is not available commercially and therefore must be purified from plant tissue. Flowers of *A. majus* contained the highest concentrations of antirrhinoside, with no antirrhide or chlorophyll to complicate purification. Flowers from mature Potomac Ivory White plants, which were grown as above, were removed from the flower spike (or inflorescence) and immersed in liquid N_2, frozen at −80°C, and then freeze-dried. Dried flowers were ground to a fine powder, loaded into a glass column (6.0×36.0 cm), and extracted as above for the leaves. The methanol extract contained antirrhinoside by HPLC analysis, and was rotary-evaporated to dryness, recovered in methanol, and dried under a stream of N_2. Finally, the methanol extract was taken up in water, frozen at −80°C, freeze-dried, and stored at −20°C before use. One gram portions of the methanol extract were fractionated on a Sephadex LH-20 column (2.5×24.0 cm) that had been equilibrated with 100% methanol and eluted with the same solvent. The column was monitored with a hand-held UV lamp at 366 nm. The first band collected was blue under UV and contained antirrhinoside in high concentration by HPLC analysis. This band was collected, concentrated by rotary evaporation, and streaked on 1,000-μm thickness, 20.0×20.0-cm silica gel preparative TLC plates (Analtech Inc., Newark, DE, USA). The blue band under UV was scraped from the plate, placed in 10.0-ml centrifuge tubes to which methanol had been added until close to the top, and centrifuged for 5 min at 3,000 rpm. The supernatants were collected, combined, and rotary-evaporated to dryness, after which, they were taken up in 2.0 ml of methanol and applied to the top of a Sephadex LH-20 column (1.5×24.0 cm) equilibrated, and eluted with 100% methanol for final purification. Purity of the isolated antirrhinoside used in the diet assays was then determined by HPLC analysis (Fig. 2b).

Preparation of Tissue for HPLC Analysis Leaf material, insects, and frass were frozen at −80°C and freeze-dried. These were placed into 1.5-ml Eppendorf tubes and two stainless steel beads (Montreal Biotech Inc. 3.17 mm) added to each tube to which 1.0 ml of methanol was added. Tissues were homogenized in a Retsch® MM300 tissue homogenizer (Newtown, PA, USA) for 5 min at 30 cycles per second, removed, centrifuged at 14,000 rpm for 3 min, and the supernatant passed through a 0.2-μm syringe filter (Whatman International Ltd., Maidstone, UK) into HPLC vials for analysis.

TLC Analyses TLC analysis of the extracts was carried out by co-chromatography with purified standards. Extracts and pure compounds were spotted onto 20.0×20.0-cm, 250 μm Avicell microcrystalline cellulose plates (Analtech Inc.) 2.0 cm from the bottom of the plates, allowed to dry, and placed in a TLC tank with *n*-butanol/acetic acid/H_2O 4:1:5 as the mobile phase. Plates were removed after the solvent front had reached within 2.0 cm of the top of the plate. After removal from the tank, plates were allowed to dry and sprayed with diphenylamine/aniline/H_3PO_4 spray (Markham 1982) and heated with a heat gun (Hejet, model HJ300 Pamran Co. Inc., Waukesha, WI, USA.) to visualize the iridoids.

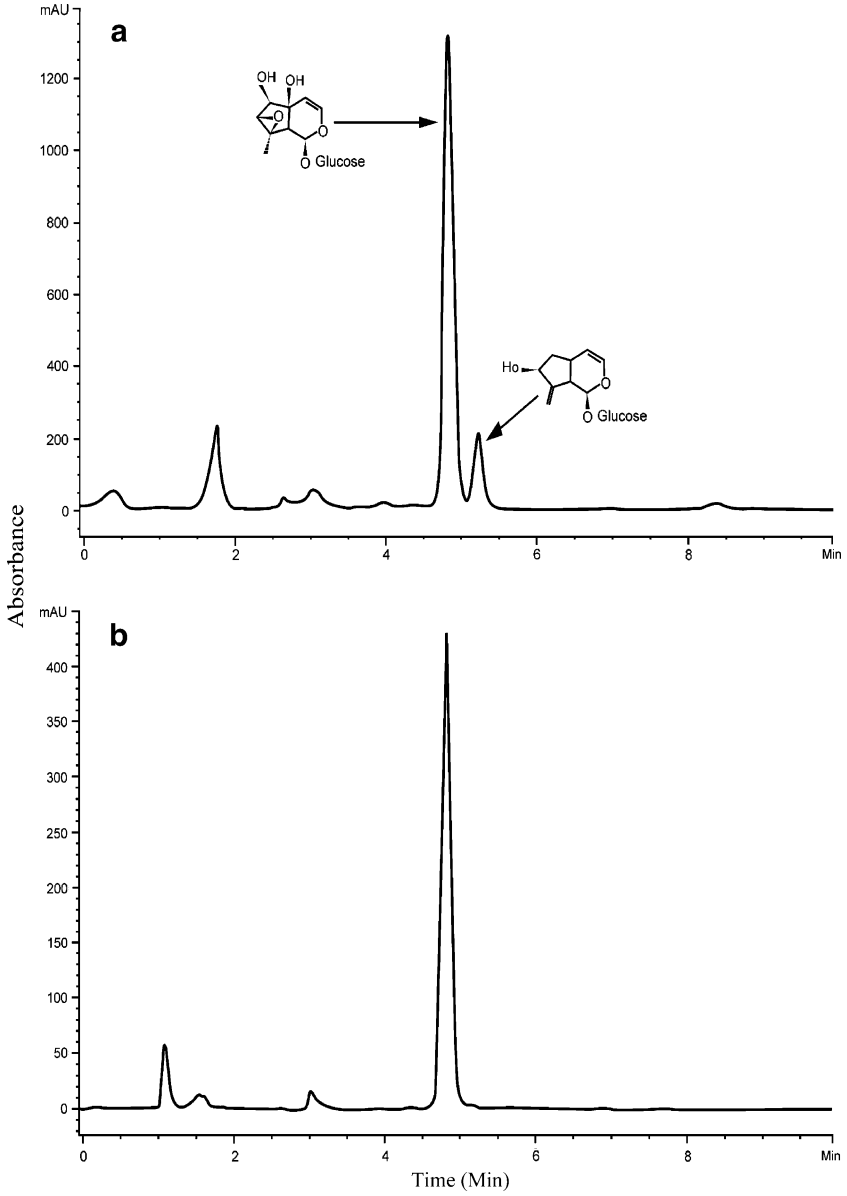

Fig. 2 **a** HPLC chromatogram of the purified iridoid fraction from *A. majus* leaves; **b** purified antirrhinoside from *A. majus* flowers

HPLC Analyses HPLC analyses were performed with an Agilent model 1100 HPLC equipped with a degasser, quad pump, autosampler, and diode array display (Agilent Technologies Canada Inc., Mississauga, ON, Canada). The column used was a CapCell Pak AG 120 C18 (4.6× 250.0 mm, 5-μm particle size; Shiseido Co. Ltd. Japan), and the HPLC had ChemStation software. The solvent system used was isocratic with 8% acetonitrile in water with a scan of 200 to 600 nm and quantification wavelength of 205 nm.

Excised Leaf-Feeding Experiments with Larvae Axillary leaves and lateral stems originate from slightly enlarged areas of the main stem of *A. majus*, defined as nodes. Nodes are numbered from the base of the stem, with the lowest node number being closest to the base. Leaves from nodes 4, 8, and 12–14 (N=5 from each node for each experiment) were excised at the point where the petiole joins the main stem. The cut ends of the petioles were placed in 1.5-ml Eppendorf tubes filled with distilled water through holes that had been cut in the lids. Eppendorf tubes and leaves were then placed on top of an empty 20.0-ml glass scintillation vial for support, and these were placed inside clear plastic Magenta® tissue culture boxes (Model GA7, 77.0×77.0×97.0 mm, Magenta Corporation, Chicago, IL, USA). Insects at the fourth instar stage were placed directly on the leaves (two per leaf) and the Magenta® boxes placed into an environmental chamber (12-hr photoperiod, day/night temperatures were approximately 23.5/18°C). Leaves from nodes opposite the

leaves that were presented to cabbage looper were analyzed by HPLC for their iridoid concentrations as an approximation of initial iridoid concentrations in leaves presented to the insects. The remaining leaf tissues that had been fed on by cabbage looper were also examined by HPLC to give a final concentration. A change would give an indication whether local leaf iridoid synthesis was altered by insect feeding over the 24-hr period. Leaves from node 10 (N=10) were also removed, one half of which was prepared as above and analyzed by HPLC to give an estimate of initial iridoid concentrations. The other half was treated exactly as leaves presented to the insects, except that no insects were placed on them. These were controls to determine what effects leaf excision may have on leaf iridoid chemistry. Ten insects for each leaf-feeding experiment were set aside and fed artificial diet for 24 hr only under the same environmental conditions as those fed leaves.

After 24 hr, insects were removed, and if leaves were fed on, they were prepared as above and analyzed by HPLC. Frass from cabbage looper leaf assays was allowed to air dry due to the difficulty in removing it from the Magenta® boxes while moist. When dry, frass was removed and subjected to HPLC analysis. Frass from artificial diet fed gypsy moth was freeze-dried before HPLC analysis.

Artificial Diet—Feeding Experiments with Larvae Gypsy moth and cabbage looper artificial diets were obtained from BioServ® (Frenchtown, NJ, USA). Extracts, fractions, and pure antirrhinoside were weighed to give the appropriate concentrations and then dissolved in 2:1 acetone/water, sonicated, and added to the artificial liquid diet. Control diet had only acetone/water 2:1. Diet was poured into Petri dishes, allowed to cool, and individual plugs were removed with cork borers of different sizes that were then placed in 29.6-ml Solo® cups to which the insect larvae (N=20 per treatment) were added. The cups were placed in an environmental chamber with the same light and temperature conditions as for the leaf assays. Diet was replaced every 2 d (or as needed), and larvae were weighed every 3 d. Gypsy moth larvae and frass from the 0.6% iridoid fraction and 3.3% antirrhinoside treatment were frozen, freeze-dried, and prepared as above for HPLC analysis.

Statistical Analyses The SAS® (2005) statistical software package was used. One-way analyses of variance with Student Newman–Keuls means differences tests were used to determine differences in iridoid concentrations in different leaf tissues, larvae, and frass using the PROC GLM, Means SNK. Differences in larval weights were analyzed in the same manner. The probability of a type I error rate was set at 0.05% for all analyses.

Results

Isolation of Iridoids Chemical structures of the compounds to be discussed are given (Fig. 1). Figure 2a,b shows the HPLC traces of the purified iridoid fraction from the leaves and antirrhinoside isolated from the flowers of *A. majus*. The bulk purification techniques outlined above gave better than 90% pure iridoid fraction and antirrhinoside.

Excised Leaf-Feeding Experiments with Larvae Cabbage looper larvae fed readily on the excised leaves of *A. majus* regardless of from where in the canopy the leaf was taken. For example, leaves from the fourth node are the oldest and contain the lowest levels of antirrhinoside and highest levels of antirrhide, whereas leaves taken from nodes 12–14 are relatively young and have the highest levels of antirrhinoside and lowest levels of antirrhide (Beninger et al. 2007). Gypsy moth did not feed on the leaves of *A. majus* and were active, moving around inside the culture boxes. Therefore, iridoid concentrations in leaves, larvae, and frass from this assay are not reported. In contrast to cabbage loopers that fed on the leaves and increased in weight (Fig. 3a), the gypsy moth larvae lost (about 13%) weight (Fig. 3b) and did not excrete frass over the 24-hr period of the experiment.

There was no significant difference in the amount of antirrhinoside, antirrhide, or total iridoids between the estimated initial and the actual final concentrations in the leaves that had been fed on by cabbage looper (Table 1). However, for the leaf taken from node 10, which was a control to examine the effect of excision on iridoid concentration, the amount of antirrhinoside increased significantly, whereas the concentration of antirrhide remained the same (Fig. 4). When the bodies and frass of cabbage looper fed *A. majus* leaves were analyzed by HPLC, antirrhinoside was not detected in either. However, antirrhide, while undetected in the body, was found at low concentrations in the frass (Table 2). This was confirmed by co-chromatography with TLC and HPLC with purified compounds and by HPLC peak UV signatures.

Artificial Diet—Feeding Experiments with Larvae Neither cabbage looper nor gypsy moth was adversely affected by methanol extract, iridoid fraction, or antirrhinoside at concentrations of 0.6% (Figs. 5a and 6a). However, cabbage looper showed significantly enhanced growth when feeding on antirrhinoside alone at the above concentration, and the same effect was seen when fed antirrhinoside at a concentration of 3.3% in diet (Fig. 5b). When fed antirrhinoside at 3.3%, gypsy moth larval weight was significantly reduced at all periods from the start of the experiment and was reduced by almost 50% by the end of the experimental period (Fig. 6b), although no larvae died.

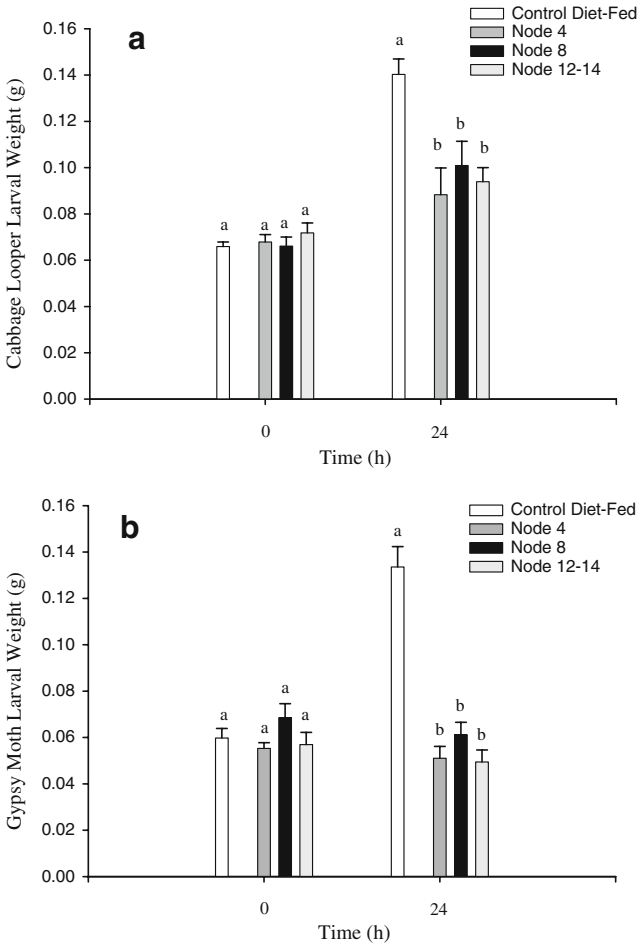

Fig. 3 **a** Larval weights of cabbage looper fed for 24 hr on artificial diet and excised *A. majus* leaves. **b** Larval weights of gypsy moth fed for 24 hr on artificial diet and excised *A. majus* leaves. Means with the *same letter above bars* at a given time period are not significantly different ($P>0.05$)

Bodies and frass of gypsy moth were also analyzed by HPLC and TLC, and antirrhinoside was not found. However, antirrhide was identified in the frass by its characteristic R_f and spot color with TLC (see Beninger et al. 2007), but the concentrations were too low for quantification.

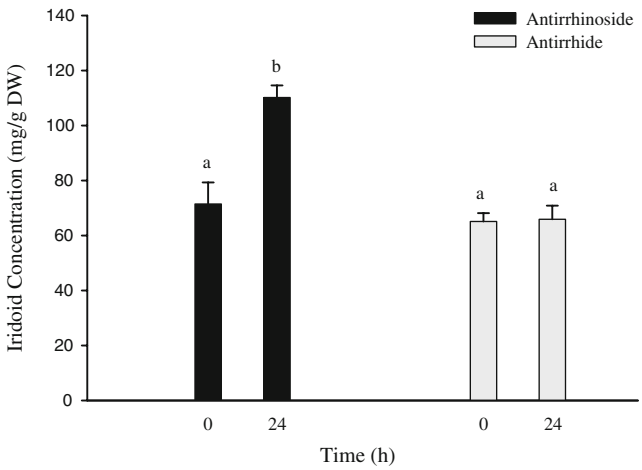

Fig. 4 Initial (based on opposite leaves) and final concentrations of iridoids in excised leaves not fed on by cabbage looper ($N=10$). Means with the *same letter above bars* at a given time period are not significantly different ($P>0.05$)

Peaks eluted with the same retention time as antirrhinoside in both cabbage looper and gypsy moth bodies; however, their UV signature from HPLC analysis and characteristic spot color on TLC was absent (data not shown). These peaks and UV signatures were found also in cabbage looper and gypsy moth bodies fed control diet, so they are likely a component of the artificial diet.

Discussion

Antirrhinoside is an iridoid glucoside that is produced constitutively in *A. majus* but whose concentration changes in leaves, depending on age (position in the canopy), relative to antirrhide, the other major iridoid found in *A. majus* (Høgedal and Mølgaard 2000; Beninger et al. 2007). A body of research has been published on the relationship between generalist and specialist insect herbivores that feed on the ribwort plantain (*Plantago lanceolata* L.; Bowers 1983, 1984; Bowers and Puttick 1988, 1989; Klockars et al. 1993; McCloud and Berenbaum 1999), which contains two major iridoids, catalpol, and aucubin (Fig. 1).

Table 1 Initial (based on opposite leaves) and final concentrations (mg/g DW) of iridoids for *A. majus* leaves fed to cabbage looper larvae

	Initial			Final		
	Node number					
	4	8	12–14	4	8	12–14
Antirrhinoside	64.07 (6.0)	106.31 (10.6)	150.51 (16.7)	74.21 (11.4)	107.69 (10.7)	125.53 (14.1)
Antirrhide	59.12 (5.8)	56.77 (5.7)	43.01 (3.3)	53.90 (1.9)	52.23 (3.0)	36.47 (1.0)
Total iridoids	123.18 (10.7)	163.09 (16.1)	193.52 (16.1)	128.1 (12.9)	159.92 (12.7)	162.01 (14.2)

Means with SE in parentheses. There were no significant differences between initial and final concentrations for leaves removed from different nodes ($P>0.05$).

Table 2 Concentration (mg/g DW) of iridoids recovered in frass of cabbage looper fed *A. majus* leaves

	Node from which Leaf Was Excised		
	4	8	12–14
Iridoid			
Antirrhinoside	nd	nd	nd
Antirrhide	6.97 (2.59)	4.32 (1.80)	6.98 (2.54)

Means with SE in parentheses. There were no significant differences in the concentration of antirrhide in the frass ($P>0.05$).
nd not detected

Because of the widespread occurrence and ease of cultivation of *P. lanceolata*, as well as the availability of the commercial standards catalpol and aucubin, this plant has become a model system to study the role of iridoids in insect–plant interactions. However, the relationship between the iridoid glucosides antirrhinoside and antirrhide in *A. majus* and their possible effects on insect herbivory are unknown. While antirrhide is found only in the leaves, antirrhinoside is found in all plant parts at 19–23% of the dry weight in new leaves and flowers, respectively (Beninger et al. 2007). The value of *A. majus* as a model system for studying the biological activity of iridoids is worthy of more careful examination because *A. majus* is a species for which a considerable amount of genetic and developmental information is known.

In the present study, we found different responses for two generalist herbivores in feeding trials with excised leaves. Fourth instar cabbage looper readily fed on leaves that contained total iridoid concentrations that varied from 12.3% to 19.4% of the dry weight of the leaf (Table 1) depending on where they were excised from in the canopy. In a study of potential host plants of cabbage looper, Soo Hoo et al. (1984) found that 60% survival to adulthood was

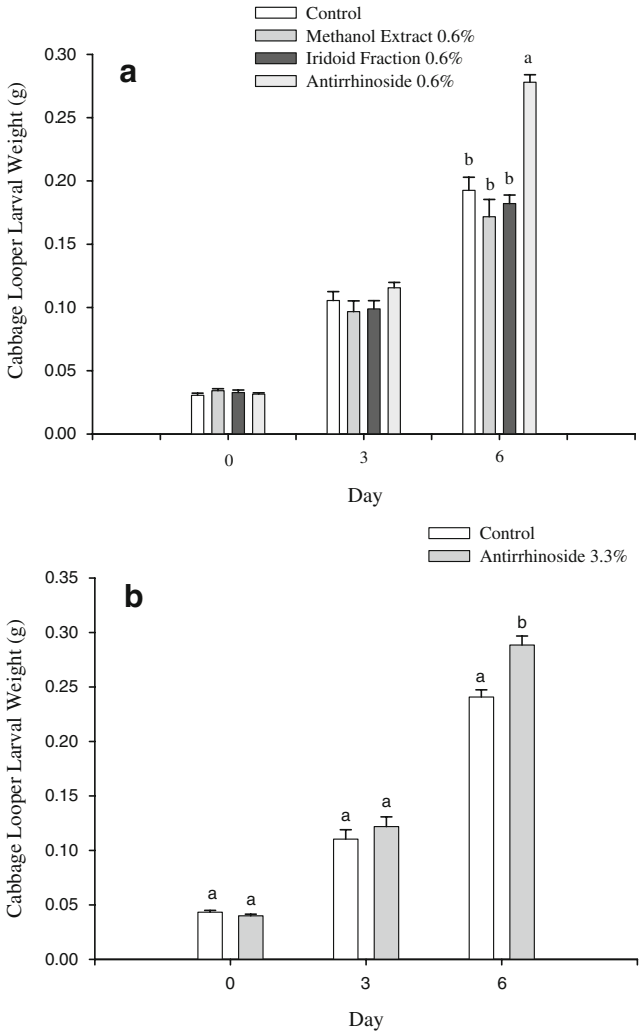

Fig. 5 **a** Larval weights for cabbage looper fed methanol extract, iridoid fraction, and pure antirrhinoside in artificial diet at 0.6%. **b** Larval weights for cabbage looper fed pure antirrhinoside at 3.3% in artificial diet. Means with the *same letter above bars* at a given time period are not significantly different ($P>0.05$)

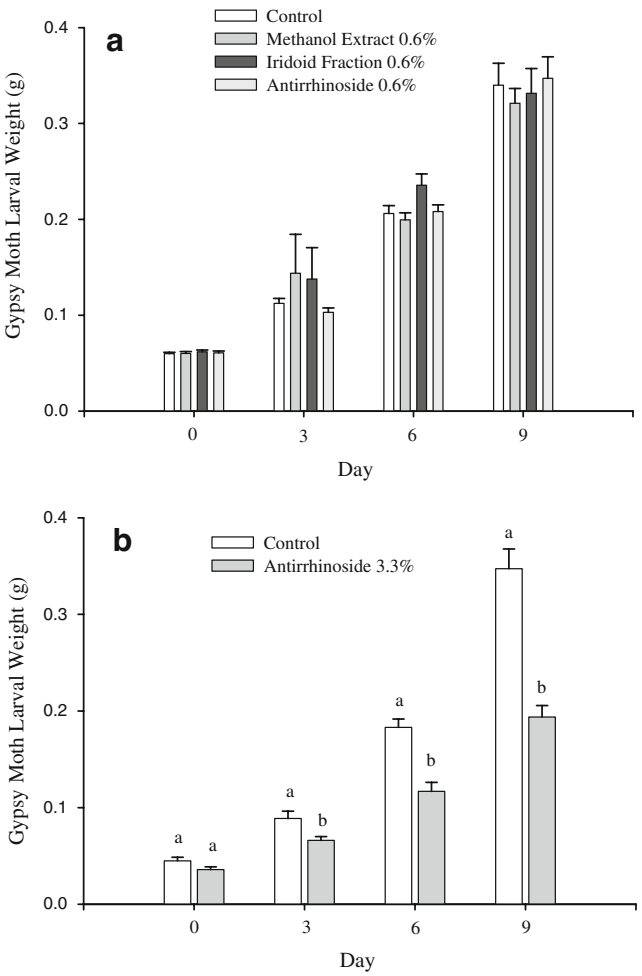

Fig. 6 **a** Larval weights for gypsy moth fed methanol extract, iridoid fraction and pure antirrhinoside in artificial diet at 0.6%. **b** Larval weights for gypsy moth fed pure antirrhinoside at 3.3% in artificial diet. Means with the *same letter above bars* at a given time period are not significantly different ($P>0.05$)

possible when fourth instars were placed on *Penstemon virgatus* Gray, which contains the iridoids catalpol, globularin, and scutellarioside-II (L'Empereur and Stermitz 1990). However, when first instars were placed on these plants, none survived to the second instar stage, which may indicate that the earliest instar of cabbage looper is more sensitive to iridoids. In our study, fourth instar cabbage looper fed leaves of different ages gained the same amount of weight, although not as much as the control larvae fed artificial diet only (Fig. 3a). This indicates that the role of antirrhide when present in leaves with antirrhinoside is unknown yet may be significant.

To our knowledge, before the present work, there has been no study that has tested directly whether antirrhinoside is anti-feedant or toxic to lepidopteran larvae. There is some evidence that it is nontoxic to specialists that feed on antirrhinoside-containing plants, as it is sequestered as a defense against predation. For example, the larvae of *Meris paradoxa* and two *Lepipolys* species are specialists on *Maurandya antirrhiniflora*, whose leaves contain concentrations of antirrhinoside similar to *A. majus*, and sequestered this compound at 3–11% dry weight of larvae (Boros et al. 1991). These larvae are aposomatic, but the adults are cryptic and do not sequester antirrhinoside. In contrast, cabbage looper is a generalist with cryptic larvae that do not sequester antirrhinoside.

In *P. lanceolata*, where the iridoids catalpol and aucubin are found, catalpol has been shown to reduce growth more than aucubin when fed to the generalist *Spodoptera eridania* (Stoll; southern armyworm; Puttick and Bowers 1988). Aucubin differs from catalpol only in the absence of the epoxide ring. Antirrhide in *A. majus* also lacks an epoxide ring (Fig. 1). Catalpol is similar to antirrhinoside, differing only in the lack of an oxygen at the 5-position and substitution of an oxygen at carbon 10. In this study, we did not feed pure antirrhide to cabbage looper because in the iridoid fraction from *A. majus*, it occurs only at low concentrations (Fig. 2a) relative to antirrhinoside and elutes very close to it in the HPLC analysis. The only method we have found to purify this compound from leaves is by semi-preparative HPLC, which gives small amounts that cannot be incorporated into artificial diet at physiologically relevant concentrations.

For leaves fed on by cabbage looper, antirrhinoside production did not appear to be induced in 24 hr, as the concentration remained unchanged (Table 1). This can be explained if *A. majus* is similar to *P. lanceolata* in which local induction of the iridoid catalpol was not apparent until 6 d after the specialist *Junonia coenia* Hübner was placed on the plants (Fuchs and Bowers 2004). As antirrhinoside in *A. majus* is likely phloem mobile, induction may have to occur at the whole plant level and result in the reallocation of antirrhinoside to damaged tissue from other leaves, a response which could not be determined within the experimental time period with excised leaves in our study. However, the excised leaves that were not fed on had significantly more antirrhinoside after 24 hr (Fig. 4). This may have been a local response to damage due to excision, but it is unclear why leaves that were fed on did not show a similar response. Cabbage looper is known to "trench" leaves to release compounds from the phloem tissue (Doussourd and Denno 1994), but this behavior was not observed in our experiments.

In diet-feeding experiments, cabbage looper fed pure antirrhinoside showed increased growth relative to controls. It should be noted that the antirrhinoside concentrations in diet are well below what can be expected in most leaves of *A. majus* (Høgedal and Mølgaard 2000; Beninger et al. 2007). However, when feeding on the concentrations of antirrhinoside and antirrhide found in leaves (Fig. 3a, Table 1), there was no deleterious effect on growth with regard to leaf position, although growth was significantly lower than larvae fed artificial diet.

Gypsy moth did not feed on the excised leaves (Fig. 3b) and was not affected when fed 0.6% antirrhinoside in diet (Fig. 6a), but at 3.3%, growth was significantly reduced (Figure 6b). In a study of the iridoids catalpol and aucubin, Bowers and Puttick (1988) found that these compounds both reduced gypsy moth final weights by approximately 50% at a concentration in diet of only 0.18% dry weight. The fact that gypsy moth was unaffected by antirrhinoside at 0.6% may be due to structural differences between catalpol and antirrhinoside or that some laboratory strains of gypsy moth are less sensitive to iridoids in their diet (Bowers and Puttick 1989).

Antirrhinoside and antirrhide were not found in the body of gypsy moth in our study, but antirrhide was present in the frass at concentrations too low for HPLC quantification. However, its characteristic R_f and spot color was found in TLC analysis. In another study, gypsy moth was found to excrete catalposide, but this was not detected in the body of the larvae (Bowers and Puttick 1986). Previously published choice studies with gypsy moth larvae either showed no preference for control vs. iridoid-containing diet or a preference for diet that contained iridoids that was dependant on the *type* of iridoid (Bowers and Puttick 1988). In another study, Bowers and Puttick (1989) found no preference for catalposide-containing diet at a concentration of 0.18% over control, but at high (7.2%) vs. low (0.18%) catalposide diet, responses varied, with the low concentration sometimes preferred and in other cases the high concentration. From these studies, the varied response of gypsy moth to iridoids in diets may indicate that it cannot reliably detect and avoid these compounds. The lack of feeding response when presented with excised leaves of *A. majus* was probably not due to gypsy moth's ability to

identify and avoid iridoids, as the leaves were not sampled by biting. In leaves, antirrhinoside accumulates mainly in vacuoles and is translocated in the phloem of leaf tissue (Voitsekhovskaja et al. 2007). It is unlikely that it is present on the leaf surface and that rejection of the leaves by gypsy moth may be a reaction to leaf volatiles or chemical components in the wax of the surface that can be detected with contact chemoreceptors on the tarsi or mouthparts (Bernays and Chapman 1994).

When feeding on *A. majus* leaves, cabbage looper is exposed to both antirrhinoside and antirrhide of differing concentrations (Table 1), dependant on the location of the leaves within the canopy. There was no effect of leaf position on growth (Fig. 3a), but growth was reduced relative to the diet-fed controls. In addition, growth of cabbage looper was unaffected when fed iridoid fraction at 0.6% in artificial diet, yet growth was enhanced when fed antirrhinoside alone (Fig. 5a,b) at 0.6% and 3.3% in diet. This indicates that when feeding on these two iridoids together in leaf or diet that the increase in weight observed when feeding on antirrhinoside alone is suppressed.

In summary, we have shown previously (Beninger et al. 2007) that antirrhinoside in *A. majus* is consistent with it being a phloem mobile compound whose distribution and concentration in various organs suggest that it functions as an allelochemical. In the current study, we have shown that one generalist herbivore, gypsy moth, rejects leaves of *A. majus*, and its growth is negatively affected when fed antirrhinoside in diet at a concentration that is lower than it would normally encounter in leaves of the plant. This defense may only be effective against some generalist herbivores, as cabbage looper growth is increased when fed on diet containing antirrhinoside alone. However, when feeding on the concentrations of antirrhinoside and antirrhide that are found in leaves, cabbage looper growth is reduced compared to control diet-fed larvae. The co-occurrence and relative concentrations of antirrhinoside and antirrhide in leaves of *A. majus* could therefore have significant effects on insect herbivores. Future research should focus on the combined effect of these two compounds.

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References

ALBACH, D. C., MEUDT, H. M., and OXELMAN, B. 2005. Piecing together the "new" Plantaginaceae. *Am J Bot*. 92:297–315.

AL-REHAILY, A. J., ABDEL-KADAR, M. S., AHMAD, M. S., and MOSSA, J. S. 2006. Iridoid glucosides from *Kickxia abhaica*. *Phytochemistry* 67:429–432.

BARBOSA, P., and KRISCHIK, V. A. 1987. Influence of alkaloids on feeding preference of eastern deciduous forest trees by the gypsy moth *Lymantria dispar*. *Am. Nat.* 130:53–69.

BENINGER, C. W., CLOUTIER, R. R., MONTEIRO, M. A., and GRODZINSKI, B. 2007. The distribution of two major iridoids in different organs of *Antirrhinum majus* at selected stages of development. *J. Chem. Ecol.* 33:731–747.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York, USA.

BIANCO, A., GUISO, M., PELLEGRINI, G., NICOLLETTI, M., and SERAFINI, M. 1997. Muralioside, an iridoid from *Cymbalaria muralis*. *Phytochemistry* 44:1515–1517.

BIANCO, A., GUISO, M., BALLERO, M., FODDAI, S., NICOLLETTI, M., PICCIN, A., SERAFINI, M., and TOMASSINI, L. 2004. Glycosidic monoterpenes from *Linaria capraria*. *Nat. Prod. Res.* 18:241.

BOROS, C. A., STERMITZ, F. R., and MCFARLAND, N. 1991. Processing of iridoid glycoside antirrhinoside from *Maurandya antirrhiniflora* (Scrophulariaceae) by *Meris paradoxa* (Geometridae) and *Lepipolys* species. *J. Chem. Ecol.* 17:1123–1133.

BOWERS, M. D. 1983. The role of iridioid glycosides in host-plant specificity of checkerspot butterflies. *J. Chem. Ecol.* 9:1983.

BOWERS, M. D. 1984. Iridoid glycosides and host-plant specificity in larvae of the buckeye butterfly, *Junonia coenia* (Nymphalidae). *J. Chem. Ecol.* 10:1567–1577.

BOWERS, M. D., and PUTTICK, G. M. 1986. Fate of ingested iridoid glycosides in Lepidopteran herbivores. *J. Chem. Ecol.* 12:169–177.

BOWERS, M. D., and PUTTICK, G. M. 1988. Response of generalist and specialist insects to qualitative allelochemical variation. *J. Chem. Ecol.* 14:319–334.

BOWERS, M. D., and PUTTICK, G. M. 1989. Iridoid glycosides and insect feeding preferences: gypsy moths (*Lymantria dispar*, Lymantriidae) and buckeyes (*Junonia coenia*, Nymphalidae). *Ecol. Entomol.* 14:247–256.

BREINHOLT, J., DAMTOFT, S., DEMETH, H., JENSEN, S. R., and NIELSON, B. J. 1992. Biosynthesis of antirrhinoside in *Antirrhinum majus*. *Phytochemistry* 31:795–797.

DAMTOFT, S., JENSEN, S. R., and JESSEN, C. U. 1993. Intermediates between 8-*epi*-deoxyloganic acid and 6,10-dideoxyaucubin in the biosynthesis of antirrhinoside. *Phytochemistry* 33:1087–1088.

DAMTOFT, S., JENSEN, S. R., and SHACHT, M. 1995. Last stages in the biosynthesis of antirrhinoside. *Phytochemistry* 39:549–551.

DOUSSOURD, D. E., and DENNO, R. F. 1994. Host range of caterpillars: Trenching permits feeding on plants with secretory canals. *Ecology* 75:69–78.

FUCHS, A., and BOWERS, M. D. 2004. Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *J. Chem. Ecol.* 30:1723–1741.

GOWAN, E., LEWIS, B. A., and TURGEON, R. 1995. Phloem transport of antirrhinoside, an iridoid glycoside, in *Asarina scandens* (Scrophulariaceae). *J. Chem. Eco.l* 21:1781–1788.

HANDJIEVA, N., POPOV, S., TERSIEVA, L., and EVSTATIEVA, L. 1995. Two iridoid glucosides, 5-*O*-menthiafoloylkickxioside and kickxin, from *Kickxia* Dum. species. *Phytochemistry* 39:925–927.

HØGEDAL, B. D., and MØLGAARD, P. 2000. HPLC analysis of the seasonal and diurnal variation of iridoids in cultivars of *Antirrhinum majus*. *Biochem. Syst. Ecol*. 28:949–962.

ILIEVA, E. I., HANDJIEVA, N. V., and POPOV, S. S. 1992. Iridoid glucosides from *Linaria vulgaris*. *Phytochemistry* 31:1040–1041.

JOHNSTONE, M., CHATTERTON, S., SUTTON, J. C., and GRODZINSKI, B. 2005. Net carbon gain and growth of bell peppers, *Capsicum annuum* 'Cubico', following root infection by *Pythium aphanidermatum*. *Phytopathology* 95:354–361.

KLOCKARS, G. K., BOWERS, M. D., and COONEY, B. 1993. Leaf variation in iridoid glycoside content of *Plantago lanceolata* (Plantaginaceae) and oviposition of the buckeye, *Junonia coenia* (Nymphalidae). *Chemoecology* 4:72–78.

KOOIMAN, P. 1970. The occurrence of iridoid glycosides in the Scrophulareaceae. *Acta. Bot. Neerl.* 19:329–340.

L'EMPEREUR, K. M., and STERMITZ, F. R. 1990. Iridoid glycoside metabolism and sequestration by *Poladryas minuta* (Lepidoptera: Nymphalidae) feeding on *Penstemon virgatis* (Scrophulariaceae). *J. Chem. Ecol.* 16:1495–1506.

MARKHAM, K. R. 1982. Techniques of Flavonoid Identification. Academic, New York.

MCCLOUD, E. S., and BERENBAUM, M. 1999. Effects of enhanced UV-B radiation on a weedy forb (*Plantago lanceolata*) and its interaction with a generalist and specialist herbivore. *Entomol. Exp. Appl*. 93:233–247.

NAEGELE, J., and JOHNSON, G. 1962. Insects. p 85, in R. Langhans (ed.). Snapdragons. A Manual of Culture, Insects and Diseases and Economics of Snapdragons. Prepared for the New York State Extension Service Snapdragon School with the cooperation of the New York State Flower Growers Association Inc.

PUTTICK, G. M., and BOWERS, M. D. 1988. Effect of qualitative and quantitative variation in allelochemicals on a generalist insect: Iridoid glycosides and the southern armyworm. *J. Chem. Ecol*. 14:335–351.

SAS Institute, Inc. 2005. SAS Procedures Guide v. 9.1. Cary, NC, USA.

SCARPATI, M. L., Guiso, M., and Esposito, P. 1968. Iridoidi (V) Struttura e configurazione dell'Antirrinoside. *Gazz. Chim. Ital.* 98:177–190.

SERAFINI, M., FODDAI, S., BALLERO, M., GUISO, M., and BIANCO, A. 2004. The occurrence of iridoid glycosides in *Cymbalaria* spp. in Italy. *Nat. Prod. Res*. 18:325–327.

SOO HOO, C. R., COUDRIET, D. L., and VAIL, P. V. 1984. *Trichoplusia ni* (Lepidoptera:Noctuidae) larval development on wild and cultivated plants. *Environ. Ent.* 13:843–846.

SUTHERLAND, D. W. S., and GREENE, G. L. 1984. Cultivated and wild host plants, pp. 1–13, in P. D. LINGREN and G. L. GREENE (eds.). Suppression and Management of Cabbage Looper Populations. USDA Tech. Bull. 1684.

VOITSEKHOVSKAJA, O. V., KOROLEVA, O. A., BATASHEY, D. R., KNOP, C., TOMOS, A. D., GAMALEI, Y. V., HELDT, H.-W., and LOHAUS, G. 2007. Phloem loading in two Scrophulariaceae species. What can drive symplastic flow via plasmodesmata? *Plant Physiol.* 140:383–395.

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RAPID COMMUNICATION

Defense-Inducing Volatiles: In Search of the Active Motif

Martin Heil · Ulrich Lion · Wilhelm Boland

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Abstract Herbivore-induced volatile organic compounds (VOCs) are widely appreciated as an indirect defense mechanism since carnivorous arthropods use VOCs as cues for host localization and then attack herbivores. Another function of VOCs is plant–plant signaling. That VOCs elicit defensive responses in neighboring plants has been reported from various species, and different compounds have been found to be active. In order to search for a structural motif that characterizes active VOCs, we used lima bean (*Phaseolus lunatus*), which responds to VOCs released from damaged plants with an increased secretion of extrafloral nectar (EFN). We exposed lima bean to (*Z*)-3-hexenyl acetate, a substance naturally released from damaged lima bean and known to induce EFN secretion, and to several structurally related compounds. (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, 5-hexenyl acetate, (*Z*)-3-hexenylisovalerate, and (*Z*)-3-hexenylbutyrate all elicited significant increases in EFN secretion, demonstrating that neither the (*Z*)-configuration nor the position of the double-bond nor the size of the acid moiety are critical for the EFN-inducing effect. Our result is not consistent with previous concepts that postulate reactive electrophile species (Michael-acceptor-systems) for defense-induction in Arabidopsis. Instead, we postulate that physicochemical processes, including interactions with odorant binding proteins and resulting in changes in transmembrane potentials, can underlie VOCs-mediated signaling processes.

Keywords Herbivore-induced volatiles · Hexenyl acetate · Indirect defense · Induced defense · Plant-plant communication · Signal

M. Heil (✉)
Departamento de Ingeniería Genética, CINVESTAV,
Km. 9.6 Libramiento Norte,
Irapuato CP 36821, Guanajuato, México
e-mail: mheil@ira.cinvestav.mx

M. Heil · U. Lion
Department of General Botany, Plant Ecology,
University of Duisburg-Essen,
Universitätsstraße 5,
45117 Essen, Germany

W. Boland
Deparment of Bioorganic Chemistry,
Max-Planck-Institute for Chemical Ecology,
Hans-Knöll-Str. 8,
07745 Jena, Germany

Introduction

Plants respond to herbivore damage with the release of volatile organic compounds (VOCs) that signal the presence of herbivore prey to predators and parasites and thereby serve as an indirect defense mechanism (e.g., Heil 2008). Research has demonstrated that these VOCs can also be perceived by neighboring plants or intact, systemic parts of the damaged plant (Baldwin et al. 2006; Heil 2008). Particularly, green-leaf volatiles (GLVs) have been associated with induced resistance in intact plants (Arimura et al. 2000; Engelberth et al. 2004; Farag et al. 2005; Ruther and Kleier 2005; Kost and Heil 2006;). However, little is known about the identity of VOCs that are active in this context or about a structural motif that active VOCs might have in common. In an attempt to explain the inducing activity of such VOCs on gene expression, it has been suggested that GLVs with an α,β-unsaturated carbonyl group such as (*E*)-2-hexenal can trigger defense responses in Arabidopsis through their activity as reactive electrophile species (Almeras et al. 2003).

In the present study, we used lima bean (*Phaseolus lunatus* L.) to search for traits that characterize defense-inducing VOCs. Lima bean responds to herbivore damage with the jasmonate-mediated production of VOCs and extrafloral nectar (EFN), an aqueous, sugar-containing secretion on nonreproductive plant organs that attracts predatory arthropods (mainly ants). The natural blend of VOCs that is released from a herbivore-damaged lima bean induces EFN secretion in intact neighboring plants (Kost and Heil 2006) and serves as a within-plant signal (Heil and Silva Bueno 2007). Among the quantitatively dominant VOCs that are released from induced lima bean, however, only (*Z*)-3-hexenyl acetate significantly induced EFN secretion when used as pure compound (Kost and Heil 2006). In the present study, we applied different structurally related esters to lima bean plants and monitored their EFN secretion.

Materials and Methods

Plants were cultivated from seeds collected in the coastal area of Puerto Escondido, Oaxaca, México (15°55.596 N and 097°09.118 W, elevation 15 m). Seedlings were cultivated under ambient conditions in 250 ml pots filled with soil from the original growing site. The plants were watered daily and fertilized 6 weeks after germination with commercial fertilizer: 10 ml per plant of a solution of 3 mg l^{-1} of "Fertilisante foliar de alta concentración" (Grupo Bioquímico Mexicano, Aaltillo, Coah., Mexico). Experiments were conducted with plants of an age of 8–10 weeks. The following compounds were dissolved in Lanolin paste (all at 0.1 μg μl^{-1}): (A) (*Z*)-3-hexenyl acetate, (B) (*E*)-3-hexenyl acetate, (C) (*E*)-2-hexenyl acetate, (D) 5-hexenyl acetate, (E) (*Z*)-3-hexenyl isovalerate, (F) (*Z*)-3-hexenyl butyrate, (G) 2-ethylhexanol, (H) (*E*)-2-hexanal, (I) decanal, and (K) nonanal (Lanolin paste and all compounds were purchased from Sigma Aldrich). Each 0.25 g paste per plant was applied on green plastic stripes (MAX Binde-band) attached to the plant in order to avoid direct contact of the paste with the plant. The plants were then packed in perforated PET foil bags (Bratenschlauch, Toppits, Minden, Germany) and in nets to protect them from EFN consumers. Amounts of VOCs released from the lanolin paste into the plants' headspace under these conditions were monitored in a parallel experiment that used a closed-loop stripping system as described previously (Kost and Heil 2006). EFN secretion was quantified from the five youngest leaves 24 h later. In short, EFN concentration was measured with a portable refractometer, and nectar volume was measured with glass capillaries (Kost and Heil 2006) to calculate the total amount of soluble solids secreted, which in the case of lima bean EFN are mainly glucose, fructose, and sucrose. Leaves were then collected and dried to calculate EFN secretion as soluble solids secreted per gram leaf dry mass and per 24 h. Plants to which Lanolin paste without any compound added had been applied served as controls (Lanolin control, LC). Per day, two groups each comprising all 11 treatments were investigated, and in total eight plants per treatment were used.

Results and Discussion

Amounts of VOCs released from the lanolin paste into the plant's headspace resembled those released from an induced plant, i.e., all individual VOCs were present at 80–130% of the amount of (*Z*)-3-hexenyl acetate that is released under comparable conditions from five induced leaves of lima

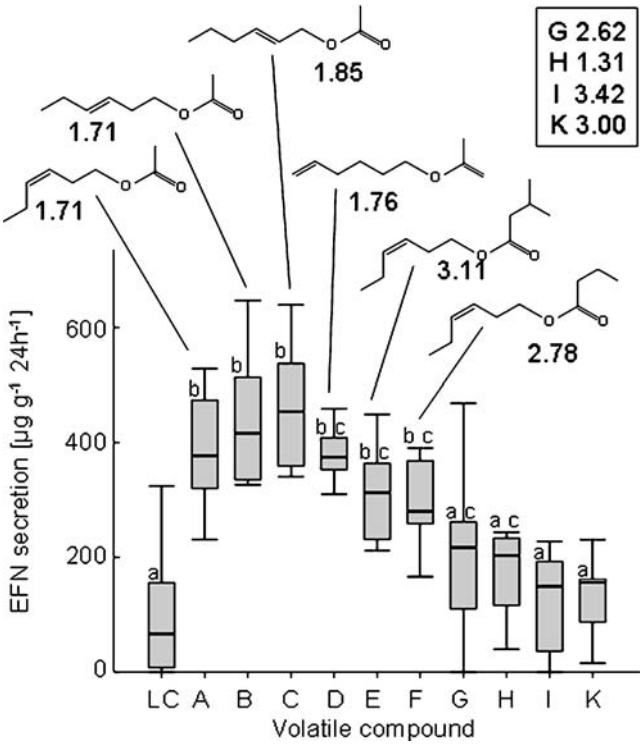

Fig. 1 EFN secretion in μg soluble solids secreted per gram leaf dry mass and per 24 h is depicted. Plants were exposed for 24 h to (*A*) (*Z*)-3-hexenyl acetate, (*B*) (*E*)-3-hexenyl acetate, (*C*) (*E*)-2-hexenyl acetate, (*D*) 5-hexenyl acetate, (*E*) (*Z*)-3-hexenyl isovalerate, (*F*) (*Z*)-3-hexenyl butyrate, (*G*) 2-ethylhexanol, (*H*) (*E*)-2-hexanal, (*I*) decanal, and (*K*) nonanal dissolved in Lanolin paste. Plants to which pure Lanolin paste had been applied served as controls (*LC*). Lower and upper whiskers represent the 5% and the 95% percentile, lower and upper margins of boxes the 25% and the 75% percentile, the *lines within boxes* indicate medians. *Different letters appearing above boxes* mark treatment effects that differ significantly from each other ($P<0.05$ according to LSD posthoc analysis), and structures of compounds leading to a significant induction of EFN secretion as compared to the control are graphically presented. LogP values of all compounds were calculated with ChemDraw Ultra 6.0 and are given under the structures (compounds *A–F*) and in the insert (*G–K*)

bean (data not shown, for amounts of VOCs released from an induced plant see Kost and Heil 2006). The different VOCs to which lima bean plants had been exposed affected their EFN secretion significantly (General linear model: $F_{77,10}=10.05$, $P<0.001$). Compounds A–F consisted of acyl hexenols in which the configuration and position of the double bond was systematically shifted from the polar head to the aliphatic terminus of the molecule. Moreover, the importance of the size of the acyl moiety was evaluated by using acetates, butyrate, and isovalerate of (*Z*)-3-hexenol. Although only one of the tested VOCs is naturally released from lima bean, exposure to the six compounds (A–F) elicited EFN secretions that were significantly higher than those of the controls (LSD posthoc analysis: $P<0.05$, see Fig. 1). These compounds were (*Z*)-3-hexenyl acetate, (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, 5-hexenyl acetate, (*Z*)-3-hexenyl isovalerate, and (*Z*)-3-hexenyl butyrate. Hence, EFN secretion by lima bean can be induced by VOCs that are not released naturally from this species, while several VOCs that are released from induced plants did not significantly change EFN secretion in previous experiments (Kost and Heil 2006).

How are VOCs perceived by plants, and via which mechanisms do they affect defense expression patterns? It has been suggested that molecules with an α,β-unsaturated carbonyl group can trigger defenses in Arabidopsis through their activity as reactive electrophile species (Almeras et al. 2003). In principle, VOCs could also be perceived by binding to specific receptor-proteins or to odorant binding proteins with a preference for a class of compounds, similar to animal olfactory systems.

Apparently, neither the configuration nor the position of the double bound is a critical factor. Compounds reported to prime or induce gene activity or phenotypic defenses in intact corn plants comprise (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenal, and (*Z*)-3-hexenyl acetate (Engelberth et al. 2004; Farag et al. 2005; Ruther and Kleier 2005). We found that (*Z*)-3-hexenyl acetate, (*E*)-3-hexenyl acetate, (*E*)-2-hexenyl acetate, and 5-hexenyl acetate all elicited particularly high EFN secretion rates in lima bean. The majority of these substances lack an α,β-unsaturated carbonyl group, which thus cannot be a generally required motif. In addition, a compound such as 5-hexenyl acetate cannot yield an electrophile such as (*E*)-2-hexenal via the sequence of ester hydrolysis, oxidation, and isomerization, as it is principally possible for (*E* or *Z*)-3-hexenyl acetate or (*E* or *Z*)-2-hexenyl acetate. Moreover, unlike (Z)-3-hexenyl acetate the potential end product of the transformation sequence, (*E*)-2-hexenal, had only a weak effect on the nectar flow. Similarly, the observation that (*Z*)-3-hexenyl acetate and (*E*)-3-hexenyl acetate elicited almost identical EFN secretion rates contradicts the involvement of classical receptor proteins, since these usually require a specific stereochemistry of the interacting molecule. We further calculated LogP values to check for putative importance of the compound's lipophilicity but did not find any clear relation between the EFN-inducing activity of a compound and its octanol-water partition coefficient (Fig. 1).

More studies are required to understand which VOCs induce plant defenses via which mechanism. However, our results point to a new mechanism. Changes in transmembrane potentials—occurring through modulations of ion fluxes—are involved in early signaling events in the cellular response to stress (Maffei et al. 2007), and exposition to VOCs indeed changes membrane potentials in intact lima bean leaves (M. Maffei, personal communication). It is thus tempting to speculate that the dissolving of VOCs in the membranes coupled to interactions with membrane proteins, similar to the odorant binding proteins of insects (Campanacci et al. 2001), leads to changes in transmembrane potentials and thereby induces gene activity. This hypothesis gains support from the observation that the induction of EFN secretion by VOCs appears to be a gradual one rather than a clear 'yes-or-know' response. Several of the compounds that are released naturally from lima bean showed a trend towards an increase in EFN secretion, although the difference was not significant: EFN secretion in response to Linalool was on average higher by 40% than in control plants, and DMNT ((3*E*)-4,8-dimethylnona-1,3,7-triene) increased EFN secretion by 50% (see Table 1 in Kost and Heil 2006). Similarly, plants exposed to ethylhexanol or (*E*)-2-hexanal in the present study showed a trend towards higher EFN secretions than controls (Fig. 1). EFN responds to a comparably wide variety of structures, and slight changes in the molecular structure gradually alter the induction effect. This observation is best explained by a comparably simple physicochemical process, whose detailed nature remains to be elucidated.

How can signals evolve that apparently lack chemical specificity? The answer might be simply that plants seldom are exposed to GLVs or chemically related compounds that are not released from attacked plants. In evolutionary terms, the probability of this situation was probably too low to cause any selection towards a more specific signal perception.

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References

ALMERAS, E., STOLZ, S., VOLLENWEIDER, S., REYMOND, P., MENE-SAFFRANE, L., and FARMER, E. E. 2003. Reactive electrophile species activate defense gene expression in Arabidopsis. *Plant J* 34:202–216.

ARIMURA, G.-I., OZAWA, R., SHIMODA, T., NISHIOKA, T., BOLAND, W., and TAKABAYASHI, J. 2000. Herbivory-induced volatiles elicit defence genes in lima bean leaves. *Nature* 406:512–515.

BALDWIN, I. T., HALITSCHKE, R., PASCHOLD, A., VON DAHL, C. C., and PRESTON, C. A. 2006. Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. *Science* 311:812–815.

CAMPANACCI, V., KRIEGER, J., BETTE, S., STURGIS, J. N., LARTIGUE, A., CAMBILLAU, C., BREER, H., and TEGONI, M. 2001. Revisiting the specificity of Mamestra brassicae and Antheraea polyphemus pheromone-binding proteins with a fluorescence binding assay. *J. Biol. Chem.* 276:20078–20084.

ENGELBERTH, J., ALBORN, H. T., SCHMELZ, E. A., and TUMLINSON, J. H. 2004. Airborne signals prime plants against insect herbivore attack. *Proc. Natl. Acad. Sci. USA* 101:1781–1785.

FARAG, M. A., FOKAR, M., ZHANG, H. A., ALLEN, R. D., and PARÉ, P. W. 2005. (Z)-3-Hexenol induces defense genes and downstream metabolites in maize. *Planta* 220:900–909.

HEIL, M. 2008. Indirect defence via tritrophic interactions. *New Phytol* 178:41–61.

HEIL, M., and SILVA BUENO, J. C. 2007. Within-plant signaling by volatiles leads to induction and priming of an indirect plant defense in nature. *Proc. Natl. Acad. Sci. USA* 104:5467–5472.

KOST, C., and HEIL, M. 2006. Herbivore-induced plant volatiles induce an indirect defence in neighbouring plants. *J. Ecol.* 94:619–628.

MAFFEI, M. E., MITHÖFER, A., and BOLAND, W. 2007. Before gene expression: early events in plant-insect interaction. *Trends Plant Sci.* 12:310–316.

RUTHER, J., and KLEIER, S. 2005. Plant-plant signaling: Ethylene synergizes volatile emission in Zea mays induced by exposure to (Z)-3-Hexen-1-ol. *J. Chem. Ecol.* 31:2217–2222.

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Olfactory Responses of the Predatory Mites (*Neoseiulus cucumeris*) and Insects (*Orius strigicollis*) to Two Different Plant Species Infested with Onion Thrips (*Thrips tabaci)*

Satoshi Tatemoto · Takeshi Shimoda

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Abstract Responses of *Neoseiulus cucumeris* (a predatory mite) and the predatory insect *Orius strigicollis* to volatiles associated with two different plant species infested with onion thrips, *Thrips tabaci*, were examined in a Y-tube olfactometer. Both predators species showed a significant preference for volatiles from infested cucumber leaves without *T. tabaci* over clean air. However, they were not attracted to volatiles from uninfested cucumber leaves, artificially damaged cucumber leaves, or volatiles from *T. tabaci* plus their visible products collected from cucumber leaves. These results suggest that both predator species are capable of exploiting herbivore-induced volatiles from *T. tabaci*-infested cucumber leaves as a foraging cue. Neither predator was attracted to volatiles from uninfested spring onion leaves, infested spring onion leaves without *T. tabaci*, or volatiles from *T. tabaci* plus their visible products collected from spring onion leaves. Interestingly, they avoided volatiles from artificially damaged spring onion leaves. A possible explanation for the non-significant olfactory responses of the predator species to spring onion plants with infestation damage of *T. tabaci* is discussed.

Keywords Spring onion · Cucumber · *Thrips tabaci* · *Neoseiulus cucumeris* · *Orius strigicollis* · Herbivore-induced volatiles · Attraction · Avoidance

S. Tatemoto
Hiroshima Prefecture Agriculture Research Center,
Hiroshima 739-0151, Japan

T. Shimoda (✉)
Insect Pest Management Research Team,
National Agricultural Research Center,
Kannon-dai 3-1-1,
Tsukuba, Ibaraki Prefecture 305-8666, Japan
e-mail: oligota@affrc.go.jp

Present address:
S. Tatemoto
Fruit Tree Research Division,
Agricultural Technology Research Center,
Hiroshima Prefectural Technology Research Institute,
Akitsu, Higashihiroshima,
Hiroshima 729-2402, Japan

Introduction

Attraction of carnivorous arthropods to herbivore-induced volatiles from favored combinations of plants and prey herbivores has been well documented in many tritrophic interactions (Takabayashi and Dicke 1996; Dicke et al. 1998; Dicke 1999a, b; Shiojiri et al. 2002). Such phenomena have received attention from the viewpoints of carnivore foraging or plant indirect defense against herbivorous arthropods. However, the olfactory responses of carnivorous arthropods to less favored plant–prey combinations have been less well documented (Takabayashi et al. 2000). What carnivore response is elicited by herbivore-induced volatiles from less favored plant species with infestation damage by a favored herbivore species remains an intriguing question. The answer should give us a better understanding of carnivore foraging decisions in response to herbivore-induced volatiles when the target prey is a polyphagous insect that can exploit a wide range of plant species.

Onion thrips (*Thrips tabaci)* are highly polyphagous and a major pest of vegetable crops (Kendall and Bjostad 1987; Edelson et al. 1989; Doederlein and Sites 1993; Shelton et al. 1998), fruits (Tsuchiya 2001, 2002), and flowers (Childers 1997) in many parts of the world. This pest is characterized as the only vector of the tospovirus, Iris Yellow Spot Virus, which causes symptoms that include straw-colored spindle-shaped lesions on host plant leaves

(Kritzman et al. 2001; Doi et al. 2003). Of the various biological control agents potentially applicable to *T. tabaci*, the generalist predatory mite *Neoseiulus cucumeris* is widely available in many countries from commercial sources (Hoy and Glenister 1991; Brødsgaard and Hansen 1992; Williams 2001; Shipp and Wang 2003). The generalist predatory bug *Orius strigicollis* is also an important natural enemy in Japan (Yasunaga 1997; Hinomoto et al. 2004) and in Taiwan (Wang et al. 2001).

The efficacy of biological control of *T. tabaci* by employing these generalist predators is influenced by plant species. For example, *N. cucumeris* is a good candidate for biological control of cucumber pests (Gillespie 1989; Brødsgaard and Hansen 1992). This also seems to be true for *O. strigicollis* since the species is commonly found on crops belonging to the Cucurbitaceae, as well as on members of the Leguminosae and Solanaceae (Yasunaga 1997; Wang et al. 2001). In contrast, attempts to use biological controls for spring onion and other *Allium* plants that use either of these predator species have largely failed (Tatemoto, unpublished data; Okazaki, personal communication). Thus, a *T. tabaci*-infested cucumber plant is a plant–prey combination favored by *N. cucumeris* and *O. strigicollis*, whereas a *T. tabaci*-infested spring onion plant is a less favored combination for predators. This relationship might be associated with the increased production of sulfur compounds by spring onion in response to physical damage, including herbivory, since these compounds have possible toxic and/or repellent effects on many insects (Dugravot et al. 2004, 2005).

The main purpose of this research was to study whether each of the two predator species shows different olfactory responses to two different plant species, i.e., cucumber and spring onion plants, infested with *T. tabaci*. Although each predator was attracted to volatiles from *T. tabaci*-infested cucumber plants, they were not attracted to volatiles from spring onion plants infested with *T. tabaci*. We give a possible explanation for the different olfactory responses to different plant–prey combinations.

Materials and Methods

Plants Cucumber (*Cucumis sativus* cv. Sharp 1) and spring onion (*Allium fistulosum* cv. Koutou) were cultivated individually in plastic pots (12-cm diam., 10-cm deep) in a climate-controlled plant growth chamber (25±2 °C and L16/D8 at approx 8,000 lux). First to third fully open leaves of 24- to 37-day-old cucumber plants were used for the experiments. Additionally, 29- to 41-day-old spring onion plants with the roots attached were used for the experiments.

Mites and insects T. tabaci were collected from spring onion plants at Higashi-Hiroshima, Hiroshima Prefecture, Japan in 2004. They had been reared on spring onion plants in a climate-controlled chamber (25±2°C and L16/D8). *N. cucumeris* were obtained from a commercial source (Ishihara Sangyo Corp., Osaka, Japan) in 2004. Insects were reared on the stored food mites *Tyrophagus putrescentiae* in wheat bran in a climate-controlled room (25±2°C and L16/D8) for more than 1 year. To obtain starved predators, adult females of *N. cucumeris* were introduced into a sealed glass tube (38 ml), containing a piece of moist filter paper and maintained at 25±2°C and L16/D8 for 20–24 h prior to the experiments. *O. strigicollis* was obtained from a commercial source (Arysta Life Science Corp., Tokyo, Japan) in 2005 and 2006. Insects were reared in a climate-controlled room (25±2°C and L16/D8) for 2–7 days on *Ephestia kuehniella* eggs and *Kalanchoe blossfeldiana* leaves as food and oviposition materials, respectively. Other conditions and procedures for obtaining starved predators were the same as those for *N. cucumeris*. Neither predator species had experienced the volatiles from cucumber, spring onion, or those from *T. tabaci* during mass rearing to assess their innate olfactory responses to the volatiles.

Olfactometer experiments All experiments were conducted in Y-tube olfactometers (Takabayashi and Dicke 1992) with 10-cm arm length, 12-cm stem length, and 4-cm i.d. The air was first cleaned by passing it through granular activated charcoal (1 l) in a glass bottle before sending it to the sample and control odor source bottles (1 l). Air (ca. 4 l/min) containing each odor was sent to either of the arms of the olfactometer. At the commencement of each bioassay, a predator was placed on the start point of a steel Y-shaped wire that was fixed at the center of the Y-tube. We regarded the observation as complete when the predator reached one end of the wire arm. Predators that did not pass the finish line of either arm within 5 min (termed "no choice" subjects) were excluded from the statistical analysis detailed below. Following every five bioassays, the arm containing the sample odor source was switched with that containing the control odor source. In each experiment, 60–70 predators were tested for more than 3 days.

Responses of predators to volatiles associated with cucumber plants We investigated the behavioral responses of *N. cucumeris* and *O. strigicollis* to the four different types of sample odor sources associated with cucumber plants discussed below.

Experiment 1 Uninfested cucumber leaves vs. clean air. Detached cucumber leaves (three to five) with the petioles attached (ca. 4 g in total) were placed onto a moist filter paper (Advantec No. 2, 9-cm diam.) within a plastic container (15×10 cm, 5-cm deep).

Leaves were kept for 20–24 h in a laboratory (25±2°C and L16/D8). Both leaves and the moist filter paper were used as the odor source. A moist filter paper was prepared as the control odor source.

Experiment 2 Infested cucumber leaves without *T. tabaci* vs. clean air. Detached cucumber leaves (three to five) with petioles attached (ca. 4 g in total) were infested with approximately 100 *T. tabaci* individuals (ca. 20–34 individuals per leaf) at the adult and larval stages (approx. 50 of these were second-instar larvae) for 20–24 h. *T. tabaci* and their visible products were removed from the infested leaves with a fine paintbrush just before the bioassays. Leaves were washed with dH$_2$O and were carefully dried with paper wipes (Kimwipe S-200). These infested leaves plus a moist filter paper were used as the sample odor source. A moist filter paper was prepared as the control odor source. Other conditions and procedures were the same as in Experiment 1.

Experiment 3 *T. tabaci* and their products from cucumber leaves vs. clean air. About 100 living *T. tabaci* individuals at the adult and larval stages (approx. 50 of these were second-instars) and their visible products were carefully removed with a fine paintbrush from *T. tabaci*-infested cucumber leaves just before the bioassays. The thrips and their products collected on a moist filter paper were used as the sample odor source. A moist filter paper was prepared as the control. Other conditions and procedures were the same as in Experiment 1.

Experiment 4 Artificially damaged cucumber leaves vs. clean air. Just before the bioassays, the detached cucumber leaves (three to five) with the petioles attached (ca. 4 g in total) were rubbed with fine sandpaper (No. AA240, Sankyo Rikagaku Corp., Saitama, Japan). The mechanically damaged leaves plus a moist filter paper were used as the sample odor source. The moistened filter paper was prepared as the control odor source. Other conditions and procedures were the same as in Experiment 1.

Responses of predators to volatiles associated with spring onion The behavioral responses of *N. cucumeris* and *O. strigicollis* to the four different types of sample odor sources associated with spring onion plants were investigated. In our preliminarily investigation, a strong sulfurous smell was noticed within several hours of detaching the roots from spring onion plants, which seemed to generate undesirable data in the experiments detailed below. Thus, to minimize physical damage, we used whole spring onion plants with the roots attached.

Experiment 5 Uninfested spring onion plants vs. clean air. Eight to 14 whole spring onion plants (approx. 15 cm in height, ca. 4 g in total), with the roots attached, were maintained for 20–24 h before the experiments. The plants plus a moist filter paper were used as the odor source. A moist filter paper was prepared as the control. Other conditions and procedures were the same as in Experiment 1.

Experiment 6 Infested spring onion plants without *T. tabaci* vs. clean air. Eight to 14 whole spring onion plants with the roots attached (ca. 4 g in total) were infested with a total of approximately 100 *T. tabaci* individuals (seven to 13 individuals per plant) in the adult and larval stages (approx. 50 of these were second-instars) for 20–24 h. *T.* tabaci and their visible products were removed from the infested leaves with a fine paintbrush just before bioassay. Infested plants and a moist filter paper were used as the sample odor source. Moistened filter paper was prepared as the control odor source. Other conditions and procedures were the same as in Experiments 2 and 5.

Experiment 7 *T. tabaci* and their products from spring onion plants vs. clean air. Immediately preceding bioassay, approximately 100 *T. tabaci* consisting of about a 50:50 mixture of adult and larval stages and their products) were removed from infested spring onion plants with a fine paint brush. The thrips and their visible products collected on a moist filter paper were used as the sample odor source. A moistened filter paper was prepared as the control odor source. Other conditions and procedures were the same as in Experiments 3 and 5.

Experiment 8 Artificially damaged spring onion plants vs. clean air. Eight to 14 whole spring onion plants except for the attached roots (ca. 4 g in total) were rubbed with fine sandpaper just before the bioassays. These mechanically damaged plants plus a moist filter paper were used as the sample odor source.

A moistened filter paper was prepared as the control. Other conditions and procedures were the same as in Experiments 4 and 5.

Chemical analysis of volatiles Each sample odor source used in Experiments 1–8 were placed into lidded glass bottles (2 l). Before collection of volatiles, a steady flow (200 ml/min) of clean air, purified by passing it through silica gel, molecular sieves, and activated charcoal (1 l each), was circulated through each glass bottle for 10 min. Headspace volatiles from each odor source were collected by using a Tenax TA Thermal Desorption Tube (180 mg, 60/80 mesh; Gerstel), connecting each lidded glass bottle (30 min) by sending clean air (100 ml/min checked by using a flow meter). Collected volatiles in each sample were analyzed by using a gas chromatography–mass spectrometry (GC–MS) system (GC: Agilent Technologies 6890 with an HP-5MS capillary column: 30-m long, 0.25-mm i.d., and 0.25-μm-file thick; injection temperature: 250°C; MS: Agilent Technologies 5973N mass selective detector, 70 eV) with Thermodesorption Autosampler System and Cold Injection System (TDS A 2 and CIS 4: Gerstel). The oven temperature of the GC–MS was programmed to rise from 40°C (9-min hold) to 280°C at 10°C/min. The TDS A 2 and CIS 4 temperatures were programmed to rise from 20°C (1-min hold) to 280°C at 60°C/min and from −150°C (0.5-min hold) to 300°C at 12°C/min, respectively. The observed compounds were tentatively identified by comparing their mass spectra and retention times with those of mass spectra in the database (Wiley Library) along with our authentic data regarding volatile compounds from herbivore-infested plants or physically damaged plants in our laboratory (e.g., Ozawa et al. 2000; Shimoda et al. 2005). However, (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, (*E*)-2-hexenal, linalool, methyl salicylate, methyl propyl disulfide and dipropyl disulfide were double-checked and positively identified by using synthetic samples of purity ranging from 97% to 99% (Wako Pure Chemical Industries).

Statistical analysis The results from each olfactometer experiment were subjected to a χ^2 test (Sokal and Rohlf 1995). The null hypothesis was that predators exhibited a 50:50 distribution over the two odor sources. The Kruskal–Wallis test followed by the Mann–Whitney *U* test, weighted by the Bonferroni correction, were used to compare the results of the chemical analysis. Each analysis was performed with JMP (version 5.0.1J for Windows, SAS Institute Inc., Cary, NC, USA) or StatView (version 5.0J for Windows, SAS Institute Inc., Cary, NC, USA).

Results

Olfactometer experiments When presented with a choice between uninfested cucumber leaves and clean air, neither predator species discriminated between the odor sources (Experiment 1; *N. cucumeris*: χ^2=0.021, *df*=1 and *P*=0.884;

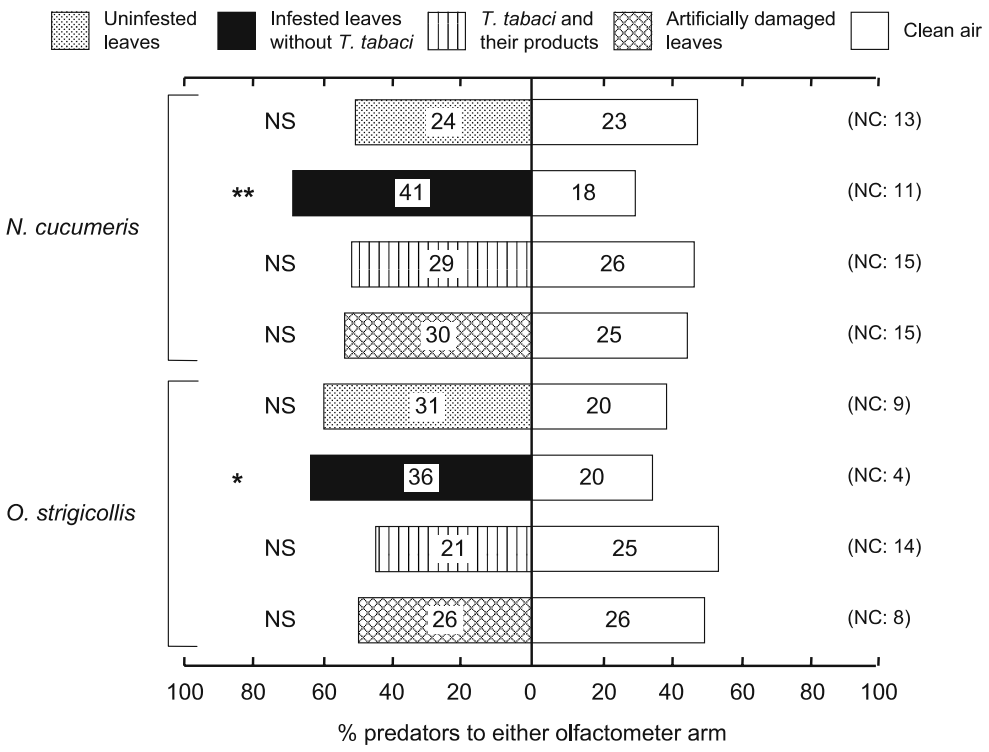

Fig. 1 Responses of *N. cucumeris* and *O. strigicollis* when offered (Experiment 1) uninfested cucumber leaves vs. clean air; (Experiment 2) infested cucumber leaves without *T. tabaci* vs. clean air, (Experiment 3) *T. tabaci* and their products from cucumber leaves vs. clean air, and (Experiment 4) artificially damaged cucumber leaves vs. clean air. The χ^2 test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (*single asterisk P*< 0.05, *double asterisk P*<0.01, *NS P*>0.05). Predators that did not reach the end of either olfactometer arm within 5 min (no choice: *NC*) were excluded from the statistical analysis. For further explanation, see the text

O. strigicollis: χ^2=2.373, *df*=1 and *P*=0.123; Fig. 1). The predators showed no significant preference for volatiles from *T. tabaci* and their products from cucumber leaves (Experiment 3; *N. cucumeris*: χ^2=0.164, *df*=1 and *P*=0.686; *O. strigicollis*: χ^2=0.348, *df*=1 and *P*=0.555) or volatiles from artificially damaged cucumber leaves (Experiment 4; *N. cucumeris*: χ^2=0.455, *df*=1 and *P*=0.5; *O. strigicollis*: χ^2=0, *df*=1 and *P*=1) over clean air. In contrast, each predator species showed an olfactory preference for infested cucumber leaves without *T. tabaci* (Experiment 2; *N. cucumeris*: χ^2=8.966, *df*=1 and *P*<0.01; *O. strigicollis*: χ^2=4.571, *df*=1 and *P*<0.05).

When presented with a choice between uninfested spring onion plants and clean air, neither predator species discriminated between these odor sources (Experiment 5; *N. cucumeris*: χ^2=0.021, *df*=1 and *P*=0.884; *O. strigicollis*: χ^2=0.077, *df*=1 and *P*=0.782; Fig. 2). The same tendency was observed in each predator species when *T. tabaci* plus visible products vs. clean air was compared (Experiment 7; *N. cucumeris*: χ^2=0.424, *df*=1 and *P*=0.515; *O. strigicollis*: χ^2=0.296, *df*=1 and *P*=0.586). Neither of the two predator species showed a significant preference for volatiles from infested spring onion plants without *T. tabaci* (Experiment 6; *N. cucumeris*: χ^2=0.276, *df*=1 and *P*=0.599; *O. strigicollis*: χ^2=0.296, *df*=1 and *P*=0.586). When artificially damaged spring onion plants were offered vs. clean air, more predators chose the latter odor source (Experiment 8; *N. cucumeris*: χ^2=16.490, *df*=1 and *P*<0.001; *O. strigicollis*: χ^2=8.963, *df*=1 and *P*<0.01).

Chemical analysis of volatiles Uninfested cucumber leaves emitted trace amounts of two green leaf volatiles (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate (Table 1). Artificially damaged cucumber leaves emitted a small amount of (*E*)-2-hexenal in addition to these two compounds. Infested cucumber leaves without *T. tabaci* emitted small amounts of (*E*)-β-ocimene, (*E*)-4,8-dimethyl-1,3,7-nonatriene, linalool, and methyl salicylate in addition to the three green leaf volatiles. There was a significant difference among the three odor sources as to the amounts of (*Z*)-3-hexen-1-ol and (*Z*)-3-hexenyl acetate.

Uninfested spring onion plants emitted small amounts of methyl propyl disulfide, dipropyl disulfide, and two unidentified volatile sulfur compounds (Table 2). Trace amounts of (*E*)-β-ocimene and linalool also were found in the headspace of the odor source due to slight infestation damage by *T. tabaci* accidentally occurring in the plant growth chamber before collection of volatiles. Artificially damaged spring onion plants also emitted these sulfur volatile compounds and herbivore-induced volatile compounds in addition to an unidentified sulfur compound. Infested spring onion plants without *T. tabaci* emitted a small amount of methyl salicylate in addition to the seven volatile compounds found in the headspace of artificially damaged spring onion plants. Artificially damaged spring onion plants emitted a significantly greater amount of dipropyl disulfide than the uninfested spring onion plants. Infested spring onion plants without *T. tabaci* were intermediate between these odor sources in the amount of this compound.

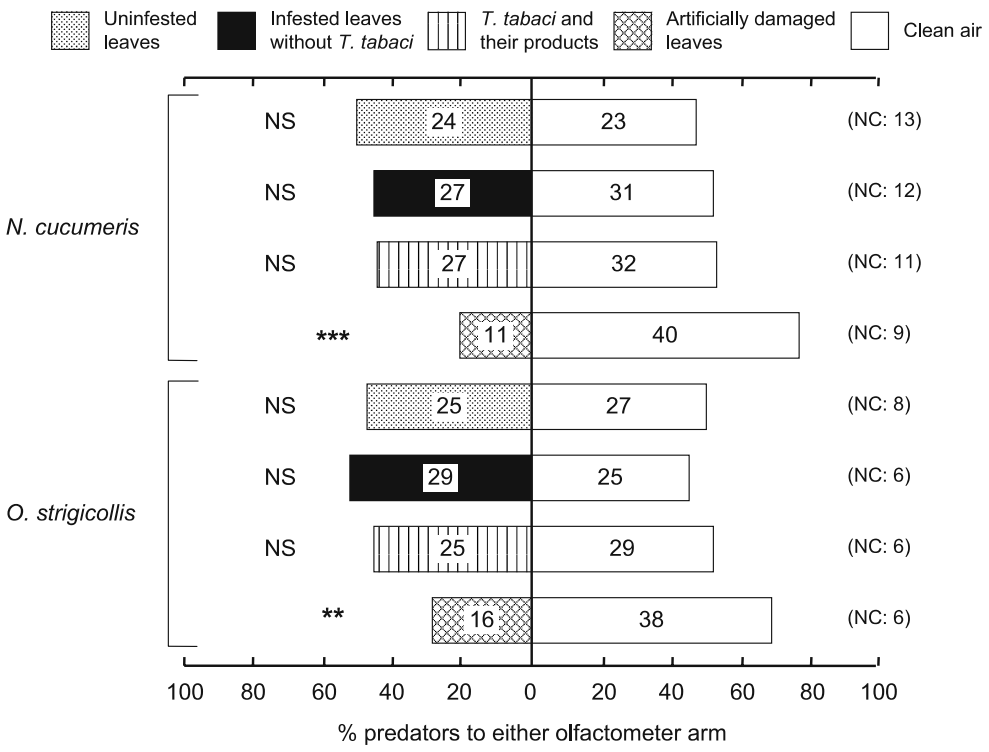

Fig. 2 Responses of *N. cucumeris* and *O. strigicollis* when offered (Experiment 5) uninfested spring onion plants vs. clean air, (Experiment 6) infested spring onion plants without *T. tabaci* vs. clean air, (Experiment 7) *T. tabaci* and their products from spring onion plants vs. clean air, and (Experiment 8) artificially damaged spring onion plants vs. clean air. The χ^2 test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (*double asterisk P*<0.01, *triple asterisk P*<0.001, *NS*:*P*>0.05). Predators that did not reach the end of either olfactometer arm within 5 min (no choice: *NC*) were excluded from the statistical analysis. For further explanation, see the text

Table 1 Mean ion intensities (±SE×10^8) of volatile compounds detected in the headspace of cucumber leaves subjected to different treatments

Compound	Uninfested (N=5)	Damaged (N=5)	Infested (N=5)	Statistics (Kruskal–Wallis test or U test)
(*E*)-2-hexenal	ND	0.07±0.03[a]	0.06±0.04[a]	z=−0.111, df=1, P=0.911
(*Z*)-3-hexen-1-ol	0.43±0.39[a]	15.02±6.97[b]	1.65±0.56[a]	H=11.260, df=2, P<0.01
(*Z*)-3-hexenyl acetate	0.10±0.07[a]	5.54±0.18[b]	0.07±0.07[a]	H=10.526, df=2, P<0.01
(*E*)-β-ocimene	ND	ND	0.18±0.02	
(*E*)-4,8-dimethyl-1,3,7-nonatriene	ND	ND	0.07±0.03	
Linalool	ND	ND	0.10±0.07	
Methyl salicylate	ND	ND	0.25±0.08	

Values with a different letter following the same compound are statistically significant (Kruskal–Wallis test followed by U test, weighted by the Bonferroni approach, P<0.01).
Uninfested Uninfested cucumber leaves, *Damaged* artificially damaged leaves, *Infested* infested cucumber leaves without *Thrips tabaci* or their products, *ND* not detected in the chemical analysis (excluded from statistical analysis)

Compared to infested cucumber leaves without *T. tabaci*, infested spring onion plants without *T. tabaci* emitted similar amounts of herbivore-induced volatile compounds (U test, linalool: z=−0.74, df=1 and P=0.459; methyl salicylate: z=−0.313, df=1 and P=0.754; (*E*)-β-ocimene: z=−0.731, df=1 and P=0.465); although the latter odor source did not produce (*E*)-4,8-dimethyl-1,3,7-nonatriene (see Tables 1 and 2).

Discussion

N. cucumeris and *O. strigicollis* showed similar responses to volatiles associated with cucumber plants. Both predators were attracted to volatiles from infested cucumber leaves without *T. tabaci*, although they had not previously experienced the volatiles. Neither insect was attracted to the volatiles from uninfested cucumber leaves, artificially damaged cucumber leaves, or *T. tabaci* plus their visible products collected from infested cucumber leaves. These results suggest that *N. cucumeris* and *O. strigicollis* may have the ability to exploit herbivore-induced volatiles from *T. tabaci*-infested cucumber leaves as a foraging cue. Olfactory attraction evoked by volatile infochemicals from herbivore-infested plants has been reported in the tritrophic interaction among *N. cucumeris*, the western flower thrip *Frankliniella occidentalis*, and cucumber plants (Janssen et al. 1998), and among *N. cucumeris*, the rust mite *Aceria tulipae*, and tulip bulbs (Aratchige et al. 2004). There are no reports of studies of the olfactory response of *O. strigicollis* to herbivore-infested plants. However, olfactory attraction to herbivore-infested plants has been reported in closely related predatory bug species such as *Orius laevigatus* (Venzon et al. 1999; Bennison et al. 2002), *Orius tristicolor* (James and Price 2004), *Anthocoris nemoralis* (Scutareane et al. 1996), and *Anthocoris nemorum* (Drukker et al. 1995).

There were only four herbivore-induced volatile compounds detected in the volatile blend of infested cucumber leaves without *T. tabaci* among the odor sources used in Experiments 1–4 (linalool, methyl salicylate, (*E*)-β-ocimene, and (*E*)-4,8-dimethyl-1,3,7-nonatriene). At least one of these volatiles probably was involved in attracting each predator

Table 2 Mean ion intensities (±SE×10^8) of volatile compounds detected in the headspace of spring onion plants subjected to different treatments

Compound	Uninfested (N=5)	Damaged (N=5)	Infested (N=5)	Statistics (Kruskal–Wallis test or U test)
(*E*)-β-ocimene	0.03±0.03[a]	0.07±0.05[a]	0.29±0.2[a]	H=2.759, df=2, P=0.252
Linalool	0.04±0.04[a]	0.16±0.1[a]	0.09±0.03[a]	H=1.689, df=2, P=0.43
Methyl salicylate	ND	ND	0.29±0.12	
Methyl propyl disulfide	0.11±0.03[a]	0.18±0.07[a]	0.15±0.09[a]	H=0.423, df=2, P=0.809
Dipropyl disulfide	0.57±0.06[a]	6.88±2.57[b]	2.07±1.02[ab]	H=7.02, df=2, P<0.05
Unidentified 1	0.03±0.02[a]	0.04±0.03[a]	0.02±0.01[a]	H=0.229, df=2, P=0.892
Unidentified 2	ND	0.03±0.02[a]	0.04±0.02[a]	z=−0.647, df=1, P=0.518
Unidentified 3	0.25±0.15[a]	0.91±0.35[a]	0.47±0.2[a]	H=3.07, df=2, P=0.215

Values with a different letter following the same compound are statistically significant (Kruskal–Wallis test followed by U test, weighted by the Bonferroni approach, P<0.01).
Uninfested Uninfested spring onion plants, *Damaged* artificially damaged spring onion plants, *Infested* infested spring onion plants without *Thrips tabaci* or their products, *Unidentified 1*, *2*, and *3* unidentified sulfur components (retention time: 9.09, 12.61, and 16.68 min respectively), *ND* not detected in the chemical analysis (excluded from statistical analysis)

species to infested cucumber leaves without *T. tabaci*. Each of these four compounds contributes to attraction of the predatory mite *Phytoseiulus persimilis* to lima bean plants infested with the two-spotted spider mite *Tetranychus urticae* (Dicke et al. 1990a; de Boer et al. 2004). Linalool and methyl salicylate are also involved in attracting the predatory mite *Neoseiulus californicus* to *T. urticae*-infested lima bean plants (Shimoda et al. 2005). Further, methyl salicylate plays a key role in attracting the anthocorid predatory bug *A. nemoralis* to psyllid-infested pear leaves (Drukker et al. 2000b). In contrast, behavioral and chemical analyses suggest that (*Z*)-3-hexen-1-ol, (*Z*)-3-hexenyl acetate, and (*E*)-2-hexenal (green leaf volatiles) emitted from infested cucumber leaves without *T. tabaci* do not play a role in the attraction of predator species to the odor source.

In *Allium* species, physical damage, including herbivory, leads to increased release of volatile sulfur compounds that can act as repellents against many insects (Auger et al. 1989; Dugravot and Thibout 2006). In the current study, volatiles from artificially damaged spring onion plants were avoided by both predator species, while those from uninfested spring onion plants did not affect their behavior. A significantly greater amount of dipropyl disulfide was a principal sulfur component in the headspace of the former odor source but less significant in the latter odor source. Thus, we consider dipropyl disulfide is a prime candidate for the predator repellent. There were at least four other sulfur compounds in the headspace of artificially damaged spring onion plants. Further studies are needed to identify the key compound(s).

Neither predator species responded positively to infested spring onion plants without *T. tabaci*. One likely explanation is that the volatiles might include both predator attractants and repellents. (*E*)-β-ocimene, linalool, and methyl salicylate were emitted from the odor source; similar amounts of the same compounds also were produced by infested cucumber plants without *T. tabaci*. Methyl salicylate was not emitted from either uninfested or artificially damaged spring onion plants. These results suggest that at least one of the three herbivore-induced volatile compounds, especially methyl salicylate, from infested spring onion plants without *T. tabaci* might be attractive to the predator species. However, this attraction might be offset by the function of dipropyl disulfide as a predator repellent because similar amounts of this compound were emitted from infested spring onion plants without *T. tabaci*, as well as from artificially damaged spring onion plants (Table 2). The attraction of carnivorous arthropods to herbivore-induced volatiles can be interfered with other volatile infochemicals from plants, herbivores, and/or their products. For example, volatiles from rosemary leaves can inhibit attraction of *O. laevigatus* to herbivore-induced volatiles from chrysanthemum buds infested with *F. occidentalis* (Bennison et al. 2002). Whether or not the predatory mite *P. persimilis* prefers or avoids volatiles from lima bean leaves infested with *Spodoptera exigua* caterpillars can be affected by the relative contribution of herbivore-induced volatiles (attractive volatiles) and fecal volatiles (repellent volatiles) in the total blend from the odor source (Shimoda and Dicke 1999).

The success of biological control against *T. tabaci* indicates that *T. tabaci*-infested cucumber is a favored plant–prey combination for *N. cucumeris* and *O. strigicollis* (Gillespie 1989; Brødsgaard and Hansen 1992). In contrast, the repeated failure of the biological control suggests that *T. tabaci*-infested spring onion may be less favored by the predators (Tatemoto unpublished data; Okazaki, personal communication). Although these two odor sources were not simultaneously compared in this study, we can infer that each generalist predator has the potential to discriminate, by olfactory means, a favored plant–prey combination from a less favored one. No positive response to spring onion plants with infestation damage by *T. tabaci* would appear to be reasonable for their successful foraging because their survival, development, and/or oviposition are presumably affected by sulfur compounds from the less favored plants, as reported in other insects adapted to *Allium* species (Venugopal and Narayanan 1981; Auger et al. 1989; Dugravot et al. 2004; Dugravot and Thibout 2006). Although the olfactory responses of several predatory mites or parasitoid wasps can be affected by their previous experience of herbivore-infested plants (Dicke et al. 1990b; Turlings et al. 1993; Vet et al. 1993; Drukker et al. 2000a; De Boer et al. 2005; Maeda et al. 2006), it is debatable whether unfavorable experience with *T. tabaci*-infested spring onion increases the olfactory response of each investigated predator species to the odor source. Further study is needed to answer the question, as well as to show their different performances on the two plant–prey combinations investigated in this study.

Our data associated with cucumber plants support previous studies that show that herbivore-induced volatiles play a role in indirect plant defenses against herbivorous arthropods, i.e., recruiting carnivorous natural enemies as bodyguards (Karban and Baldwin 1997; Dicke et al. 2003; Choh et al. 2004), as well as in predators' foraging decisions. However, this indirect defense via herbivore-induced volatiles was not seemingly observed in spring onion plants, which might be associated with their direct defense. Sulfur compounds produced by spring onions, as well as other *Allium* plants, may prevent herbivory by many generalist herbivore insects (Auger et al. 1989; Hori and Harada 1995; Dugravot and Thibout 2006). Because *T. tabaci* can easily exploit spring onions, it would appear to be important for the plant to provide an efficient indirect defense to cope with herbivores. However, our results

suggest that defensive sulfur volatiles in spring onion may inhibit the activities of generalist carnivore insects as biological agents against *T. tabaci*. This opens the interesting question of whether there is an efficient indirect defense (e.g., by the aid of specialized predators or parasitoids) against *T. tabaci* in spring onion plants. This remains to be investigated.

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References

ARATCHIGE, N. S., LESNA, I., and SABELIS, M. W. 2004. Belowground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. *Exp. Appl. Acarol.* 33:21–30.

AUGER, J., DUGRAVOT, S., and THIBOUT, E. 1989. Leek odor analysis by gas chromatography and identification of the most active substance for the leek moth, *Acrolepiopsis assectella*. *J. Chem. Ecol.* 15:1847–1854.

BENNISON, J., MAULDEN, K., DEWHIRST, S., POW, E., SLATTER, P., and WADHAMS, L. 2002. Towards the development of a push–pull strategy for improving biological control of western flower thrips on chrysanthemum, pp. 199–206, in R. Marullo and L. Mound (eds.). Thrips and Tospoviruses. Proceedings of the 7th International Symposium on Thysanoptera, Reggio Calabria, Italy.

BRØDSGAARD, H. F., and HANSEN, L. S. 1992. Effect of *Amblyseius cucumeris* and *Amblyseius barkeri* as biological control agents of *Thrips tabaci* on glasshouse cucumbers. *Biocontrol Sci. Technol.* 2:215–223.

CHILDERS, C. C. 1997. Feeding and oviposition injuries to plants, pp. 505–537, in T. Lewis (ed.). Thrips as Crop Pests. CAB International, New York.

CHOH, Y., SHIMODA, T., OZAWA., R., DICKE, M., and TAKABAYASHI, J. 2004. Exposure of lima bean leaves to volatiles from herbivore-infested conspecific plants results in emission of carnivore attractants: active of passive process. *J. Chem. Ecol.* 30:1305–1317.

DE BOER, J. G., POSTHUMUS, M. A., and DICKE, M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or non–prey herbivores by a predatory mite. *J. Chem. Ecol.* 30:2215–2230.

DE BOER, J. G., SNOEREN, T. A., and DICKE, M. 2005. Predatory mites learn to discriminate between plant volatiles induced by prey and nonprey herbivores. *Anim. Behav.* 69:869–879.

DICKE, M. 1999a. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol. Exp. Appl.* 91:131–142.

DICKE, M. 1999b. Evolution of induced indirect defense of plants, pp. 63–88, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible Defense, Princeton University Press, Princeton.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DON, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990a. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions: Involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DICKE, M., VAN DER MAAS, K. J., TAKABAYASHI, J., and VET, L. E. M. 1990b. Learning affects response of volatile allelochemicals by predatory mites. *Proc. Exp. Appl. Entomol.* 1:31–36.

DICKE, M., TAKABAYASHI, J., POSTHUMUS, M. A., SCHÜTTE, C., and OLGA, E. K. 1998. Plant-phytoseiid interactions mediated by herbivore-induced plant volatiles: Variation in production of cues and in responses of predatory mites. *Exp. Appl. Acarol.* 22: 311–333.

DICKE, M., AGRAWAL, A. A., and BRUIN, J. 2003. Plant talk, but are they deaf. *Trends Pl. Sci.* 8:403–405.

DOEDERLEIN, T. A., and SITES, R. W. 1993. Host plant preferences of *Frankliniella occidentalis* and *Thrips tabaci* (Thysanoptera: Thripidae) for onions and associated weeds on the Souther High Plains. *J. Econ. Entomol.* 86:1706–1713.

DOI, M., ZEN, S., OKUDA, M., NAKAMURA, H., KATO, K., and HANADA, K. 2003. Leaf necrosis disease of lisianthus (*Eustoma ganadiflorum*) caused by Iris yellow spots virus. *Jpn. J. Phytopathol.* 69:181–188. (in Japanese with English summary).

DRUKKER, B., SCUTAREANU, P., and SABELIS, M. W. 1995. Do anthocorid predators respond to synomones from *Psylla*-infested pear trees under field conditions? *Entomol. Exp. Appl.* 77: 193–203.

DRUKKER, B., BRUIN, J., JACOBS, G., KROON, A., and SABELIS, M. W. 2000a. How predatory mites learn to cope with variability in volatile plant signals in the environment of their herbivorous prey. *Exp. Appl. Acarol.* 24:881–895.

DRUKKER, B., BRUIN, J., and SABELIS, M. W. 2000b. Anthocorid predators learn to associate herbivore-induced plant volatiles with presence or absence of prey. *Physiol. Entomol.* 25:260–265.

DUGRAVOT, S., and THIBOUT, E. 2006. Consequences for a specialist insect and its parasitoid of the response of *Allium porrum* to conspecific herbivore attack. *Physiol. Entomol.* 31:73–79.

DUGRAVOT, S., THIBOUT, E., ABO-GHALIA, A., and HUIGNARD, J. 2004. How a specialist and a non-specialist insect cope with dimethyl disulfide produced by *Allium porrum*? *Entomol. Exp. Appl.* 113:173–179.

DUGRAVOT, S., MONDY, N., MANDON, N., and THIBOUT, E. 2005. Increased sulfur precursors and volatiles production by the leek *Allium porrum* in response to specialist insect attack. *J. Chem. Ecol.* 31:1299–1314.

EDELSON, J. V., CARTWRIGHT, B., and ROYER, T. A. 1989. Economics of controlling onion thrips (Thysanoptera: Thripidae) on onions with insecticides in south Texas. *J. Econ. Entomol.* 82:561–564.

GILLESPIE, D. R. 1989. Biological control of thrips [Thysanoptera: Thripidae] on greenhouse cucumbers by *Amblyseius cucumeris*. *Entomophaga* 34:185–192.

HINOMOTO, N., MURAJI, M., NODA, T., SHIMIZU, T., and KAWASAKI, K. 2004. Identification of five *Orius* in Japan by multiplex polymerase chain reaction. *Biol. Contol.* 31:276–279.

HORI, M., and HARADA, H. 1995. Screening plants resistant to green peach aphid, *Myzus persicae* (Sulzer) (Homoptera: Aphididae). *Appl. Entomol. Zool.* 30:246–249.

HOY., C. W., and GLENISTER., C. S. 1991. Releasing *Amblyseius* spp. (Acarina: Phytoseiidae) to control *Thrips tabaci* (Thysanoptera: Thripidae) on cabbage. *Entomophaga* 36:561–573.

JAMES, D. G., and PRICE, T. S. 2004. Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. *J. Chem. Ecol.* 30:1613–1628.

JANSSEN, A., PALLINI, A., VENZON, M., and SABELIS, M. W. 1998. Behaviour and indirect interactions in food webs of plant-inhabiting arthropods. *Exp. Appl. Acarol.* 22:497–521.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. Chicago University Press, Chicago, IL, USA.

KENDALL., D. M., and BJOSTAD, L. B. 1987. Susceptibility of onion growth stages to onion thrips (Thysanoptera: thripidae) damage and mechanical defoliation. *Environ. Entomol.* 16:859–863.

KRITZMAN., A., LAMPEL, M., RACCAH, B., and GERA, A. 2001. Distribution and transmission of Iris yellow spot virus. *Plant Disease* 85:838–842.

MAEDA, T., LIU, Y., ISHIWARI, H., and SHIMODA, T. 2006. Conditioned olfactory responses of a predatory mite, *Neoseiulus womersleyi*, to volatiles from prey-infested plants. *Entomol. Exp. Appl.* 121:167–175.

OZAWA, R., ARIMURA, G., TAKABAYASHI, J., SHIMODA, T., and NISHIOKA, T. 2000. Involvement of jasmonic- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. *Plant Cell Physiol.* 41: 391–398.

SCUTAREANU, P., DRUKKER, B., BRUIN, J., POSTHUMUS, M. A., and SABELIS, M. W. 1996. Leaf volatiles and polyphenols in pear trees infested by *Psylla pyricola*. Evidence of simultaneously induced responses. *Chemoeology* 7:34–38.

SHELTON, A. M., WILSEY, W. T., and SCHMAEDICK, M. A. 1998. Management of onion thrips (Thysanoptera: Thripidae) on cabbage by using plant resistance and insecticides. *J. Econ. Entomol.* 91:329–333.

SHIMODA, T., and DICKE, M. 1999. Volatile stimuli related to feeding activity of nonprey caterpillars, *Spodoptera exigua*, affect olfactory response of the predatory mite, *Phytoseiulus persimilis*. *J. Chem. Ecol.* 23:2033–2048.

SHIMODA, T., OZAWA, R., SANO, K., YANO, E., and TAKABAYASHI, J. 2005. The involvement of volatile infochemicals from spider mites and from food-plants in prey location of the generalist predatory mite, *Neoseiulus californicus*. *J. Chem. Ecol.* 31: 2019–2032.

SHIOJIRI, K., MAEDA, T., ARIMURA, G., OZAWA, R., SHIMODA, T., and TAKABAYASHI, J. 2002. Functions of plant infochemicals in tritrophic interactions between plants, herbivores and carnivorous natural enemies. *Jpn. J. Appl. Entomol. Zool.* 46:117–133 (in Japanese with English summary).

SHIPP, J. L., and WANG, K. 2003. Evaluation of *Amblyseius cucumeris* (Acari: Phytoseiidae) and *Orius insidiosus* (Hemiptera: Anthocoridae) for control of *Frankliniella occidentalis* (Thysanoptera: Thripidae) on greenhouse tomatoes. *Biol. Contol.* 28:271–281.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry. Freeman, New York. p. 887.

TAKABAYASHI, J., and DICKE, M. 1992. Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. Exp. Appl.* 64:187–193.

TAKABAYASHI, J., and DICKE, M. 1996. Plant–carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1:109–113.

TAKABAYASHI, J., SHIMODA, T., DICKE, M., ASHIHARA, W., and TAKAFUJI, A. 2000. Induced response of tomato plants to injury by green and red strains of *Tetranychus urticae*. *Exp. Appl. Acarol.* 24:377–383.

TSUCHIYA, M. 2001. Occurrence of blemished fruits caused by *Thrips tabaci* on Satsuma mandarin cultivated in the greenhouses in Shizuoka Prefecture. *Ann. Rept. Kanto Pl. Plot. Soc.* 48:153–155 (in Japanese with English summary).

TSUCHIYA, M. 2002. Infestation and oviposition of *Thrips tabaci* (Lindeman) on Satsuma mandarin (*Citrus unshiu* Marc.) *Jpn.. J. Appl. Entomol. Zool.* 46:217–224 (in Japanese with English summary).

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1993. Learning of host-finding cues by Hymenopterous parasitoids, pp. 51–78, in D. R. Papaj, and A. C. Lewis (eds.). Insect Learning. Chapman and Hall, New York.

VENUGOPAL, M. S., and NARAYANAN, V. 1981. Effects of allition on the green peach ahid (*Myzus persicae* Sulzer). *Int. Pest. Cont.* 93: 130–131.

VENZON, M., JANSSEN, A., and SABELIS, M. W. 1999. Attraction of a generalist predator towards herbivore-infested plants. *Entomol. Exp. Appl.* 93:305–314.

VET, L. E. M., LEWIS, W. J., and CARDE, R. T. 1993. Parasitoid foraging and learning, pp. 65–101, in R. T. Carde, and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall, New York.

WANG, C. L., LEE, P. C., and WU, Y. J. 2001. Field augmentation of *Orius strigicollis* for the control of thrips in Taiwan. *International Seminar on Biological Control of IPEC* 141–152.

WILLIAMS, M. D. 2001. Biological control of thrips on ornamental crops: interactions between the predatory mite *Neoseiulus cucumeris* (Acari: Phytoseiidae) and western flower thrips, *Frankliniella occidentalis* (Thysanoptera: Thripidae), on cyclamen. *Biocontrol Sci. Technol.* 11:41–55.

YASUNAGA, T. 1997. The flower bug genus *Orius* Wolff (Heteroptera: Anthocoridae) from Japan and Taiwan, Part II. *Appl. Entomol. Zool.* 32:379–386.

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Attraction to Herbivore-induced Plant Volatiles by the Host-foraging Parasitoid Fly *Exorista japonica*

Ryoko T. Ichiki · Yooichi Kainoh · Soichi Kugimiya · Junji Takabayashi · Satoshi Nakamura

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Abstract Responses of the tachinid fly *Exorista japonica* Townsend to odors from corn plants infested with the fly's host, the larvae of the noctuid moth *Mythimna separata* (Walker), were examined in a wind tunnel. Naïve female flies showed a higher rate of landing on *M. separata*-infested corn plants from which the host larvae had been removed than on artificially damaged or intact corn plants. When paper impregnated with a solution of headspace volatiles collected from host-infested plants was attached to intact plants, females landed on the plants at a high rate. Females also responded to intact plants to which had been attached with paper impregnated with a synthetic blend of nine chemicals identified previously in host-infested plants. There was an optimum concentration of the synthetic blend for the females' landing. Of the nine chemicals identified previously, four [(*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, 3-hydroxy-2-butanone, and 2-methyl-1-propanol] released only by host-infested plants were classified as a host-induced blend. The other five [(*Z*)-3-hexen-1-yl acetate, (*E*)-2-hexenal, hexanal, (*Z*)-3-hexen-1-ol, and linalool] were classified as a non-specific blend released not only by infested plants but also by artificially damaged or intact plants. In the wind tunnel, *E. japonica* females did not respond to intact plants to which paper containing a solution of non-specific blend or host-induced blend was attached. However, they showed a high level of response to a mixture of the non-specific and host-induced blends. These results indicate that naïve *E. japonica* use a combination of non-specific and host-induced blends as an olfactory cue for locating host-infested plants.

Keywords *Exorista japonica* · Herbivore-induced plant volatiles · Host selection · *Mythimna separata* · Tachinid

R. T. Ichiki (✉) · S. Nakamura
Japan International Research Center for Agricultural Sciences,
Ohwashi 1-1, Tsukuba,
Ibaraki 305-8686, Japan
e-mail: richiki@mail.goo.ne.jp

Y. Kainoh
Doctoral Program of Biosphere Resource Science and Technology,
Graduate School of Life and Environmental Sciences,
University of Tsukuba,
Tennodai 1-1-1,
Ibaraki 305-8572, Japan

S. Kugimiya
National Institute for Agro-Environmental Science,
Kannondai 3-1-3, Tsukuba,
Ibaraki 305-8604, Japan

J. Takabayashi
Center for Ecological Research, Kyoto University,
Hirano 2 509-3, Otsu,
Shiga 520-2113, Japan

Introduction

Olfactory cues play a major role in the host-finding behavior of parasitoids (Vinson 1976; Van Alphen and Vet 1986; Godfray 1994). Particularly, the volatiles produced by plants infested with herbivorous hosts are important cues for host finding by a number of hymenopteran parasitoids (e.g., Turlings et al. 1990; Vet and Dicke 1992; Potting et al. 1995; Takabayashi et al. 1995; Fukushima et al. 2002; Shiojiri et al. 2006) and by several types of dipteran parasitoid (Roth et al. 1982; Roland et al. 1989, 1995; Mondor and Roland 1997; Kainoh et al. 1999; Stireman 2002). These so-called herbivore-induced plant

volatiles (HIPVs), which are released in response to herbivore attacks, are considered to be an indirect plant defense against herbivores (for review see Turlings and Wäckers 2004) because the feeding of the parasitoids that are attracted reduces the damage caused by the herbivores. However, it remains largely unknown which specific chemicals, or chemical blends, in HIPVs actively attract parasitic insects, except in the case of several hymenopteran species such as *Encarsia formosa* Gahan (Birkett et al. 2003) and *Opius dissitus* Muesebeck (Wei and Kang 2006). The chemical attractant of only one of the species of tachinid fly, *Cyzenis albicans* (Fallén), has been identified (Roland et al. 1995).

The tachinid fly *Exorista japonica* Townsend is a gregarious and polyphagous endoparasitoid that attacks a wide range of lepidopteran pests in Japan, including the noctuid moth *Mythimna separata* (Walker), the fall webworm *Hyphantria cunea* Drury, and the gypsy moth *Lymantria dispar* (Linnaeus) (Shima 2006). The female fly lays a macrotype egg directly on the host's cuticle. The hatched larva enters the host's body cavity, develops within the body, and finally kills the host (Nakamura 1994). A previous study showed that *E. japonica* females were attracted to corn plants placed together with the larvae of *M. separata* in a wind tunnel (Kainoh et al. 1999). We hypothesized that the flies use chemical cues from host-infested plants. To test this hypothesis, we investigated the attraction of *E. japonica* to intact, artificially damaged, or *M. separata*-infested corn plants in a wind tunnel. We then examined the flies' attraction to the headspace volatiles of an *M. separata*-infested plant. Previously, we had identified a series of volatile chemicals in headspace samples from *M. separata*-infested corn plants (Takabayashi et al. 1995). We classified these volatiles into two groups: (1) the "host-induced blend" of chemicals released from corn plants infested by the host larvae and (2) the "non-specific blend" of chemicals released not only from plants infested by the hosts but also from artificially damaged or intact plants. Here, we investigated which synthetic blend was responded to by *E. japonica* in a wind tunnel.

Methods and Materials

Insects

Larvae of *M. separata* were obtained from a stock culture and reared on an artificial diet in accordance with the method of Hattori and Atsusawa (1980). The colony of *E. japonica* originated from parasitized final-instar larvae of *H. cunea*, which had been collected at Tsukuba, Ibaraki, Japan (36°2′ N, 140°5′ E) in September and October 2004. This colony was maintained by using final instars of *M. separata* as the hosts. Within 2 days after adult emergence, a female was mated once with a male in a cage (30×28×25 cm) with two windows of nylon mesh. The mated female was transferred to a cylindrical plastic cage (8-cm diameter, 14-cm high) with a sugar cube and water and kept alone until the experiments were begun. Because the active time of oviposition by *E. japonica* is 5–10 days after emergence (Nakamura 1994), females aged 5–10 days were used in the experiments. To eliminate the effects of oviposition experience and learning of odors, naïve females were used. All procedures were conducted at 25°C under a photoperiod of 16:8 h (L/D) and 60% relative humidity (RH).

Plants

Corn plants (*Zea mays* Linnaeus, 'Honey-bantam Peter 619', Sakata Seed Co., Kanagawa, Japan) used for the wind tunnel bioassay were individually grown from seeds in soil in plastic pots (300 ml) in a greenhouse for more than 1 month (27±2°C, 50–70% RH, 16 L:8 h D). Corn plants used for collecting the headspace volatiles of host-infested plants were grown in a field from seeds during the summer for more than 1 month. Seedlings 30- to 40-cm high were used for the experiments.

Collection of Headspace Volatiles

Corn plants were cut at 5-cm high from the ground. To avoid desiccation, the cut ends of the stem were covered with moist cotton wool and then aluminum foil. Corn plants were individually provided with ten final-instar larvae of *M. separata* in the cage in a laboratory. After 5 h, the host larvae had consumed approximately 20% of the total surface area of the leaves. After the larvae and frass had been removed from the plants, headspace volatiles from the infested plants were collected in accordance with methods of the previous studies (Takabayashi et al. 1991, 1995), as follows. One glass tube (4-mm diameter×160-mm long) containing 100 mg Tenax TA (GL Science, Tokyo, Japan) was attached to the inlet, and one to the outlet, of a glass bottle (2 l) in which the five infested corn plants (100 g) were placed. A water aspirator was connected to the glass tube of the outlet and sucked for 1 h to collect the corn headspace volatiles. Volatiles trapped on the Tenax TA in the glass tube attached to the outlet of the glass bottle were extracted with ether (5 ml). Collection of headspace volatiles of infested plants was replicated five times using 25 corn plants (500 g) and 250 hosts in total, and all the extracts were mixed together as a headspace volatile solution (20 g leaves/ml). All sample solutions were kept in a freezer until use for bioassay.

Chemicals

Nine chemicals were used to prepare the synthetic blends (Table 1). The nine had been identified as being released in relatively large amounts from corn plants infested by the final (sixth) instars of *M. separata* (Takabayashi et al. 1995). We used gas chromatography-mass spectrometry (GC-MS) analysis to confirm that the nine chemicals were recorded in the headspace of infested corn plants (data not shown).

(*E*)-4,8-dimethyl-1,3,7-nonatriene, indole, 3-hydroxy-2-butanone, and 2-methyl-1-propanol were found only in corn plants infested by final instars (Takabayashi et al. 1995). We called the blend of these four chemicals the host-induced blend. (*Z*)-3-hexen-1-yl acetate, (*E*)-2-hexenal, hexanal, (*Z*)-3-hexen-1-ol, and linalool were compounds commonly found in infested, artificially damaged, or intact plants at relative amounts of at least 5% (Takabayashi et al. 1995). We called the blend of these five chemicals the non-specific blend. Additionally, we called the blend of all nine chemicals the "mixed blend." These three blends were prepared by dissolving each of the synthetic chemicals in 1 ml of hexane at the amounts (in μg) listed in Table 1. The amounts of each chemical were determined based on their relative percentage amounts detected in the headspace of host-infested plants (Takabayashi et al. 1995).

Linalool has two stereoisomers, (*R*)-(−)-linalool and (*S*)-(+)-linalool. Our enantioselective GC analysis by chiral column (β-DEX 225, 30 m×0.25 mm ID, 0.25-μm film thickness; Supelco, Sigma-Aldrich, St. Louis, MO, USA) of the headspace volatiles from *M. separata*-infested corn plants revealed that the plants released mainly (*S*)-(+)-linalool, with only a marginal amount of (*R*)-(−)-linalool. Because pure (*S*)-(+)-linalool was not available, we used racemic linalool (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) here. (*E*)-4,8-dimethyl-1,3,7-nonatriene was synthesized by a known reaction (Maurer et al. 1986). The other chemicals were purchased from Wako Pure Chemical Industries, Ltd., Osaka, Japan.

Table 1 Chemical composition and purity of synthetic non-specific, host-induced, and mixed blends

Compounds	Amount (μg)	Purity (%)
Non-specific blend[a]		
Linalool	8.3	95
(*Z*)-3-hexen-1-ol	8.4	97
Hexanal	9.2	95
(*E*)-2-hexenal	16.6	97
(*Z*)-3-hexen-1-yl acetate	19.7	98
Total	62.2	
Host-induced blend[b]		
2-methyl-1-propanol	1.2	99
Indole	3.2	98
(*E*)-4,8-dimethyl-1,3,7-nonatriene	3.4	>95
3-hydroxy-2-butanone	7.4	95
Total	15.2	
Mixed blend[c]		
Total	77.4	

[a] Five chemicals are released not only from *Mythimna separata*-infested corn plants but also from artificially damaged or intact plants (Takabayashi et al. 1995). A total of 62.2 μg was dissolved in 1 ml of hexane

[b] Four chemicals are released only from *M. separata*-infested corn plants (Takabayashi et al. 1995). A total of 15.2 μg was dissolved in 1 ml of hexane

[c] All the nine chemicals were mixed together as a mixed blend. A total of 77.4 μg was dissolved in 1 ml of hexane

Wind Tunnel Bioassay

The flight response of female flies to several target plants was examined in a wind tunnel. The tunnel was 50-cm high×50-cm wide, 1.5-m long, and 5-mm thick. Air was drawn through the tunnel at 30 cm/s, and the light intensity on the tunnel floor was 2,600 lx. A target plant was placed on the upwind side of the tunnel. A mated female fly was introduced to the tunnel on a sugar cube, which was placed on a platform (9-cm diameter, 20-cm high) 1 m downwind from the target. When the female did not take off from the platform within 5 min or did not land on the plant within 5 min after having taken off, we regarded her as not having been attracted to the target plant and replaced her with a new female. The target plant and the odor source were replaced with fresh ones after every two or three females. The following four experiments were conducted in the wind tunnel.

Experiment 1: Response to Infested, Artificially Damaged, or Intact Corn Plants

A corn plant was cut 5 cm above the ground, and the cut end was placed in an Erlenmeyer flask (200 ml) with 150 ml water. The opening of the flask was filled with cotton wool. To make an infested plant sample, the cut corn in the flask was provided with ten final instars of *M. separata*. After 5 h, we removed the larvae and their frass. To make an artificially damaged plant sample, the leaves of the cut corn in the flask were damaged with a hole punch (5 mm diameter) 30 min before the bioassay. We made 30 holes per plant to damage approximately 20% of the total surface area of the leaves. To make an intact plant sample, the cut corn in the flask was kept without hosts for 5 h after cutting. The flight responses of the female flies to the 3 target plants were assayed on five separate days using a total of 75 females.

Experiment 2: Response to Headspace Volatiles of Infested Corn Plants

The headspace volatile solution (20 g leaves/ml) was diluted to concentrations of 10^{-1} and 10^{-2} with ether. Each diluted solution (100 μl) was separately absorbed onto green drawing paper (2×10 cm). After solvent evaporation (30 s), the paper was fixed to the intact plant with a clip. As a control, 100 μl of ether was similarly tested. The flight responses of females to three doses (0.02, 0.2, and 2 g leaves) of the headspace volatiles and the control were assayed on six separate days using a total of 104 females.

Experiment 3: Response to Synthetic Mixed Blends at Different Doses

To examine whether the parasitoid could detect synthetic volatiles and to determine the optimum dose, we assayed the responses of *E. japonica* to the mixed blend. The original mixed blend solution (7.7×10^{-2} μg/μl) was diluted to concentrations of 10^{-1} and 10^{-2} with hexane. Each diluted solution (100 μl) was absorbed separately onto the green paper and tested in the same way. As a control, 100 μl of hexane was similarly tested. The flight responses of females to three doses of the mixed blends (7.7×10^{-2}, 7.7×10^{-1}, and 7.7 μg) and a control were examined on seven separate days using a total of 100 females. GC-MS analysis showed that a synthetic blend dose of 7.7×10^{-1} μg gave an ion intensity roughly identical to that in the headspace volatile from between 2 and 20 g of leaves.

Experiment 4: Response to Synthetic Host-induced and Non-specific Blends

The host-induced and non-specific blends were prepared. Each of the two blends was diluted to a concentration (host-induced blend: 1.5×10^{-3} μg/μl; non-specific blend: 6.2×10^{-3} μg/μl) the same as the one determined to be the most attractive as a component of the mixed blend in experiment 3 (Fig. 3). Each diluted solution (100 μl) was absorbed onto the green paper to give a dose of 1.5×10^{-1} or 6.2×10^{-1} μg, respectively. The host-induced and non-specific blends also were combined to make a different mixed blend (3.87×10^{-3} μg/μl); this mixed blend solution (200 μl) was absorbed onto the green paper to give a dose of 7.7×10^{-1} μg. As controls, 100 μl of hexane was used for the host-induced and non-specific blends and 200 μl of hexane for the mixed blend. The landing rates of the females to the three types of synthetic blend and the controls were examined on eight separate days using a total of 150 females.

Statistical Analyses Differences in the rates at which flies landed on the three types of test plant, the plants with papers containing headspace volatiles, and the plants with papers containing the different doses of synthetic mixed blends were subjected to the χ^2 test and then compared by Ryan's multiple-range test for proportions. Landing rates on the plants by flies were also compared among the infested plants, the headspace volatiles, and two types of synthetic mixed blends by using the χ^2 test. Fisher's exact probability test was used to compare the landing rates of flies between the plant treated with the three types of synthetic blend and the controls. We used the software package R, version 2.4.1 (R Development Core Team 2006) to do these statistical analyses.

Results

Experiment 1: Response to Infested, Artificially Damaged, or Intact Corn Plants

A significantly higher proportion of naïve female flies (80.0%) landed on the *M. separata*-infested corn plants than on the intact corn plants (32.0%; Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$; Fig. 1). The females responded in an intermediate way to the artificially damaged plants (56.0%), but this result was not significantly different from that with intact plants ($P>0.05$).

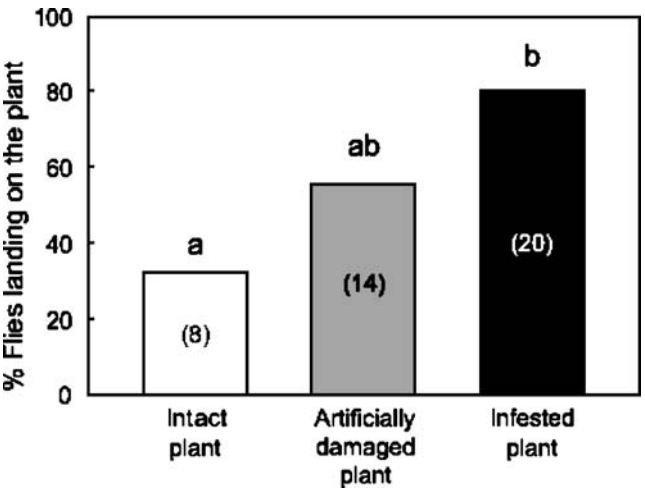

Fig. 1 Flight response of naïve females of *E. japonica* to intact, artificially damaged, and *M. separata*-infested corn plants in the wind tunnel. For each target plant, 25 females were used. *Numbers in bars* indicate the numbers of flies that reached the target plants. *Different letters above bars* indicate significant differences between values (Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$)

Experiment 2: Response to Headspace Volatiles of Infested Corn Plants

When the intact plant was treated with volatile solution prepared from the headspace volatiles of the host-infested plants, more naïve females landed on the treated targets than on the controls (Fig. 2). The responses to the 0.2- and 2-g doses of the volatile solutions (73.1% and 76.9% of females landing on the plant) were significantly greater than that to the control (38.5%; Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$). At the lowest dose of the volatile solution (0.02 g), the response rate declined slightly (69.2%) and was not significantly different from the response to the control ($P>0.05$).

Experiment 3: Response to Synthetic Mixed blends at Different Doses

Naïve females were attracted to the intact plants treated with the synthetic mixed blend solution prepared from the nine chemicals but not to the control plants (Fig. 3). Among the three doses of the blend, the middle dose of 7.7×10^{-1} μg was most attractive (72.0% of females landing on the plant). The response of the females to the middle dose was significantly higher than that to the control (32.0%; Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$). The responses were lower to the other two doses, 7.7×10^{-2} (56.0%) and 7.7 μg (48.0%); these responses were not significantly different from the response to the control ($P>0.05$).

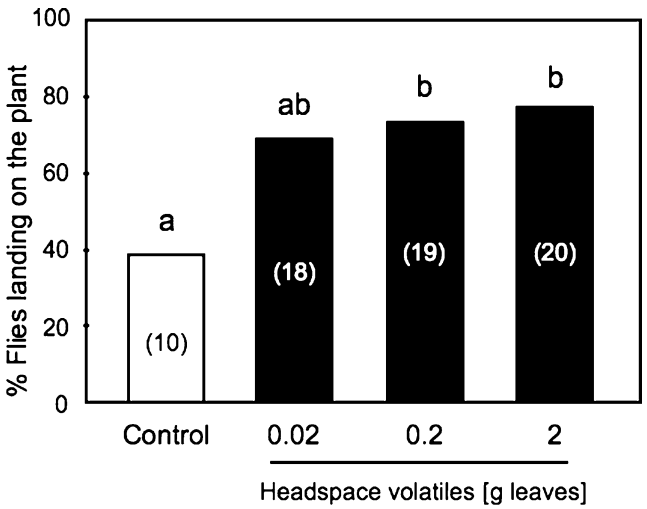

Fig. 2 Flight response of naïve females of *E. japonica* in the wind tunnel to plant volatiles collected from the headspace of *M. separata*-infested corn plants. For each odor source, 26 females were used. *Numbers in bars* indicate the numbers of flies that reached the target plants. *Different letters above bars* indicate significant differences between values (Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$)

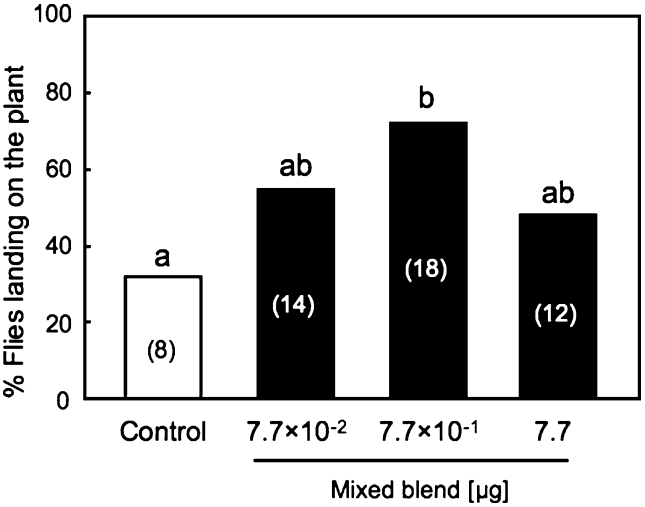

Fig. 3 Flight response of naïve females of *E. japonica* to mixed blend of nine chemicals from *M. separata*-infested corn plants in the wind tunnel. For each odor source, 25 females were used. *Numbers in bars* indicate the numbers of flies that reached the target plants. *Different letters above bars* indicate significant differences between values (Ryan's multiple-range test for proportions after χ^2 test, $P<0.05$)

Experiment 4: Response to Synthetic Host-induced and Non-specific Blends

When the intact plant was treated with the synthetic non-specific blend, naïve females showed a low landing rate (40.0%; Fig. 4). The landing rate in response to the non-specific blend was not significantly different from that to the control (20.0%; Fisher's exact probability test, $P=0.217$). The females also did not respond significantly more to intact plants treated with the synthetic host-induced blend (40.0%) than to the control (28.0%; $P=0.551$). In contrast, the females showed a high landing rate (76.0%) in

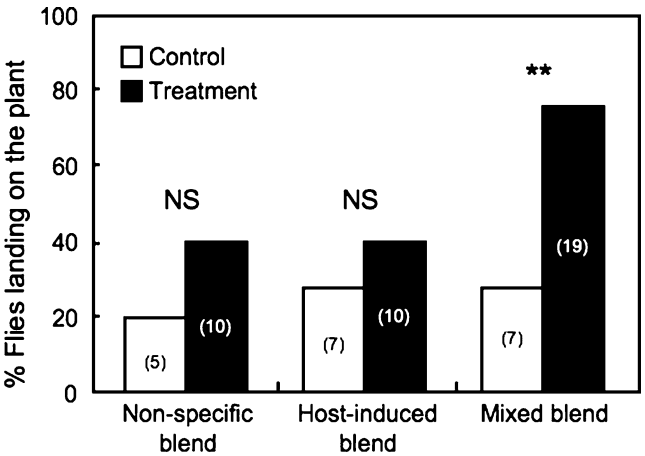

Fig. 4 Flight response of naïve females of *E. japonica* to non-specific blend (1.5×10^{-1} μg), host-induced blend (6.2×10^{-1} μg), and mixed blend (7.7×10^{-1} μg) in the wind tunnel. In each case, 25 females were used. *Numbers in bars* indicate the numbers of flies that reached the target plants. *Asterisks* indicate significant differences between treatment and control (Fisher's exact probability test, $P<0.05$)

response to intact plants treated with the mixed blend that combined the non-specific and host-induced blends. The landing rate in response to the mixed blend was significantly greater than that to the control (28.0%; P=0.002).

Comparison of Responses to Infested Corn Plants, Headspace Volatiles, and Synthetic Mixed Blends We compared the responses of the flies to four odor sources: *M. separata*-infested corn plants (Fig. 1), the most attractive dose of headspace volatiles (2 g; Fig. 2), the most attractive dose of synthetic mixed blend composed of the nine chemicals (7.7×10^{-1} μg; Fig. 3), and the synthetic mixed blend combined with the non-specific and host-induced blends (Fig. 4). The responses were not significantly different from each other (χ^2 test, χ^2=0.45, df=3, P=0.93).

Discussion

Our results demonstrated that the naïve females of *E. japonica* were attracted to volatiles emitted from corn plants infested by *M. separata* larvae. Our previous study (Kainoh et al. 1999) reported that naïve *E. japonica* females had lower responses to infested plants than did oviposition-experienced females: When female flies were tested with *M. separata*-infested corn plants together with the host larvae in a wind tunnel, the rate of landing on the plants within 2 min after takeoff was 33.3% (5 of 15) in naïve females and 70.6% (12 of 17) in females conditioned by oviposition on hosts. However, when naïve females were observed for 5 min after takeoff and tested on infested plants from which the host larvae had recently been removed, the rate of landing on infested plants by naïve females was found to be 80.0% (Fig. 1). Therefore, the observation period used in our previous bioassays may have been too short for us to detect fully the reactivity of naïve females to the infested plants. *E. japonica* females appear to have innate sensitivity to the plant volatiles, but this sensitivity may be still lower in naïve females than in oviposition-experienced females. In hymenopteran parasitoids, the sensitivity of females generally increases with exposure to the host or a host product (e.g., McAuslane et al. 1991; Turlings et al. 1992). To eliminate the effects of oviposition experience and learning of odors, we used naïve females in these experiments.

Naïve females of *E. japonica* responded to intact corn plants attached only with headspace volatiles or only with synthetic volatiles that reflected the infested plants (Figs. 2, 3, and 4); they responded in the absence of other potential host-searching cues such as frass or other host secretions, suggesting that the volatiles alone from the host–plant complex are adequate olfactory cues for this tachinid to locate host-infested plants.

In the Tachinidae, attraction to herbivore-infested plants themselves or to their extracts has been demonstrated in several species (Roth et al. 1982; Roland et al. 1989, 1995; Mondor and Roland 1997; Kainoh et al. 1999; Stireman 2002). However, attraction to a synthetic chemical has been confirmed in only one species of microtype tachinid. *C. albicans* had a high rate of response to borneol among the ten volatile compounds identified from a crude extract of garry oak (*Quercus garryana* Dougl. ex Hook.) foliage, which was known to be attractive to this tachinid fly (Roland et al. 1995). Here, we demonstrated that *E. japonica* was attracted to a mixture of several synthetic chemicals. A mixed blend of nine synthetic chemicals identified from the *M. separata*-infested plants was attractive to naïve females of *E. japonica* (Fig. 3). The middle dose of the mixed blend (7.7×10^{-1} μg) was most attractive among the three doses tested, and the landing rate in response to this dose of the mixed blend did not differ from that in response to the infested plants or the headspace extract, suggesting that this dose was optimal for attracting *E. japonica*. In addition, *E. japonica* responded to neither the non-specific blend nor the host-induced blend but showed a high rate of response to a mixture of the non-specific and host-induced blends at the optimum dose (Fig. 4). These results indicate that a combination of non-specific and host-induced blends is essential for the attraction of *E. japonica*. An example of the reciprocal effects of non-specific and host-induced blends had been reported in the learned response of *Cotesia kariyai* Watanabe to plant volatiles (Fukushima et al. 2002).

E. japonica attacks 49 lepidopteran species in Japan (Shima 2006). Some host species such as *H. cunea* and *L. dispar* are polyphagous and attack many plant species (see Japanese Society of Applied Entomology and Zoology 2006). In order for this generalist parasitoid to locate hosts on a multitude of species, it seems to utilize volatile compounds common to various species. Components of the non-specific blend, namely (*Z*)-3-hexen-1-yl acetate, (*E*)-2-hexenal, hexanal, and (*Z*)-3-hexen-1-ol, called green leaf volatiles (GLV), are commonly released from various plants after physical damage, as well as by herbivory (e.g., Takabayashi et al. 1995; Turlings et al. 1998; Hoballah et al. 2002; Chamberlain et al. 2006). Some parasitic wasps are attracted by these GLV (Takabayashi et al. 1991; Wickremasinghe and Van Emden 1992). Our data suggest that the presence of the non-specific blend, composed mainly of GLV, was essential for *E. japonica* to land on the infested plants. However, it is likely that the GLV alone are insufficient to attract *E. japonica* because this parasitoid showed moderate responses to both the artificially damaged plants (Fig. 1) and the non-specific blend (Fig. 4). The GLV appear to have an additive effect on the attraction of *E. japonica* with the host-induced blend. Our data suggest that

the presence of the host-induced blend was also important for *E. japonica* to land on the infested plants. The homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene in the host-induced blend is released from several plant species, including corn (Turlings and Tumlinson 1992; Takabayashi et al. 1995; Hoballah et al. 2002; Birkett et al. 2003), bean (Dicke et al. 1990; Wei and Kang 2006), and cotton (Röse et al. 1996), in response to herbivory, and it attracts some parasitic wasps such as *E. formosa* (Birkett et al. 2003) and *O. dissitus* (Wei and Kang 2006). This homoterpene is one of common HIPVs found in several host–plant complexes and is a highly reliable indicator of host presence. Therefore, it may be the most likely candidate for the chemical that attracts the generalist *E. japonica*. By exploiting mixtures of such common HIPVs and GLV as chemical cues, in nature, *E. japonica* may efficiently search for a variety of hosts on plants.

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References

BIRKETT, M. A., CHAMBERLAIN, K., GUERRIERI, E., PICKETT, J. A., WADHAMS, L. J., and YASUDA, T. 2003. Volatiles from whitefly-infested plants elicit a host-locating response in the parasitoid *Encarsia formosa*. *J. Chem. Ecol.* 29:1589–1600.

CHAMBERLAIN, K., KHAN, Z. R., PICKETT, J. A., TOSHOVA, T., and WADHAMS, L. J. 2006. Diel periodicity in the production of green leaf volatiles by wild and cultivated host plants of stemborer moths, *Chilo partellus* and *Busseola fusca*. *J. Chem. Ecol.* 32:565–577.

DICKE, M., VAN BEEK, T. A., POSTHUMUS, M. A., BEN DOM, N., VAN BOKHOVEN, H., and DE GROOT, A. E. 1990. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions: involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

FUKUSHIMA, J., KAINOH, Y., HONDA, H., and TAKABAYASHI, J. 2002. Learning of herbivore-induced and nonspecific plant volatiles by a parasitoid, *Cotesia kariyai*. *J. Chem. Ecol.* 28:579–586.

GODFRAY, H. C. J. 1994. Parasitoids: behavioural and evolutionary ecology. Princeton University Press, Princeton, New Jersey.

HATTORI, M., and ATSUSAWA, S. 1980. Mass-rearing of the cabbage armyworm, *Mamestra brassicae* Linné and the common armyworm, *Mythimna separata* Walker (Lepidoptera: Noctuidae) on a simple artificial diet. *Jpn. J. Appl. Entomol. Zool.* 24:36–38, (in Japanese with English summary).

HOBALLAH, M. E. F., TAMO, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality of quantity important? *J. Chem. Ecol.* 28:951–962.

JAPANESE SOCIETY OF APPLIED ENTOMOLOGY AND ZOOLOGY 2006. Major insect and other pests of economic plants in Japan (revised edition). The Japanese Society of Applied Entomology and Zoology, Tokyo.

KAINOH, Y., TANAKA, C., and NAKAMURA, S. 1999. Odor from herbivore-damaged plant attracts the parasitoid fly *Exorista japonica* Townsend (Diptera: Tachinidae). *Appl. Entomol. Zool.* 34:463–467.

MAURER, B., HAUSER, A., and FROIDEVAUX, J. C. 1986. (*E*)-4,8-dimethyl-1,3,7-nonatriene and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, two unusual hydrocarbons from cardamom oil. *Tetrahedron Lett.* 27:2111–2112.

MCAUSLANE, H. J., VINSON, S. B., and WILLIAMS, H. J. 1991. Stimuli influencing host microhabitat location in the parasitoid *Campoletis sonorensis*. *Entomol. Exp. Appl.* 58:267–277.

MONDOR, E. B., and ROLAND, J. 1997. Host locating behaviour of *Leschenaultia exul* and *Patelloa pachypyga*: two tachinid parasitoids of the forest tent caterpillar, *Malacosoma disstria*. *Entomol. Exp. Appl.* 85:161–168.

NAKAMURA, S. 1994. Parasitization and life history parameters of *Exorista japonica* (Diptera: Tachinidae) using the common armyworm, *Pseudaletia separata* (Lepidoptera: Noctuidae) as a host. *Appl. Entomol. Zool.* 29:133–140.

POTTING, R. P. J., VET, L. E. M., and DICKE, M. 1995. Host microhabitat location by stem-borer parasitoid *Cotesia flavipes*: the role of herbivore volatiles and locally and systemically induced plant volatiles. *J. Chem. Ecol.* 21:525–539.

R DEVELOPMENT CORE TEAM. 2006. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. <http://www.R-project.org>.

ROLAND, J., EVANS, W. G., and MYERS, J. H. 1989. Manipulation of oviposition patterns of the parasitoid *Cyzenis albicans* (Tachinidae) in the field using plant extracts. *J. Insect Behav.* 2:487–503.

ROLAND, J., DENFORD, K. E., and JIMENEZ, L. 1995. Borneol as an attractant for *Cyzenis albicans*, a tachinid parasitoid of the winter moth, *Operopthera brumata* L. (Lepidoptera: Geometridae). *Can. Entomol.* 127:413–421.

RÖSE, U. S. R., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1996. Volatile semiochemicals released from undamaged cotton leaves. *Plant Physiol.* 111:487–495.

ROTH, J. P., KING, E. G., and HENSLEY, S. D. 1982. Plant, host, and parasite interactions in the host selection sequence of the tachinid *Lixophaga diatraeae*. *Environ. Entomol.* 11:273–277.

SHIMA, H. 2006. A host–parasite catalog of Tachinidae (Diptera) of Japan. *Makunagi, Acta Dipt.* 31:1–171.

SHIOJIRI, K., OZAWA, R., Matsui, K., Kishimoto, K., Kugimiya, S., and Takabayashi, J. 2006. Role of the lipoxygenase/lyase pathway of host–food plants in the host searching behavior of two parasitoid species, *Cotesia glomerata* and *Cotesia plutellae*. *J. Chem. Ecol.* 32:969–979.

STIREMAN, J. O. III. 2002. Host location and selection cues in a generalist tachinid parasitoid. *Entomol. Exp. Appl.* 103:23–34.

TAKABAYASHI, J., NODA, T., and TAKAHASHI, S. 1991. Plants produce attractants for *Apanteles kariyai*, a parasitoid of *Pseudaletia separata*; cases of 'communication' and 'misunderstanding' in parasitoid–plant interactions. *Appl. Entomol. Zool.* 26:237–243.

TAKABAYASHI, J., TAKAHASHI, S., DICKE, M., and POSTHUMUS, M. A. 1995. Developmental stage of the herbivore *Pseudaletia separata* affects production of herbivore-induced synomone by corn plants. *J. Chem. Ecol.* 21:273–287.

TURLINGS, T. C. J., and TUMLINSON, J. H. 1992. Systemic release of chemical signals by herbivore-injured corn. *Proc. Natl. Acad. Sci. U.S.A.* 89:8399–8402.

TURLINGS, T. C. J., and WÄCKERS, F. 2004. Recruitment of predators and parasitoids by herbivore-induced plants, pp. 21–75, in R. T. Cardé, and J. G. Millar (eds.). Advances in insect chemical ecologyCambridge University Press, Cambridge.

TURLINGS, T. C. J., TUMLINSON, J. H., and LEWIS, W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science.* 250:1251–1253.

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1992. Learning of host-location cues by hymenopterous parasitoids, pp. 51–78, in A. C. Lewis, and D. R. Papaj (eds.). Insect learning: ecological and evolutionary perspectives. Chapman and Hall, New York.

TURLINGS, T. C. J., LENGWILER, U. B., BERNASCONI, M. L., and WECHSLER, D. 1998. Timing of induced volatile emissions in maize seedlings. *Planta.* 207:146–152.

VAN ALPHEN, J. J. M., and VET, L. E. M. 1986. An evolutionary approach to host finding and selection, pp. 23–61, in J. K. Waage, and D. J. Greathead (eds.). Insect ParasitoidsAcademic, London.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VINSON, S. B. 1976. Host selection by insect parasitoids. *Annu. Rev. Entomol.* 21:109–133.

WEI, J.-N., and KANG, L. 2006. Electrophysiological and behavioral responses of a parasitic wasp to plant volatiles induced by two leaf miner species. *Chem. Senses.* 31:467–477.

WICKREMASINGHE, M. G. V., and VAN EMDEN, H. F. 1992. Reactions of adult female parasitoids, particularly *Aphidius rhopalosiphi*, to volatile chemical cues from the host plants of their aphid prey. *Physiol. Entomol.* 17:297–304.

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Flight Tunnel Responses of Female Grape Berry Moth (*Paralobesia viteana*) to Host Plants

Dong H. Cha · Stephen P. Hesler · Charles L. Moser · Satoshi Nojima · Charles E. Linn Jr. · Wendell L. Roelofs · Gregory M. Loeb

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Abstract Semiochemicals play important roles in mate and host recognition of herbivorous insects, such as moths, and flight tunnels have been an effective tool in the identification of these bioactive compounds. However, more work has been carried out on pheromones than on host plant cues, and few examples exist where flight tunnel evaluations of host cues have resulted in a lure that is attractive under field conditions. Our goal was to determine whether the flight tunnel could be used to evaluate the response of a specialist moth, grape berry moth (GBM), to its host plant (grapevines), by incorporating ecological and physiological aspects of GBM biology. We found grape shoot tips and mature leaves were more attractive to female GBM than unripe and ripe berries or flowers. Under optimized flight tunnel conditions, approximately 80% of tested females flew upwind and closely approached or landed on the most preferred target. Mating status, wind speed, the time of day, and the presence/absence of patterns that resemble grape tissues on the top of the flight tunnel all significantly affected the responses of female GBM. Consideration of these factors in flight tunnel assays will aid in the development of a synthetic lure that can be used to monitor female moths in the field.

D. H. Cha · S. P. Hesler · C. L. Moser · S. Nojima · C. E. Linn Jr. · W. L. Roelofs · G. M. Loeb (✉)
Department of Entomology, Cornell University,
630 W. North St., NYS Agricultural Experimental Station,
Geneva, NY 14456, USA
e-mail: gme1@cornell.edu

Present address:
S. Nojima
Department of Entomology,
North Carolina State University,
Raleigh, NC 27695, USA

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Introduction

Moth species in the family Tortricidae cause economic losses in agricultural and horticultural crops and forests in temperate regions worldwide (Van der Geest and Evenhuis 1991). Semiochemicals play important roles in both mate and host recognition in these moths (Ridgway et al. 1990; Bruce et al. 2005). Sex pheromones have been identified in over 60 tortricids (Arn 1991) and used in the field for control or monitoring purposes. Similarly, host plant volatiles that are involved in host recognition or oviposition have been studied with a focus on female tortricids (Reed and Landolt 2002; Ansebo et al. 2004; Hern and Dorn 2004; Natale et al. 2004; Tasin et al. 2005, 2006a, b, 2007). Most of the behavioral components of these studies have been carried out in flight tunnels since this type of bioassay is a useful method to evaluate the responses of flying insects to semiochemicals (Howse et al. 1998). However, most of the research using flight tunnels has been looking at responses to sex pheromones, and there is considerably less on responses to host plant cues (e.g., Cardé 1984). This could be because male moths generally have more obvious behavioral responses to pheromone, whereas females have rather subtle behavioral responses to host cues (Tasin et al. 2006b), thus making identification of such bioactive compounds difficult. This may explain why few flight tunnel evaluations of host cues, especially for lepidopterans, have resulted in a lure that works in the field (e.g., Ansebo et al. 2004). In addition, plants generally emit a greater number of volatile compounds than found in insect pheromones, making identification of key compounds more difficult. This situation has been improved

recently with the development of volatile collection systems, such as solid phase microextraction in combination with gas chromatographic-electroantennographic detection (e.g., Zhang et al. 1999; Nojima et al. 2003a, b).

Grape berry moth (*Paralobesia viteana*) (GBM), a major specialist pest of cultivated grape in the Eastern USA, is crepuscular in activity (Taschenberg 1945). Although the female-produced sex pheromone of GBM has been known for several decades (Roelofs et al. 1971) and used to monitor male flight activity, male captures in these pheromone traps have proven to be poorly correlated with female activity (Hoffman 1990; Weigle et al. 1999). Consequently, trap catch data from pheromone traps have not been useful for timing management decisions. As a first step towards developing a host-plant-based lure that is effective in the field, our goal was to determine whether female GBM respond to plant sources in the flight tunnel by comparing response to different grape tissues (flowers, unripe berries, ripe berries, mature leaves, and shoot tips) since knowing the most attractive plant part would help in future studies to identify bioactive volatile cues used for host location. We also examined the effects of time of day, ceiling pattern, wind speed, and mating status on female behavioral responses.

Materials and Methods

Insect Grape berry moths were reared in cages placed in walk-in environmental chambers at 26°C and 60% RH under an 18:6 (L:D) photoperiod. Adult moths were fed with 50% honey and water impregnated in cotton. They mated freely in rearing cages (45 cm height × 77 cm width × 45 cm diameter) and oviposited on seedless grape (*Vitis vinifera*, red flame variety). First and second instars were transferred to diet cups (30 ml, WinCup Inc., Stone Mountain, GA, USA) and reared on semi-synthetic diet (Nagarkatti et al. 2000) consisting of grapes, pinto beans, and commercially available tobacco hornworm diet (Bio-Serve, Frenchtown, NJ, USA). Pupae were sexed and set up in cohorts for flight tunnel bioassays. Each cohort was set up with 10–15 female pupae in a Plexiglas cage (30 cm height × 30 cm width × 30 cm diameter) and provided with 50% honey and water. For mated cohorts, females were housed in cages with 15 to 20 males (one antenna clipped for distinction from females) and a grape cluster to increase mating. Both 3-day-old virgin females and 5-day-old mated females were used for flight tunnel bioassays.

Flight Tunnel The response of female GBM to host plants under different conditions was assessed by using two different, but similarly designed flight tunnels. Both flight tunnels were 2 m in length by 0.6 m in width and 0.6 m in height with a fan installed at the upwind end to create a steady airflow into the tunnel and an exhaust hood at the downwind end to evacuate odor from the flight tunnel and laboratory through a vent. The upwind and downwind ends of the tunnel consisted of two layers of cheesecloth to prevent escape of moths. The first tunnel was constructed with glass doors on one long side and a white panel on the other and a Plexiglas floor and ceiling. A black stripe floor pattern (1.5 cm width stripes every 19 cm perpendicular to the direction of airflow) over a white surface was installed under the Plexiglas floor. Light was provided from above with eight 40-watt incandescent bulbs. Light intensity was reduced to 25 lx (using a rheostat and measured with a Sunlight Illumination Meter, Weston Instruments Inc., Archbald, PA, USA) to mimic dusk conditions. We used this tunnel initially to quantify the most attractive grape tissue type to female GBM, and to investigate the importance of diel periodicity, additional optomotor cues, and wind speed. We conducted subsequent bioassays in an all-glass flight tunnel to minimize volatile contamination in anticipation of future work to identify specific volatile cues used by female GBM. The second flight tunnel was used to determine the effect of mating status on the flight response of female GBM. Light (again at 25 lx) was provided by eight 25-watt incandescent bulbs, and the pattern on the glass floor was made up of randomly distributed dark green paper circles (10 cm diameter) on a white background. During the experiments, temperature and relative humidity of the first flight tunnel was 23.3°C (±0.64 STD) and 30.6% (±7.71 STD), and 23.0°C (±0.32 STD) and 23.6% (±0.16 STD) in the second.

We tested the response of small groups (four to six) of females in the trials conducted in the first flight tunnel, recording the behavior of each moth in the group for 15 min. Flight behavior was made by two observers. We tested single female moths for trials conducted in the second flight tunnel, recording behavior for 8 min per moth. We noted the time it took each moth to respond, and the behavior expressed: leaving the release cage or making an orientation flight (more than 50 cm of tight zigzag flight to within 10 cm of the target, similar behavior as observed in flights with pheromone lure) or contacting the target. For data analysis, we categorized each moth based on the best behavior that the moth displayed within the duration of each assay. Thus, moths that flew from the release cage were categorized as “no orientation” (no directed flight toward the target), “orientation,” or “landing”. The target for a particular trial was introduced at the upwind end in the center of tunnel (30 cm from upwind end) affixed on an acetone-washed copper tube stand, while females were introduced in the plume about 1.5 m downwind of the target in small metal screen cages. The pattern and movement of smoke from incense placed at the target end

of the tunnel was used to determine the female moth release point.

Response of Female Moths to Host plants We compared the response of female GBM to flowers, unripe berries, ripe berries, mature leaves, or shoot tips (three to four immature leaves with tendrils) of *Vitis labrusca* (variety concord). All grape plants were grown from cuttings in 1-gal pots under greenhouse conditions at 21–26°C, with supplemental light extending the day length to 14 to 16 h. They were fertilized weekly with water-soluble fertilizer (Peters 20–20–20, Scotts-Sierra Horticultural Products Co., Marysville, OH, USA). Grape tissue was harvested and immediately placed into a water pick just prior to the start of assays. The volume of each tissue type presented had an approximate area of 10×10×10 cm. A portion of the grapevine stem was retained for all types of tissue used in the flight tunnel. Control flights were performed by using the acetone-washed copper stand without water pick as a target. The effect of mating status on flight response of female GBM was examined by using concord shoot tips as the target. We performed both experiments (tissue type and mating status) under optimized flight tunnel conditions defined by experiments described below.

Effect of Environmental and Physiological Factors on Flight Tunnel Responses In all tests, except where noted, we used live concord shoot tips as a target. The effects of diel periodicity on female flight were tested by comparing flight responses of mated and unmated females to shoot tips at dusk (in our experiment, this was a 2-h period including the hour before and after the start of the scotophase) and 2–4 h before dusk. The response of female GBM with a ceiling pattern was assessed by adding pieces of dark green paper, cut in the shape of grape leaves, on the glass ceiling below the light source, thereby creating a pattern of dark shapes that filled approximately 25% of the surface. We tested the effect of wind speed by adjusting the fan with a rheostat to create velocities of 0.25, 0.5, or 0.66 m/s (measured with a vaneometer from Dwyer Instruments, Inc., Michigan City, IN, USA) at the point of release for the moths.

Statistical Analyses The effect of tissue type (N=238), time of day (N=46), wind speed (N=25), ceiling pattern (N=30), and mating status (N=127) was analyzed by using generalized linear models with orientation or landing as dependent variables and time, wind speed, ceiling pattern, tissue type, or mating status as fixed independent variables by using binomial distribution with logit link function and maximum likelihood estimation (Proc Glimmix, SAS Institute 2006). Test effects were compared by using contrast statements and mean % response and standard errors were estimated with lsmeans statement with ilink option and presented in the figures (Proc Glimmix, SAS Institute 2006). We interpreted our results as odds ratios where odds are defined as the ratio between probability of event and probability of no event (Allison 2001). Odds were calculated by exponentiating coefficients of independent variables in Proc Glimmix output.

Results

Orientation to Different Tissue Types Females responded differently to different tissue types in terms of orientation (P<0.001) and landing (P<0.001) (Fig. 1). Females displayed the greatest level of orientation and landing to shoot tips, but this was not statistically different from the response to mature leaves (P=0.27 for orientation, P=0.17 for landing). Shoot tips were more attractive to females than unripe berries (P=0.014 for orientation, P=0.051 for landing), which in turn were more attractive than ripe berries (P=0.012 for orientation, P< 0.001 for landing) and flowers (P=0.004 for orientation, P=0.011 for landing). Ripe berries were not different from flowers (P=0.13 for orientation, P=0.55 for landing) although they were more attractive from control with respect to orientation (P=0.014) but not landing. Attractiveness of flowers to females was not different than control (P=0.26 for orientation, P=0.63 for landing).

Effects of Diel Periodicity on Flight Behavior The proportion of females that oriented to (P=0.010) and landed on

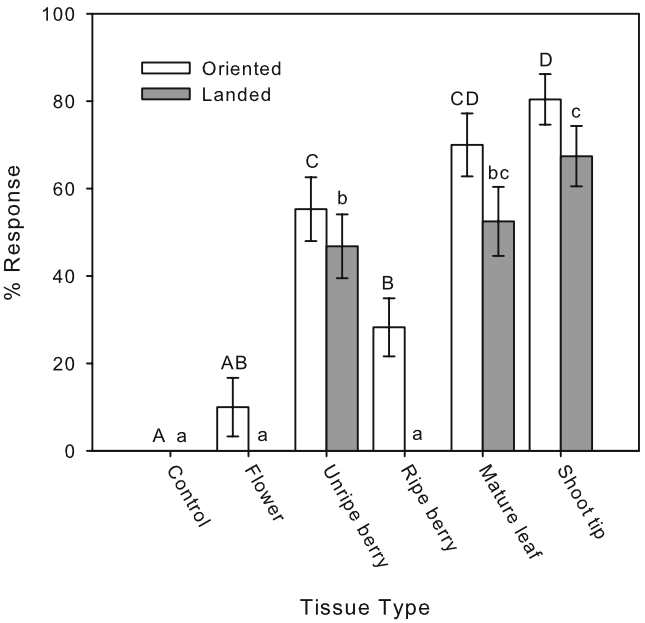

Fig. 1 Flight tunnel response (%) of female GBMs to different parts of the host plant, *V. labrusca*. Different *letters* (*capital letters* for orientation response and *small letters* for landing response) on *bars* indicate significant differences (P<0.05). Error bars=±1 SE

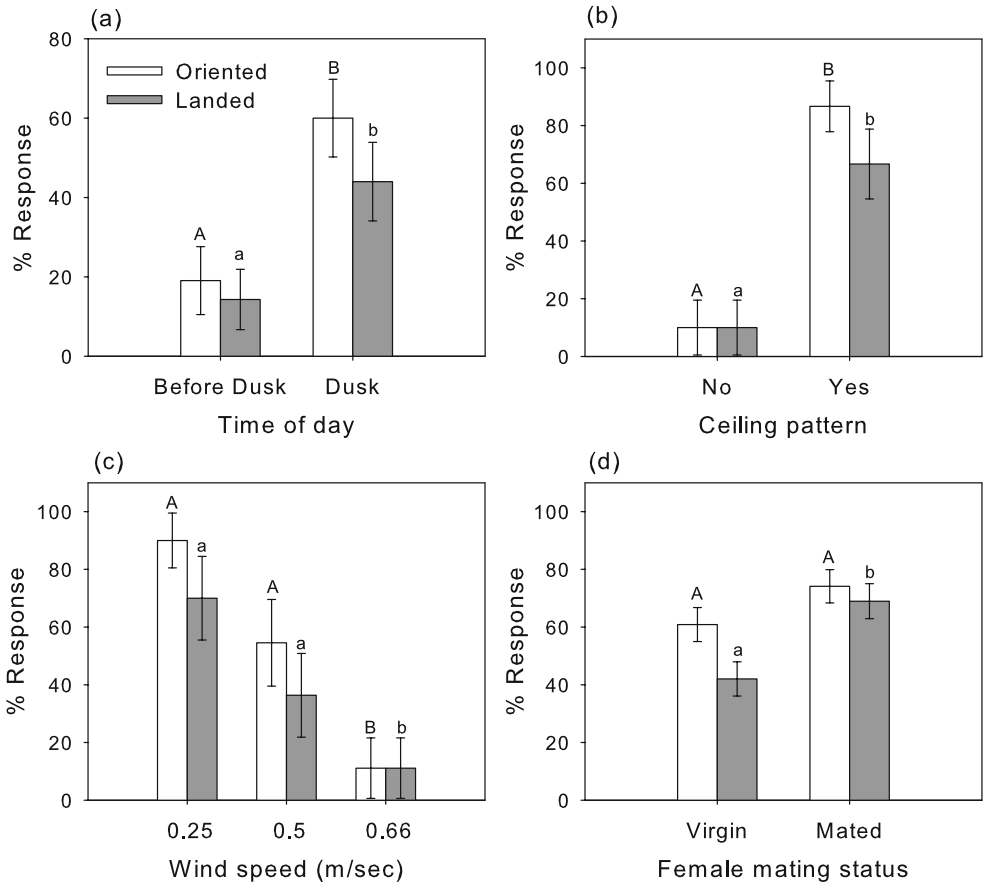

Fig. 2 Flight tunnel response (%) of female GBMs to shoot tips of *V. labrusca* when **a** flown at dusk (between 1 h before and after dusk time) or 2–4 h before dusk, **b** flown with or without a ceiling pattern, **c** flown at different wind speeds (0.25, 0.5, and 0.66 m/s), and **d** either virgin or mated. Different *letters* (*capital letters* for orientation response and *small letters* for landing response) on *bars* indicate significant differences ($P<0.05$). Error bars=±1 SE

shoots ($P=0.043$) was significantly higher at dusk than moths flown 2–4 h before dusk (Fig. 2a).

Effects of Flight Tunnel Environment The addition of a ceiling pattern to the flight tunnel increased the proportion of females orientating ($P=0.014$) to and landing on ($P=0.041$) grape shoots compared to the control (Fig. 2b). The effect of wind speed was marginally important for both orientation ($P=0.050$) and landing ($P=0.103$) responses of females to grape shoots (Fig. 2c) with significantly fewer females responding at 0.66 m/s than at the lower wind speeds.

Effect of Mating Status on Female Flight Behavior The mating status of females did not affect the orientation response ($P=0.150$) but more mated females landed at the source than virgins ($P=0.015$) (Fig. 2d).

Discussion

We compared attractiveness of different grape tissue types to female GBM as a first step in our effort to identify attractive host volatiles. Under the optimized flight tunnel condition determined by this study, we found that shoot tips and mature leaves are the two most attractive tissue types to female GBM. This appears somewhat counter intuitive, given that females lay eggs mostly on grape clusters. However, it is possible that they use shoot odors (shoot tips and mature leaves) to orient to the vines over long distances (several meters or more) but use different cues, including visual and chemotactile cues, to locate oviposition sites once within the canopy. We found that adjusting flight tunnel factors, such as wind speed and optomotor cues, along with consideration of biological factors, such as diel periodicity and mating status, can significantly impact on the orientation and landing responses of females. We observed that females had four to six times greater chance of locating the target around dusk than 2–4 h before dusk, which is not really surprising given that GBM is a crepuscular species and oviposition activity also peaks at dusk (Taschenberg 1945; Greg Loeb, unpublished data). Our findings are similar to those reported for the European grapevine moth, where responses to the host plant in the flight tunnel were significantly lower 6 h prior to dusk than at dusk (Hurtrel and Thiery 1999).

The addition of a ceiling pattern in addition to the existing one on the floor significantly increased orientation and landing female responses. In a previous study, male

spruce budworm moths responded better to sex pheromone in a flight tunnel with a ceiling compared to a floor pattern (Sanders et al. 1981). However, we did not compare the response of females when only a ceiling pattern was used.

In our study, the behavior of females improved as wind speed decreased. This might be because wind speed affects plume structure, which is important in the flight of moths (Willis and Baker 1984; Vickers and Baker 1992). In addition, the size of the odor active space and the maximum distance of communication decrease at higher wind speed (reviewed in Elkinton and Cardé 1984). Moreover, GBM is small and does not appear to be a strong flyer, and may have difficulty maintaining upwind flight in even moderate wind. Indeed, Botero-Garces and Isaacs (2004) found that movement of marked GBM in the field was greatly reduced at wind speeds of 0.6 m/s.

Mating is an important event in a moth's life and brings about many physiological and behavioral changes (Thompson and Pellmyr 1991). Our interest was to test whether mated and unmated females showed different flight responses to host plants in the flight tunnel. We found that mated females had a better chance of landing on grape shoots compared to unmated female moths, although there was no difference in the incidence of orientation. There is the potential that our results from the mating status experiment are confounded because mated moths were 5 days old while unmated moths were 3 days old. However, in separate experiments evaluating orientation to volatile blends, unmated 3-day-old females were not different than unmated 5-day-old females (Dong Cha, unpublished data), suggesting a minimal confounding effect between age and mating status in our experiment. In addition, the importance of mating status in the behavior of other Tortricidae moths (e.g., oviposition site selection of eastern spruce budworm moth described by Wallace et al. (2004)) supports our result.

Other studies with tortricid moths also have shown a similar increase in landing response by mated females (Hurtrel and Thiery 1999; Yang et al. 2005; Masante-Roca et al. 2007). This might be because mated females are ready to oviposit, and landing may indicate a transition from host searching to oviposition-related behaviors. Increased levels of landings by mated females could have resulted from being previously exposed to odors of grape clusters present in our cohort mating cages. Improved flight response in mated females pre-exposed to tansy flower volatile has been reported in European grapevine moth (Hurtrel and Thiery 1999). However, in our environmental chamber, air moved freely among rearing and cohort chambers, so mated and unmated moths should have shared all the volatiles in the air. Thus, it would appear probable that physiological and neurological changes that follow mating could explain the increased probability of females landing.

The flight tunnel has proved to be an essential tool in developing a synthetic lure for the sex pheromone where the bioassay system relates well to what is happening in the field. Our finding showing that female GBM respond to host plant volatiles, especially under optimized flight tunnel conditions, suggests it has potential to develop synthetic host plant lures. We will be using this approach for future tests on isolated volatiles, as these tools will help in our efforts to improve a GBM monitoring system for use in management of this key grape pest.

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References

ALLISON, P. D. 2001. Logistic Regression Using the SAS System: Theory and Application. SAS Institute, Cary, N.C.

ANSEBO, L., CORACINI, M. D. A., BENGTSSON, M., LIBLIKAS, I., RAMIREZ, M., BORG-KARLSON, A. K., TASIN, M., and WITZGALL, P. 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J. Appl. Entomol.* 128:488–493.

ARN, H. 1991. Sex pheromones, pp. 187– 208 in L. P. S. Van der Geest and H. H. Evenhuis (eds.). Tortricid Pests. Their Biology, Natural Enemies and Control. Elsevier, New York, NY.

BOTERO-GARCES, N., and ISAACS, R. 2004. Movement of the grape berry moth, *Endopiza viteana*: displacement distance and direction. *Physiol. Entomol.* 29:443–452.

BRUCE, T. J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2005. Insect host location: a volatile situation. *Trends. Plant Sci.* 10:269–274.

CARDÉ, R. T. 1984. Chemo-orientation in flying insects. pp. 111–124, in W. J. BELL and R.T. CARDÉ (ed.). Chemical Ecology of InsectsChapman and Hall, London, UK.

ELKINTON, J. S., and CARDÉ, R. T. 1984. Odor dispersion, pp. 73–91, in W. J BELL, and R.T. CARDÉ (eds.). Chemical Ecology of InsectsChapman and Hall, London, UK.

HERN, A., and DORN, S. 2004. A female-specific attractant for the codling moth, *Cydia pomonella*, from apple fruit volatiles. *Naturwissenschaften* 91:77–80.

HOFFMAN, C. J. 1990. Development and validation of a risk assessment program for the management of grape berry moth, *Endopiza viteana* (Clemens), in New York state. PhD Dissertation, Cornell University, Ithaca, NY.

HOWSE P., STEVENS, I., and JONES, O. 1998. Insect Pheromones and Their Use in Pest Management. Chapman & Hall, London, UK.

HURTREL, B., and THIERY, D. 1999. Modulation of flight activity in *Lobesia botrana* Den. and Schiff. (Lepidoptera: Tortricidae) females studied in a wind tunnel. *J. Insect Behav.* 12:199–211.

MASANTE-ROCA, I., ANTON, S., DELBAC, L., DUFOUR, M.-C., and GADENNE, C. 2007. Attraction of the grapevine moth to host and non-host plant parts in the wind tunnel: effects of plant phenology, sex, and mating status. *Entomol. Exp. Appl.* 122:239–245.

NAGARKATTI, S., MUZA, A., and SAUNDERS, M. 2000. Meridic diet for *Endopiza viteana* (Lepidoptera: Tortricidae). *Can. Entomol.* 132:259–261.

NATALE, D., MATTIACCI, L., PASQUALINI, E., and DORN, S. 2004. Apple and peach fruit volatiles and the apple constituent butyl hexanoate attract female oriental fruit moth, *Cydia molesta*, in the laboratory. *J. Appl. Entomol.* 128:22–27.

NOJIMA, S., LINN, C, and ROELOFS, W. 2003a. Identification of host fruit volatiles from flowering dogwood (*Cornus florida*) attractive to dogwood-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol.* 29:2347–2357.

NOJIMA, S., LINN, C., MORRIS, B., ZHANG, A. J, and ROELOFS, W. 2003b. Identification of host fruit volatiles from hawthorn (*Crataegus* spp.) attractive to hawthorn-origin *Rhagoletis* pomonella flies. *J. Chem. Ecol.* 29:321–336.

REED, H. C., and LANDOLT, P. J. 2002. Attraction of mated female codling moths (Lepidoptera: Tortricidae) to apples and apple odor in a flight tunnel. *Florida Entomol.* 85:324–329.

RIDGWAY, R. L., SILVERSTEIN, R. M., and INSCOE, M. N. 1990. Behavior-modifying Chemicals for Insect Management: Applications of Pheromones and Other Attractants. Marcel Dekker, New York, NY.

ROELOFS, W. L., TETTE, J. P., TASCHENBERG, E. F. , and COMEAU, A. 1971. Sex pheromone of the grape berry moth: identification by classical and electroantennogram methods, and field tests. *J. Insect Physiol.* 17:2235–2243.

SANDERS, C. J., LUCUIK, G. S., and FLETCHER, R. M. 1981. Response of male spruce budworm (Lepidoptera: Tortricidae) to different concentrations of sex pheromone as measured in a sustained-flight wind tunnel. *Can. Entomol.* 113:943–948.

Sas Institute. 2006. The GLIMMIX Procedure. <http://www.sas.com>.

TASCHENBERG, E. F. 1945. The biology and control of the grape berry moth *Polychrosis vineana* (Clemens). PhD Dissertation, Cornell University, Ithaca, NY.

TASIN, M., ANFORA, G., IORIATTI, C., CARLIN, S., DE CRISTOFARO, A., SCHMIDT, S., BENGTSSON, M., VERSINI, G., and WITZGALL, P. 2005. Antennal and behavioral responses of grapevine moth *Lobesia botrana* females to volatiles from grapevine. *J. Chem. Ecol.* 31:77–87.

TASIN, M., BACKMAN, A. C., BENGTSSON, M., IORIATTI, C., and WITZGALL, P. 2006a. Essential host plant cues in the grapevine moth. *Naturwissenschaften* 93:141–144.

TASIN, M., BACKMAN, A. C., BENGTSSON, M., VARELA, N., IORIATTI, C., and WITZGALL, P. 2006b. Wind tunnel attraction of grapevine moth females, *Lobesia botrana*, to natural and artificial grape odour. *Chemoecology* 16:87–92.

TASIN, M., BACKMAN, A.-C., CORACINI, M., CASADO, D., IORIATTI, C., and WITZGALL, P. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. *Phytochemistry* 68:203–209.

THOMPSON, J. N., and PELLMYR, O. 1991. Evolution of oviposition behavior and host preference in Lepidoptera. *Annu. Rev. Entomol.* 36:65–89.

VAN DER GEEST, L. P. S., and EVENHUIS, H. H. 1991. Tortricid pests. Their Biology, Natural Enemies and Control. Elsevier, New York, NY.

VICKERS, N. J., and BAKER, T. C. 1992. Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone (Lepidoptera, Noctuidae). *J. Insect Behavior* 5:669–687.

WALLACE, E. K., ALBERT, P. J., and MCNEIL, J. N. 2004. Oviposition behavior of the eastern spruce budworm *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *J. Insect Behavior* 17:145–154.

WEIGLE, T., BIXBY, J., and ENGLISH-LOEB, G. 1999. Reexamination of grape berry moth management practices in the Lake Erie region. 1998 New York State Fruit Project Reports Relating to IPM. NYS IPM Publication #216. Cornell University Cooperative Extension.

WILLIS, M. A., and BAKER, T. C. 1984. Effects of intermittent and continuous pheromone stimulation on the flight behavior of the oriental fruit moth, *Grapholita molesta*. *Physiol. Entomol.* 9:341–358.

YANG, Z. H., CASADO, D., IORIATTI, C., BENGTSSON, M., and WITZGALL, P. 2005. Pheromone pre-exposure and mating modulate codling moth (Lepidoptera: Tortricidae) response to host plant volatiles. *Agric. For. Entomol.* 7:231–236.

ZHANG, A. J., LINN, C., WRIGHT, S., PROKOPY, R., REISSIG, W., and ROELOFS, W. 1999. Identification of a new blend of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella*. *J. Chem. Ecol.* 25:1221–1232.

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Belowground Chemical Signaling in Maize: When Simplicity Rhymes with Efficiency

Ivan Hiltpold · Ted C. J. Turlings

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Abstract Maize roots respond to feeding by larvae of the beetle *Diabrotica virgifera virgifera* by releasing (*E*)-β-caryophyllene. This sesquiterpene, which is not found in healthy maize roots, attracts the entomopathogenic nematode *Heterorhabditis megidis*. In sharp contrast to the emission of virtually only this single compound by damaged roots, maize leaves emit a blend of numerous volatile organic compounds in response to herbivory. To try to explain this difference between roots and leaves, we studied the diffusion properties of various maize volatiles in sand and soil. The best diffusing compounds were found to be terpenes. Only one other sesquiterpene known for maize, α-copaene, diffused better than (*E*)-β-caryophyllene, but biosynthesis of the former is far more costly for the plant than the latter. The diffusion of (*E*)-β-caryophyllene occurs through the gaseous rather than the aqueous phase, as it was found to diffuse faster and further at low moisture level. However, a water layer is needed to prevent complete loss through vertical diffusion, as was found for totally dry sand. Hence, it appears that maize has adapted to emit a readily diffusing and cost-effective belowground signal from its insect-damaged roots.

Keywords Belowground tritrophic interactions · (*E*)-β-caryophyllene · Roots · Diffusion · Entomopathogenic nematodes · Indirect plant defense · Plant–insect interactions

I. Hiltpold · T. C. J. Turlings (✉)
University of Neuchâtel, Institute of Biology, FARCE,
Case Postale 158,
CH-2009 Neuchâtel, Switzerland
e-mail: ted.turlings@unine.ch

Introduction

Plants defend themselves against herbivores either directly with the use of toxins, repellents, or morphological structures (Karban et al. 1997; Karban and Baldwin 1997; Schoonhoven et al. 1998; Agrawal and Rutter 1998; Baldwin and Preston 1999; Dicke et al. 2003), or indirectly by attracting the enemies of herbivores (Dicke and Sabelis 1988; Agrawal 1998; Dicke and Hilker 2003; Turlings and Wäcker 2004). The role of volatiles in the attraction of such natural enemies was first brought to light in studies on the interactions between arthropods and plant leaves. Results revealed that herbivore attack induces emissions of volatile organic compounds (VOC) attractive to parasitoids and predators (Dicke and Sabelis 1988, Turlings et al. 1995; De Moraes et al. 1998), and this role of induced volatiles in tritrophic interactions has also been demonstrated under realistic field conditions (Bernasconi et al. 1998; De Moraes et al. 1998; Thaler 1999; Kessler and Baldwin 2001). While the aboveground portion of a plant is obviously essential for photosynthesis and reproduction, the root system is also of vital importance. Little is known about possible indirect defense mechanisms against root feeders, but several studies now have demonstrated that roots also are able to recruit enemies of herbivores by releasing chemical cues into soil. These chemicals can attract entomopathogenic nematodes (Boff et al. 2001; van Tol et al. 2001; Bertin et al. 2003), predatory mites (Aratchige et al. 2004), and even parasitoids (Neveu et al. 2002). In *Zea mais* L., feeding by larvae of the western corn rootworm (WCR), *Diabrotica virgifera virgifera* LeConte (Coleoptera: Chrysomelidae), results in the release of a few sesquiterpenoids, dominated by (*E*)-β-caryophyllene (EβC), which is attractive to the entomopathogenic nema-

tode (EPN) *Heterorhabditis megidis* Poinar (Rhabditida: Heterorhabditidae) (Rasmann et al. 2005).

Western corn rootworm is the most destructive pest of maize in the United States and its introduction in Europe (Miller et al. 2005) also has generated concern among maize growers in the Old World (Vidal et al. 2005). Several strategies have been used worldwide to control WCR populations, of which crop rotation has thus far been the most effective (Levine et al. 1992). However, certain US populations have developed an extended diapause (Tollefson 1988; Levine et al. 1992) or the ability to develop on soybean roots (O'Neil et al. 2002), and other control measures are being considered. Some potential biological control agents have been identified (Toepfer and Kuhlmann 2004). Field results with such agents have been variable (Jackson 1996; Journey and Ostlie 2000; McCoy et al. 2002), but EPNs are the most promising (Gaugler et al. 1997; Kuhlmann and Burgt 1998). EPNs are obligate parasites that kill insect hosts with the aid of mutualistic bacteria (Forst and Nealson 1996; Burnell and Stock 2000). The effectiveness of *H. megidis*, a promising nematodes against WCR, is strongly correlated with the emission of EβC (Rasmann et al. 2005). A good understanding of the mechanism by which EβC attracts EPNs could help to improve their efficacy.

The release by the roots of only one dominant compound in response to herbivory contrasts strongly with insect-damaged maize leaves, which release a complex blend of green leaf volatiles, aromatic compounds, and various terpenoids (Degen et al. 2004). We hypothesized that this difference between roots and leaves is due to the chemical properties of EβC, which might make it particularly suitable for belowground diffusion. To test this, we compared the diffusion of EβC with those of other typical maize volatiles that the roots could potentially emit.

Materials and Methods

Experiments were carried out in clean sand that had been passed through a 2mm sieve and autoclaved to obtain a homogeneous, air-dried, and VOC-free medium. Deionized water was added in precise quantities to obtain specific humidity levels. Humidity was 10% in all initial experiments, and was obtained by adding 50ml deionized water to 450g of sand in a Teflon-box (12 × 10 × 4cm; 480cm^3, internal dimensions), maintaining constant porosity at about 90%. The box was put on a thermal tray, maintaining the temperature at 12°C. A 0.2mm diameter cylinder made of ultra-fine metal mesh (2300 mesh; Small Parts Inc., USA) was inserted into the sand, thereby creating a hole in which a solid-phase microextraction (SPME) fiber could be safely inserted. Automated sampling was performed with a 100μm polydimethylsiloxane SPME fiber (Supelco, Buchs, Switzerland) within 12h with a multipurpose sampler (MPS2, Gerstel GmbH & Co. KG, Germany) (Koziel et al. 2000; Gorecki and Namiesnik 2002; Vas and Vekey 2004). At 30min intervals, the adsorbed compounds were analyzed by retracting the fiber from the sand and inserting it for 3min in the injector of an Agilent 6890 Series gas chromatograph heated at 230°C (G1530A) coupled to a quadrupole-type mass-selective detector (Agilent 5973;

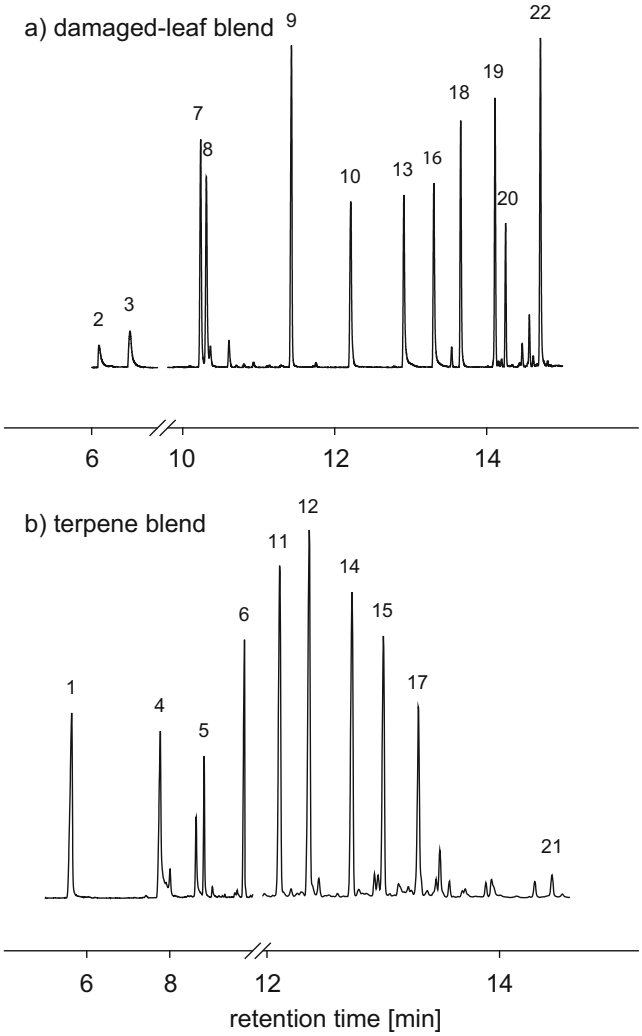

Fig. 1 Representative GC-MS chromatograms obtained by sampling just above the two synthetic blends that were used for the diffusion experiments. **a** analysis after collection with a 100μm polydimethylsiloxane SPME fiber of a synthetic mix of typical leaf volatiles. **b** analysis after collection with a 75μm corboxen™-polydimethylsiloxane SPME fiber of a blend of selected terpenoids. Labeled peaks are as follow: (*1*) (+)-α-pinene, (*2*) (E)-2-hexenal, (*3*) (Z)-3-hexenol, (*4*) β-myrcene, (*5*) cis-ocimene, (*6*) and (*9*) linalool, (*7*) β-myrcene, (*8*) (Z)-3-hexenyl acetate, (*10*) methyl salicylate, (*11*) (−)-α-cubebene, (*12*) (−)-α-copaene, (*13*) indole, (*14*) and (*19*) EβC, (*15*) α-humulene, (*16*) methyl anthranilate, (*17*) valencene, (*18*) geranyl acetate, (*20*) (E)-β-farnesene, (*21*) caryophyllene oxide and (*22*) ($^+/_-$)-trans-nerolidol

transfer line 230°C, source 230°C, ionization potential 70eV). The desorbed volatiles were separated on a polar column (HP1-MS, 30m, 0.25mm ID, 0.25μm film; Agilent Technologies, USA) using helium as a carrier gas (constant pressure of 127.9kPa). Following injection, the column temperature was maintained at 40°C for 1min and then increased 20°C min^{-1} to 250°C, where it was held for another 12min. After the first 30min sampling period, a synthetic mixture of typical caterpillar-induced leaf volatiles (0.2μg for each compound) (Turlings and Ton 2006) dissolved in 5μl of pentane (>99%; Acros Organic) (Fig. 1a) was injected 3cm into the sand. The injection site was located 0.5cm from the edge of the tray, opposite the fiber insertion site. Measurements with the fiber were done at four distances (1.5, 3, 6, and 10cm) from the injection point, and were replicated 5 times for each distance.

The results of the first experiment prompted an additional similar diffusion test with a blend that mainly comprised terpenoids (Fig. 1b). The set-up was the same as described above except that we sampled only at 10cm from the source and a 75μm Corboxen™-polydimethylsiloxane SPME fiber was used, because of its higher affinity for terpenes.

Diffusion also was assessed in a standard soil (type 5M, LUFA Speyer, Germany) that was first autoclaved. Experiments were conducted with the two synthetic blends described above (Fig. 1) following the same experimental set-ups as for the sand experiments, but sampling was only done at 10cm of the release point.

The effect of moisture level on EβC diffusion was determined by measuring horizontal diffusion of a synthetic version injected at 0.2μg/5μl pentane. Porosity of sand was maintained constant by adding a fixed mass of medium (500g) into the Teflon box. The ratio between sand and water was adjusted to obtain moisture levels of 0%, 1%, 5%, or 10% water.

All experiments were replicated five times. Chromatograms were analyzed with ChemStation (version D.00.00.38, Agilent Technologies), and peak areas of VOCs were compared with analysis of variance (ANOVA) and *t*-test. Differences among compounds and humidity levels were determined by using a Bonferroni post hoc test. All analyses were run on SigmaStat (version 2.03, Access Softek Inc.).

Results

Diffusion of Typical Maize Leaf Volatiles At concentrations of 0.2μg/5μl, the amount and number of VOCs adsorbed onto the SPME fiber decreased drastically with sampling distance (Fig. 2). At 10cm, only three terpenes EβC, β-myrcene, and (*E*)-β-farnesene were detected (Fig. 3). Of these, EβC diffused best (one-way ANOVA, $N = 5$, $F_{2,14} = 6.95$, $P = 0.01$) and was detected longer after the injection.

Diffusion of Terpenoids in Sand All terpenoids, with the exception of caryophyllene oxide diffused readily through sand and were detected 10cm from the source, but the amounts detected were considerably different for the different compounds. Data shown in Fig. 4 include only the four terpenes that are emitted by WCR-damaged maize roots (Rasmann et al. 2005). Of these, β-copaene diffused best, followed by EβC, which diffused equally well as the structurally similar α-humulene (one-way ANOVA, $N = 5$, $F_{2,14} = 21.57$, $P < 0.001$). As we did not detect any diffusion of caryophyllene oxide, we omitted this compound from the statistical analyses.

Diffusion of Terpenes in Soil Differences in how the terpenes diffused in soil were similar to those in sand, but the amount of each chemical recovered at 10cm from the source was considerably lower (Fig. 4). Again, β-copaene

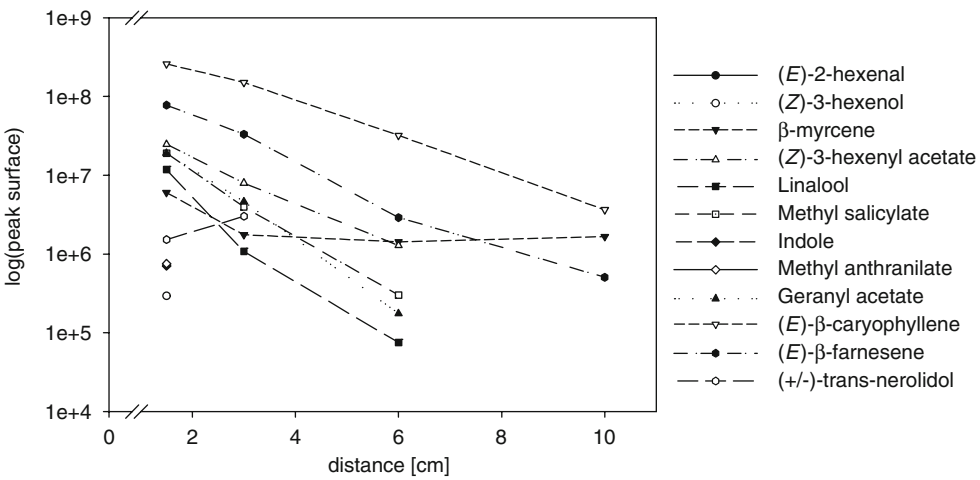

Fig. 2 Diffusion of a synthetic blend along 10cm in a sand medium. VOCs were sampled 1.5, 3, 6, and 10cm from the odor source. Only terpenes were diffused as far as 10cm. All the other compounds were not detected at distances of more than 6cm. Trans-nerolidol, methyl anthranilate and indole were detected only 1.5cm from the source. Detection directly at the release point was not possible because of fiber saturation

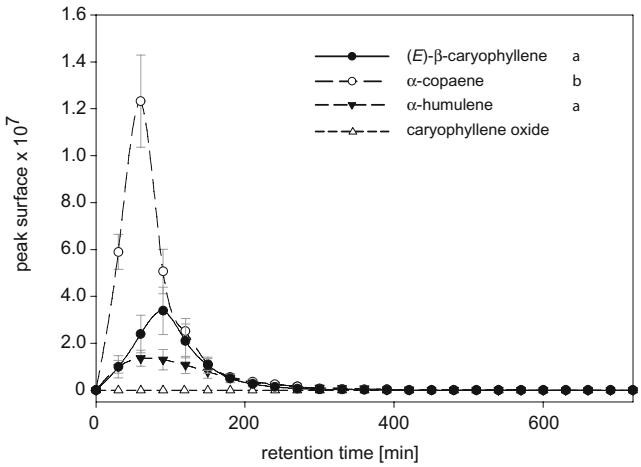

Fig. 3 Diffusion of an authentic terpenoids blend measured at 10 cm from the source. GC-MS peak surfaces of EβC, α-copaene and α-humulene reached their maximum at 72, 30, and 128 min (mean time) after injection, respectively. Maximum GC-MS peak surfaces differed significantly (one-way ANOVA, N=5, $F_{2,14}$=6.95, P=0.01). Letters next to the compound names indicate significant differences between compounds (Bonferroni posthoc test)

diffused better than EβC (*t*-test, $N = 5$, $P = 0.039$). α-Humulene and caryophyllene oxide were not detected in soil.

Moisture Level and EβC Diffusion The amount of water present in the sand strongly affected EβC diffusion. An almost twofold larger amount of EβC was detected at a humidity level of 1% than at 10% humidity. However, a water layer is needed to avoid loss by vertical diffusion: no EβC was detected when the sand was completely free of water (Fig. 5, one-way ANOVA, $N = 5$, $F_{2,14} = 21.47$, $P < 0.001$). As we did not detect EβC in dry sand, results for this treatment were omitted from the statistical analyses.

Discussion

The results support our hypothesis that EβC is particularly well-suited as a belowground signal because of its diffusion properties. The only other maize-produced compound that diffused better than EβC was α-copaene. All other compounds were consistently detected at lower amounts away from the release point or not detected at all. The limited detection of these other compounds could be explained by early evaporation (vertical diffusion), which is likely to be the case for compounds with low molecular weight (Lowell and Eklund 2004) such as (*E*)-2-hexanal and (*Z*)-3-hexanol. Besides vertical diffusion, adsorption onto colloids (polar particles) within the media could slow down or stop both vertical and horizontal diffusion. Although adsorption is expected to be relatively low in sand (Ruiz et al. 1998), silicates on the surface of sand particles, and also the aqueous phase of the medium, could adsorb many of the compounds that were tested. This was evident from a study in which a blend of induced maize volatiles was pushed through a silica-filter, and several of the volatiles that did not diffuse in the current study were found trapped on the filter (D'Alessandro and Turlings 2005). As adsorption does not alter chemical properties, adsorption of VOCs onto static silicates could enhance the establishment of a chemical gradient over a longer period of time (McGechan and Lewis 2002), thus allowing EPNs to follow a chemical trail towards potential hosts.

Terpenes were the best diffusing compounds, possibly explaining why WCR-damaged maize roots exude mainly terpenes, even though maize is able to synthesize many other VOCs (Figs. 2 and 4). EβC clearly diffuses better than α-humulene, the other sesquiterpene released by maize roots, but we detected up to two times more β-copaene in

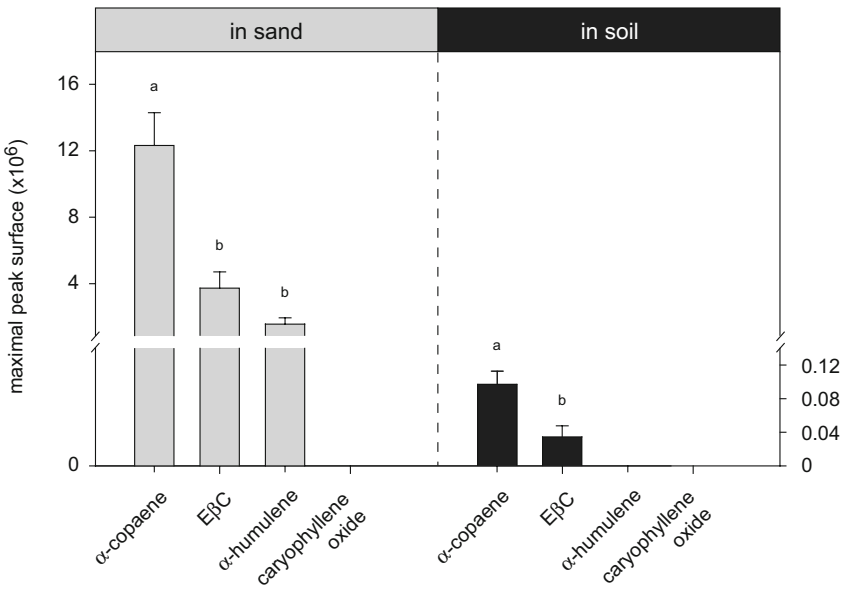

Fig. 4 Diffusion of terpenoids in sand and in soil. VOCs were sampled 10 cm from the odor source. In sand, α-copaene diffused significantly better than the other compounds (one-way ANOVA, N=5, $F_{2,14}$=21.47, P<0.001). The same pattern of diffusion was observed in soil (*t*-test, N=5, P=0.039). Undetected compounds were excluded from the statistic

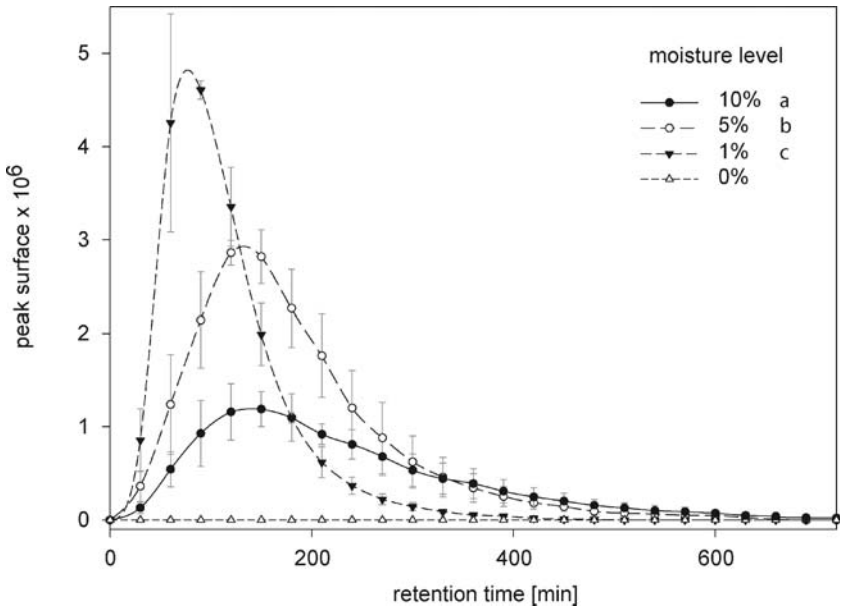

Fig. 5 Diffusion of EβC under different moisture levels measured at 10 cm from the source. Peak surfaces of EβC with 1%, 5%, and 10% of water in sand reached their maximum at 80, 140, and 160 min after injection, respectively. Diffusion velocity tended to increase with lower moisture levels, but there was no statistical difference among the three moisture levels (data not shown) (one-way ANOVA, N=5, $F_{2,14}$=2.17, P=0.19). Maximum GC-MS peak surfaces differed significantly (one-way ANOVA, N=5, $F_{2,14}$=21.47, P<0.001). *Letters* indicate significant differences between compounds (Bonferroni post hoc test). As no diffusion was detected in dry sand (0%), it was excluded from the statistical analyses

sand 10cm from the source than EβC, and this difference was similar in soil (Fig. 4, in soil). That β-copaene diffuses better than EβC (Fig. 4) may reflect their respective adsorption strengths onto the substrate. The most likely explanation why roots emit EβC rather than β-copaene as a belowground signal comes from what is known about their respective biosyntheses. While there is much speculation and some disagreement about the evolution and function of VOCs (Firn and Jones 2000; Peñuelas and Llusia 2004; Owen and Penuelas 2005, 2006; Firn and Jones 2006), researchers agree on at least one principle: production of such herbivore-induced plant volatiles can require considerable resource investment. It has been reported that some plants may allocate up to 10% of their carbon for the production of VOCs (Firn and Jones 2006). Considering this potential cost, plants can be expected to have adapted the energetically cheapest solutions. The precursor for the maize sesquiterpenes is the same, farnesyl diphosphate (FPP). When WCR larvae feed on roots, this triggers a cascade of reactions that lead to the production of two different enzymes. The first reacts with FPP to catalyze the production of EβC and α-humulene, and the second reacts with the same precursor, but forms β-copaene and approximately 50 other compounds (T. Köllner, personal communication), (Fig. 6). Because of the production of these additional compounds, a plant produces nine times more EβC than β-copaene with the same number of FPP molecules. Hence, even if EβC diffusion is half as

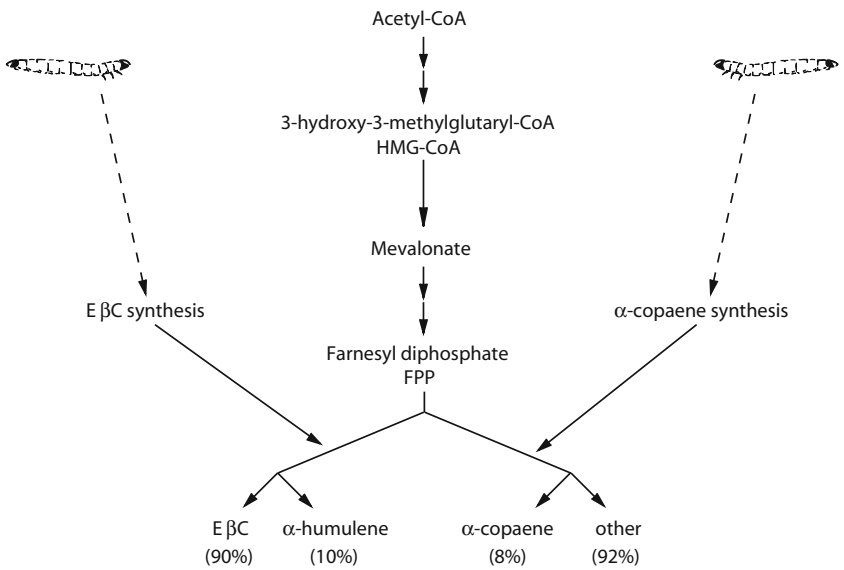

Fig. 6 Schematic model of the metabolic pathway for EβC synthesis. When WCR larvae feed on roots this triggers a cascade of reactions resulting in the production of two different enzymes that lead to two separate pathways of volatile synthesis. The ratio in which the volatiles are produced from these pathways implies a much more cost-effective production of EβC than of β-copaene (T. Köllner, personal communication)

efficient as β-copaene, the fact that it is far less expensive for the plant to produce should counterbalance this difference.

The moisture level of the substrate strongly affected EβC diffusion (Fig. 5). The detected abundance and the velocity of horizontal diffusion were negatively correlated with the water volume present in the sand. Porosity was maintained constant. Therefore, the addition of water reduced the gaseous phase volume. Thus, the results confirm the notion that the diffusion of EβC occurs in the gaseous phase, which was expected because of its poor solubility in water. Low moisture levels enhance horizontal diffusion of EβC, but a complete lack of water dramatically reduces it. This is best explained by a need for a thin layer of water to avoid quick vertical evaporation of the sesquiterpene.

EβC is a common compound and has been identified from various plant species (e.g., Rodriguez-Saona et al. 2001; Tholl et al. 2005; Calyecac-Cortero et al. 2007; Cheng et al. 2007; Helmig et al. 2007). Its function, as for most plant volatiles, remains unclear. Sesquiterpenes in general may have anti-microbial or insecticidal effects (e.g., Erasto et al. 2006; Liu et al. 2006; Sabulal et al. 2006; Ji et al. 2007), but at the dose emitted by maize roots it is unlikely to be effective against insects (Rasmann et al. 2005). Interestingly, EβC is also emitted from maize silk and has been implicated in the attraction of adult *Diabrotica* beetles (Hammack 2001), but recent behavioral assays suggest that other plant compounds are considerably more important for adult attraction (Tòth et al. 2007). Belowground sesquiterpenes (strigolactone) also have been found to play a role in the symbiosis between roots and arbuscular mycorrhiza as fungi branching factor (Akiyama et al. 2005) and in germination of *Striga*, *Alectra*, and *Orobanche* species (Butler 1995). Hence, it is important to stress that multiple functions and modes of selection for EβC or other sesquiterpenes must be considered. The recently identified sequiterpene-synthase gene TPS23, which is responsible for EβC production in maize (Köllner et al. 2008), was found to be highly conserved, implying an important function for the plant. There is little information about herbivore-induced root volatiles from other plants. A comparison among the emissions from maize, cotton, and cowpea roots in response to feeding by *Diabrotica balteata* larvae has revealed the typical release of EβC by maize roots, a more complex mixture of terpenoids emitted from cotton roots, including α-copaene, but no detectable amounts of EβC, whereas no volatiles were detected from attacked cowpea roots (Rasmann and Turlings 2008). Nematodes are most attracted to damaged maize roots and far less to damaged cowpea roots (Rasmann and Turlings 2008), which is in accordance with an important role for EβC in the attraction.

This study set out to explain why the induced emission of volatiles in maize roots is basically limited to just one dominant sesquiterpene, EβC. The hypothesized explanation that this compound would be particularly suited as a belowground signal because of its diffusion properties was confirmed. The only maize-produced compound that appeared to be even better at diffusing in the substrates tested was β-copaene, another sesquiterpene. A plausible reason why the plant has evolved to emit EβC is that β-copaene is more costly to produce. Given the logic behind these explanations, we should perhaps now turn the question around and wonder why the leaves emit such complex blends if they could be so much simpler.

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References

AGRAWAL, A. A., and RUTTER, M. T. 1998. Dynamic anti-herbivore defense in ant-plants: the role of induced responses. *Oikos* 83:227–236.

AGRAWAL, A. A. 1998. Induced responses to herbivory and increased plant performance. *Science* 29:1201–1202.

AKIYAMA, K., MATSUZAKI, K., and HAYASHI, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435:824–827.

ARATCHIGE, N. S., LESNA, I., and SABELIS, M. W. 2004. Belowground plant parts emit herbivore-induced volatiles: olfactory responses of a predatory mite to tulip bulbs infested by rust mites. *Exper. Appl. Acarology* 33:21–30.

BALDWIN, I. T., and PRESTON, C. A. 1999. The eco-physiological complexity of plant responses to insect herbivores. *Planta* 208:137–145.

BERNASCONI, M. L., TURLINGS, T. C. J., AMBROSETTI, L., BASSETTI, P., and DORN, S. 1998. Herbivore-induced emissions of maize volatiles repel the corn leaf aphid, *Rhopalosiphum maidis*. *Entomol. Exp. Appl.* 87:133–142.

BERTIN, C., YANG, X., and WESTON, L. A. 2003. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256:67–83.

BOFF, M. I. C., ZOON, F. C., and SMITS, P. H. 2001. Orientation of *Heterorhabditis megidis* to insect hosts and plant roots in a Y-tube sand olfactometer. *Entomol. Exp. Appl.* 98:329–337.

BURNELL, A. M., and STOCK, S. P. 2000. *Heterorhabditis*, *Steinernema* and their bacterial symbionts—lethal pathogens of insects. *Nematology* 2:31–42.

BUTLER, L. G. 1995. Chemical communication between the parasitic weed Striga and its crop host—a new dimension in allelochemistry. pp. 158–168, in KINDERJIT , and FAEINHELLIG (eds.). Insights into Allelopathy, ACS Symposium SeriesACS Books, Washington, DC.

CALYECAC-CORTERO, H. G., CIBRIAN-TOVAR, J., SOTO-HERNANDEZ, M., and GARCIA-VELASCO, R. 2007. Isolation and identification

of *Physalis philadelphica Lam.* volatiles. *Agrociencia* 41:337–346.

CHENG, A. X., XIANG, C. Y., LI, J. X., YANG, C. Q., HU, W. L., WANG, L. J., LOU, Y. G., and CHEN, X. Y. 2007. The rice (*E*)-beta-caryophyllene synthase (OsTPS3) accounts for the major inducible volatile sesquiterpenes. *Phytochemistry* 68:1632–1641.

D'ALESSANDRO, M., and TURLINGS, T. C. J. 2005. In Situ modification of herbivore-induced plant odors: a novel approach to study the attractiveness of volatile organic compounds to parasitic wasps. *Chem. Senses* 30:739–753.

DEGEN, T., DILLMANN, C., MARION-POLL, F., and TURLINGS, T. C. J. 2004. High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135:1928–1938.

DE, MORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., and HILKER, M. 2003. Induced plant defences: from molecular biology to evolutionary ecology. *Basic Appl. Ecol.* 4:3–14.

DICKE, M., VAN POECKE, R. M. P., and DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* 4:27–42.

ERASTO, P., GRIERSON, D. S., and AFOLAYAN, A. J. 2006. Bioactive sesquiterpene lactones from the leaves of *Vernonia amygdalina*. *J. Ethnopharmacol.* 106:117–120.

FIRN, R. D., and JONES, C. G. 2000. The evolution of secondary metabolism - a unifying model. *Mol. Microbiol.* 37:989–994.

FIRN, R. D., and JONES, C. G. 2006. Do we need a new hypothesis to explain plant VOC emissions? *Trends Plant. Sci.* 11:112–113.

FORST, S., and NEALSON, K. 1996. Molecular biology of the symbiotic pathogenic bacteria *Xenorhabdus* spp and *Photorhabdus* spp. *Microbiol. Rev.* 60:21–43.

GAUGLER, R., LEWIS, E., and STUART, R. J. 1997. Ecology in the service of biological control: The case of entomopathogenic nematodes. *Oecologia* 109:483–489.

GORECKI, T., and NAMIESNIK, J. 2002. Passive sampling. *Trends Analyt. Chem.* 21:276–291.

HAMMACK, L. 2001. Single and blended maize volatiles as attractants for diabroticite corn rootworm beetles. *J. Chem. Ecol.* 27:1373–1390.

HELMIG, D., ORTEGA, J., DUHL, T., TANNER, D., GUENTHER, A., HARLEY, P., WIEDINMYER, C., MILFORD, J., and SAKULYANONTVITTAY, T. 2007. Sesquiterpene emissions from pine trees - Identifications, emission rates and flux estimates for the contiguous United States. *Environ. Sci. Technol.* 41:1545–1553.

JACKSON, J. J. 1996. Field performance of entomopathogenic nematodes for suppression of western corn rootworm (Coleoptera: Chrysomelidae). *J. Econ. Entomol.* 89:366–372.

JI, Z. Q., WU, W. J., YANG, H., SHI, B. J., and WANG, M. G. 2007. Four novel insecticidal sesquiterpene esters from *Celastrus angulatus*. *Nat. Prod. Res.* 21:334–342.

JOURNEY, A. M., and OSTLIE, K. R. 2000. Biological control of the western corn rootworm (Coleoptera: Chrysomelidae) using the entomopathogenic nematodes, *Steinernema carpocapsae*. *Environ. Entomol.* 29:822–831.

KARBAN, R., AGRAWAL, A. A., and MANGEL, M. 1997. The benefits of induced defenses against herbivores. *Ecology* 78:1351–1355.

KARBAN, R., and BALDWIN, I. 1997. Induced Responses to Herbivory. University Press of Chicago, Chicago.

KESSLER, A., and BALDWIN, I. T. 2001. Defensive function of herbivore-induced plant volatile emissions in nature. *Science* 291:2141–2144.

KÖLLNER, T., HELD, M., LENK, C., HILTPOLD, I., TURLINGS, T. C. J., GERSGENZON, J., and DEGENHARDT, J. 2008. A maize (*E*)-β-caryophyllene synthase implicated in indirect defense responses against herbivores is not expressed in most American maize varieties. *Plant Cell* 20:482–494.

KOZIEL, J., JIA, M. Y., and PAWLISZYN, J. 2000. Air sampling with porous solid-phase microextraction fibers. *Anal. Chem.* 72:5178–5186.

KUHLMANN, U., and van der BURGT, W. A. C. M. 1998. Possibilities for biological control of the western corn rootworm, *Diabrotica virgifera virgifera* LeConte, in Central Europe. *Biocontrol* 19:59N–68N.

LEVINE, E., OLOUMI, S. H., and FISHER, J. R. 1992. Discovery of multiyear diapause in Illinois and South Dakota northern corn rootworm (Coleoptera: Chrysomelidae) eggs and incidence of the prolonged diapause trait in Illinois. *J. Econ. Entomol.* 85:262–267.

LIU, C. H., MISHRA, A. K., and TAN, R. X. 2006. Repellent, insecticidal and phytotoxic activities of isoalantolactone from *Inula racemosa*. *Crop Prot.* 25:508–511.

LOWELL, P. S., and EKLUND, B. 2004. VOC emission fluxes as a function of lateral distance from the source. *Environ. Prog.* 23:52–58.

MCCOY, C. W., STUART, R. J., DUNCAN, L. W., and NGUYEN, K. 2002. Field efficacy of two commercial preparations of entomopathogenic nematodes against larvae of *Diaprepes abbreviatus* (Coleoptera: Curculionidae) in ALFISOL type soil. *Fla. Entomol.* 85:537–544.

MCGECHAN, M. B., and LEWIS, D. R. 2002. Transport of particulate and colloid-sorbed contaminants through soil, part 1: General principles. *Biosystems Engineering* 83:255–273.

MILLER, N., ESTOUP, A., TOEPFER, S., BOURGUET, D., LAPCHIN, L., DERRIDJ, S., KIM, K. S., REYNAUD, P., FURLAN, L., and GUILLEMAUD, T. 2005. Multiple transatlantic introductions of the western corn rootworm. *Science* 310:992–992.

NEVEU, N., GRANDGIRARD, J., NENON, J. P., and CORTESERO, A. M. 2002. Systemic release of herbivore-induced plant volatiles by turnips infested by concealed root-feeding larvae *Delia radicum* L. *J. Chem. Ecol.* 28:1717–1732.

O'NEIL, M. E., DIFONZO, C. D., and LANDIS, D. A. 2002. Western corn rootworm (Coleptera: Chrysomelidae) feeding on corn and soybean leaves affected by corn phenology. *J. Econ. Entomol.* 31:285–292.

OWEN, S. M., and PEÑUELAS, J. 2005. Opportunistic emissions of volatile isoprenoids. *Trends Plant Sci.* 10:420–426.

OWEN, S. M., and PENUELAS, J. 2006. Response to Firn and Jones: Volatile isoprenoids, a special case of secondary metabolism. *Trends Plant Sci* 11:113–114.

PEÑUELAS, J., and LLUSIÀ, J. 2004. Plant VOC emissions: making use of the unavoidable. *Trends Ecol. Evol.* 19:402–404.

RASMANN, S., KÖLLNER, T. G., DEGENHARDT, J., HILTPOLD, I., TOEPFER, S., KUHLMANN, U., GERSHENZON, J., and TURLINGS, T. C. J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434:732–737.

RASMANN, S., and TURLINGS, T. C. J. 2008. First insights into specificity of belowground tritrophic interactions. *Oikos* 117:362–369.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDER, S. J., PARE, P. W., and HENNEBERRY, T. J. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27:679–695.

RUIZ, J., BOLBAO, R., and MURILLO, M. B. 1998. Adsorption of different VOC onto soil minerals from gas phase: Influence of mineral, type of VOC, and air humidity. *Environ. Sci. Technol.* 32:1079–1084.

SABULAL, B., DAN, M., ANIL, J. J., KURUP, R., PRADEEP, N. S., VALSAMMA, R. K., and GEORGE, V. 2006. Caryophyllene-rich

rhizome oil of *Zingiber nimmonii* from South India: Chemical characterization and antimicrobial activity. *Phytochemistry* 67:2469–2473.

SCHOONHOVEN, L. M., JERMY, T., and VAN, LOON, J. J. A. 1998. Insect-Plant Biology: From Physiology to Evolution. Chapman and Hall, New York.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–688.

THOLL, D., CCHEN, F., PETRI, J., GERSHENZON, J., and PICHERSKY, E. 2005. Two sesquiterpene synthases are responsible for the complex mixture of sesquiterpenes emitted from *Arabidopsis* flowers. *Plant J.* 42:757–771.

TOEPFER, S., and KUHLMANN, U. 2004. Survey for natural enemies of the invasive alien chrysomelid, *Diabrotica virgifera virgifera*, in Central Europe. *Biocontrol* 49:385–395.

TOLLEFSON, J. J. 1998. A pest insect adapts to the cultural control of crop rotation; Brighton Crop Protection Conference. *Pests and Diseases* 3:1029–1033.

TÒTH, M., VUTS, J., SZARUKÁN, I., JUHÁSZ, I., and MANAJLOVICS, F. 2007. Preliminary study of female-targeted semiochemical baits for the western corn rootworm in Europe. *J. Appl. Entomol.* 131:416–419.

TURLINGS, T. C. J., LOUGHRIN, J. H., MCCALL, P. J., RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1995. How caterpillar-damaged plants protect themselves by attracting parsitic wasps. *Proc. Natl. Acad. Sci. USA* 92:4169–4174.

TURLINGS, T. C. J., and TON, J. 2006. Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Curr. Opin.Plant Biol.* 9:421–427.

TURLINGS, T. C. J., and WACKER, F. 2004. Recruitment of predators and parasitoids by herbivore injured-plants.. pp. 21–75, in R. T. CARDÉ, and J. G.MILLAR (eds.). Advances in Insect Chemical EcologyCambridge University Press, Cambridge.

VAN TOL, R. W. H. M., VAN DER SOMMEN, A. T. C., BOFF, M. I. C., VAN BEZOOIJEN, J., SABELIS, M. W., and SMITS, P. H. 2001. Plants protect their roots by alerting the enemies of grubs. *Ecology Lett.* 4:292–294.

VAS, G., and VEKEY, K. 2004. Solid-phase microextraction: a powerful sample preparation tool prior to mass spectrometric analysis. *J. Mass Spectrom.* 39:233–254.

VIDAL, S., KUHLMANN, U., and EDWARDS, R. 2005. Western Corn Rootworm: Ecology and Management. p. 324. CABI, Wallingford, United Kingdom.

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Sex Pheromone of the American Warble Fly, *Dermatobia hominis*: The Role of Cuticular Hydrocarbons

Claudia Cristina Gulias Gomes · José Roberto Trigo · Álvaro Eduardo Eiras

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Abstract Chemical communication between adults of the American warble fly, *Dermatobia hominis* (Diptera: Oestridae), was investigated by electroantennography and behavioral bioassays. Significant electroantennographic responses were recorded from both sexes to hexane-soluble cuticular lipids from either sex. Olfactometer tests indicated an attraction between males and females, and between females. Copulatory behavior of males with a white knotted string treated with female extract confirmed production of a sexual stimulant by females. Such behavior was not observed in tests with male extract, demonstrating that the pheromone acts also as a sex recognition factor. Cuticular hydrocarbons of sexually mature female and male *D. hominis* were identified by Gas chromatography–mass spectrometry and consist of a mixture of saturated *n*-, monomethyl-, and dimethylalkanes in both sexes. Sexual dimorphism was characterized by a higher relative concentration of dimethylalkanes in males and the presence of alkenes only in females.

C. C. Gulias Gomes (✉)
Departamento de Parasitologia Animal,
Instituto de Veterinária, UFRRJ,
Seropédica, BR 465, Km 07,
Rio de Janeiro 23890-000, Brazil
e-mail: guliasgomes@yahoo.com.br

J. R. Trigo
Laboratório de Ecologia Química, Departamento de Zoologia,
Instituto de Biologia, UNICAMP,
Caixa Postal 6109,
13083-970 Campinas, SP, Brazil

Á. E. Eiras
Laboratório de Ecologia Química de Insetos Vetores,
Departamento de Parasitologia,
Instituto de Ciências Biológicas, UFMG,
Av. Presidente Antônio Carlos, 6627, Bloco I4, sala 158,
31270-901 Belo Horizonte, MG, Brazil

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Introduction

During the last five decades, pheromone production has been confirmed in more than ten species of muscoid Diptera of importance in human and animal health within the families Muscidae, Calliphoridae, and Sarcophagidae (Howard and Blomquist 1982; Eiras 2001). Most of these compounds are sex pheromones isolated from the cuticle (Lockey 1980; Howard and Blomquist 1982). Such information has improved understanding of the biology of these taxa and has provided new insight for control methods. Similar research, however, is lacking for many other Diptera, including the American warble fly, *Dermatobia hominis* (L.) (Diptera: Oestridae), an important mammal parasite in Neotropical regions.

Dermatobia hominis has an unusual biology. Gravid females use other dipterous species to transport their eggs to suitable hosts, and adults have non-functional mouthparts and live just a few days, surviving on nutritional reserves from the larval stage (Hall and Wall 1995). Larvae are able to penetrate intact mammalian skin and develop in subcutaneous tissue until the third instar. Mature larvae leave the host and pupate below ground (Guimarães and Papavero 1999). Adults emerge after 4 to 8 wk; males emerge 1 to 2 days before females (Barbosa et al. 2000). The highest percentage of female insemination was measured in the laboratory with flies between 24–30 h after emergence, and sexual vigor of males decreases after 3 days of age (Banegas and Mourier 1967).

Parasitism by the American warble fly affects cattle production, with economic losses in Brazil estimated between

US $200–260 million per year, mainly as a consequence of leather devaluation and control costs (Guimarães and Papavero 1999; Grisi et al. 2002). Application of broad-spectrum injectable or topical insecticides is the main control strategy employed to reduce larval infestation (Hall and Wall 1995; Oliveira et al. 1999). Alternative methods of management of *D. hominis* are desired because excessive use of insecticides represents a risk to environmental, human, and animal health.

The process of orientation and copulatory behavior of *D. hominis* adults in the field is still poorly understood. Aggregation behavior is common among species in the Oestridae (Catts et al. 1965; Anderson et al. 1994), and males of *D. hominis* have been observed to aggregate in trees (Guimarães 1966). Olfactometer tests suggested that female odors do not regulate male orientation or play a minor role in this behavior (Banegas and Mourier 1967). However, most sex pheromones produced by muscoids have been characterized as short-range attractants, sexual stimulants, or contact sex/species recognition factors. Usually, they are alkanes or alkenes and are produced by females (Uebel et al. 1975a, b; 1978a, b; Sonnet et al. 1979; Bolton et al. 1980; Carlson et al. 1974, 1998, 2007). In this study, we investigated the role of intraspecific chemical communication in the copulatory behavior of *D. hominis*. The cuticular lipids of each sex were identified to assist in the future identification of the sex pheromone.

Methods and Materials

Insects Third instars of *D. hominis* were collected from cattle hides in two slaughter houses located in Contagem and Betim, Minas Gerais, Brazil. Larvae and pupae were maintained in humidified sawdust at 28°C, 60%±10% RH, and L/D 12:12 h. Pupae were placed individually in sawdust in plastic vials (3-cm height×2.5-cm diameter) and covered with a thin layer of white polystyrene beads (1-2 mm diameter) to provide contrast with adult fly color. Emergence time was monitored with a video camera to determine the age of each adult. Insects were removed from the sawdust within a few hours after emergence and kept separate until the beginning of tests. All insects used in experimental trials were 1 to 3 days old.

Cuticular Hydrocarbon Extracts Insects were anesthetized by using low temperature (−20°C) and then immersed individually in 500 μl *n*-hexane (Merck, 99% purity, Darmstadt, Germany) for 10 min. All samples were stored at −20°C and used within a maximal period of 1 week unless specified otherwise. Combined extracts used for the olfactometer bioassay (three insect equivalents) and mass spectrometric analyses (nine insect equivalents) were concentrated to 100 μl after they were pooled. The remaining individual extracts were kept separately for later chemical analysis. All samples were concentrated just before each test.

Electroantennography Hexane extracts of one male equivalent (1 ME) or one female equivalent (1 FE) were concentrated under a stream of N_2 to ~100 μl and transferred onto a strip of filter paper (40×5 mm). After solvent evaporation, the filter paper was placed into a 15-cm long Pasteur pipette. Glass electrodes were filled with 0.1 N aqueous KCl solution, and silver electrodes fitted within. The head and tip of one arista of *D. hominis* was cut off and mounted between the reference and recording glass electrodes, respectively. A purified and humidified air stream of 30 cm/s was directed continually over the preparation, and a pulse stimulus of 0.3-s duration was added to the air stream every 2 min (Stimulus Controller C5-05, Synthec, Hilversum, The Netherlands). Electroantennographic signals were amplified and transferred to a personal computer (IDAC Box Model ID-01B, Synthec). The electroantennography (EAG) analyses were performed sequentially by using the following stimuli: air, hexane, 1 ME of hexane extract, and 1 FE of hexane extract (ten females and ten males analyzed in response to each stimulus). Stimuli were tested three times per insect, and baseline was also recorded. EAG deflections were measured and stored numerically as millivolts. Results were normalized as a percentage of the control (hexane), which was by definition set at 100% (Synthec program for Windows version 2.6) and compared with the Kruskal–Wallis test (Zar 1999), followed by Dunn's post-test (Siegel and Castellan 1988).

Behavioral Bioassays Attractiveness of male and female *D. hominis* to conspecific adults was evaluated in a four-arm linear olfactometer system. Each olfactometer arm was composed of three chambers: (1) a transparent central tube (plexiglass tube 500 mm long×90-mm diameter), (2) another transparent plexiglass tube at the downwind end (100 mm long) with a rotating screen to control insect release, and (3) a polyvinylchloride (PVC) tube at the upwind end (200 mm long×100-mm diameter), with an entrance for the stimulus located above. The PVC tube was isolated from the central tube by gauze screen. Each olfactometer arm received air from a single air conducting system (PVC tubes), which involved air pumped from outside by an extractor fan, purified by an activated charcoal filter, heated, and finally, humidified before passing simultaneously into each arm of the olfactometer (Fig. 1).

The olfactometer was arranged over a metal stand covered with white-striped paper. Each olfactometer arm was shielded on both sides from visual stimulation by white

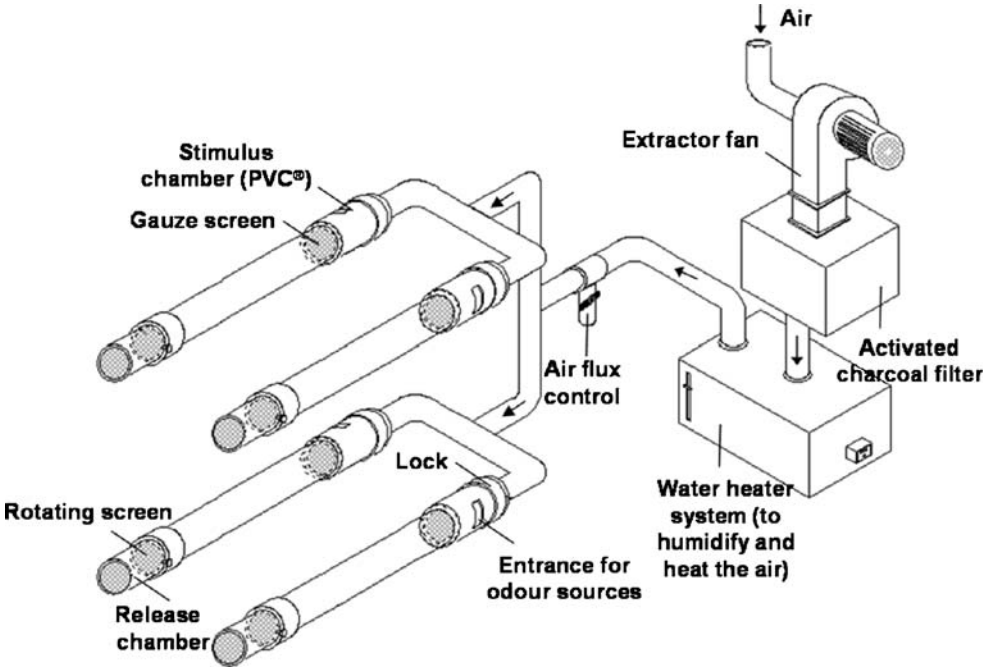

Fig. 1 Schematic drawing of the four-arm linear olfactometer system

and yellow cardboard. They were used in sequence, and after four replications, all the equipment was cleaned with water and 5% Extran® (MA 02, Neutral, Merck) detergent solution before starting a new battery of tests. Although not confirmed by chemical analysis, the capacity of the detergent solution to remove contaminants from the equipment was considered adequate because the responses to control stimuli remained low and uniform among the battery of tests. The experimental area was illuminated by two 20-W light bulbs above each set of two olfactometer arms, providing 1,200-lx light intensity. The experimental area was maintained at 27°C±1°C, 60%±10% RH, L/D 12:12 h, and 0.1–0.15 m/s wind speed.

Behavioral stimuli consisted of male and female adults of *D. hominis*, as well as hexane extracts (1 FE, 1 ME, 3 FE, and 3 ME). Before presentation of the stimulus, each responding adult was acclimated 15 min in the release chamber in the olfactometer air stream. Stimulus insects were placed in a screened cage (20×20×20 mm) and allowed to become stationary before being transferred to the PVC chamber. Extracts were concentrated under a gentle stream of N_2 to 100 µl and transferred onto a strip of filter paper (20×20 mm). After solvent evaporation, the filter paper was placed on a support in the middle of the PVC chamber. After the release chamber was opened, the maximum linear location of the test fly in either direction in the central tube was recorded during a period of 5 min. The maximal distance (centimeter) attained by each fly in the direction of the stimulus was taken as a measure of attraction. Responses to each stimulus and its control (pure air) were recorded from 30 different insects of each sex. Results were analyzed by the Kruskal–Wallis test and compared by the Mann–Whitney test (Zar 1999).

Production of a short-range pheromone by females was investigated in behavioral tests by using fly models (dummies) in a glass tube (400 mm long×50 mm diameter). Dummies were constructed of male and female *D. hominis* anaesthetized at –20°C or a white knotted string impregnated with conspecific body extracts (<24 hours old). One test male and one dummy were released at opposite ends of the glass tube. The side of each dummy was sealed with Parafilm® to keep dispersion of the odor plume toward the direction of the test male. Male behavior was observed during an initial 5 min period (phase 1). Behavioral data recorded were (1) contact, characterized as a short pouncing contact with the dummy, and (2) copulation attempt, characterized by mounting and exposure of the genitalic surstyli. In the absence of an orientated mount, contact between dummy and test male was induced by bending the tube during a subsequent 5-min period (phase 2). The only behavioral event recorded during phase 2 was copulation attempt. The effects of visual and tactile stimuli, which could induce copulatory behavior, were controlled by testing flies with white knotted string washed previously with hexane. Thirty replications of each treatment and control were conducted. Statistical analyses of the number of contacts and copulatory attempts were performed by using Fisher's test (Zar 1999).

Analysis and Identification of Cuticular Hydrocarbons

Chemical analyses were carried out with extracts of nine females and nine males concentrated to 100 µl in a N_2

stream. Aliquots of 1 µl of each sample were injected in splitless mode into an HP6890 gas chromatograph coupled to an HP5973 mass selective detector (GC–MS; Hewlett Packard, Palo Alto, CA, USA). The GC was equipped with an HP-5MS capillary column (Hewlett Packard; 30-m×0.25-mm×0.25-µm film thickness). The oven temperature was increased from 60°C to 230°C at 20°C/min and then from 230°C to 320°C at 2°C/min, where it was maintained for 10 min. The injector and detector temperatures were 250°C and 300°C, respectively. Helium was used as the carrier gas (1.2 ml min^{-1}). All samples were analyzed separately and by co-injection with consecutive *n*-alkane standards for determination of Kovats Retention Index (KI). Individual components were identified by their KI (Carlson et al. 1998) and by fragmentation patterns (Nelson et al. 1981; Pomonis 1989; Carlson et al. 1999). Peaks with areas less than 0.5% relative to the major peak were not considerated in GC–MS analysis. Alkenes were derivatized with dimethyldisulfide for determination of double-bond position (Francis and Veland 1981). The relative percentages of hydrocarbons were calculated by analysis of individual extracts of ten males and ten females with a Shimadzu 17A GC (Shimadzu, Osaka, Japan) equipped with a DB-1 capillary column (J&W, Folsom, CA, USA; 30-m×0.5-mm×0.5-µm film thickness) and a flame ionization detector. The temperature program was the same as described above; injector and detector temperatures were 330°C and 340°C, respectively. Hydrogen was used as the carrier gas (1.5 ml/min). Data acquisition and processing were performed with an HP G1030 data system (version B.00.01).

Volatile Entrapment To confirm the volatility of cuticular hydrocarbons identified from hexane extracts of *D. hominis*, volatiles were collected from ten living specimens of each sex during a 12-h photophase period, considering that the species has diurnal habits. The insects used in the bioassay were 24–48 h old to ensure that specimens were sexually mature and active. A blank test was run as a control. Volatiles were collected from *D. hominis* in a glass chamber (135-mm high×60-mm-diameter base×45-mm-diameter top) with two lateral arms (30-mm long×10-mm diameter), which served as the entrance and exit for the air stream. This air stream was produced by an air compressor system (1 l/min) and was purified by using an activated charcoal and silica filter (130-mm long×30-mm diameter) before entering the aeration chamber. The air stream exited through a glass column containing 0.1 g of Super Q adsorbent (100–120 mesh, Alltech, Deerfield, IL, USA). The experimental area was maintained at 24°C and 23%± 5% RH. Adsorption samples were extracted with diethyl ether (99% chemical purity, Merck) to a volume of 1 ml and concentrated under a stream of N_2 to 100 µl. One microliter of each sample was analyzed by GC–MS, as described above.

Results

Electroantennography Hexane extracts of conspecifics elicited significant EAG responses from female and male *D. hominis* (Kruskal–Wallis test; female, $H=41.95$, $df=4$, $P<0.001$; male, $H=43.71$, $df=4$, $P<0.001$; Figs. 2 and 3). Responses were higher in females than in males (Figs. 2 and 3).

Behavioral Bioassays Olfactometer assays showed significant responses by females and males to females, as well as by females to males. The mean distances attained by insects in these treatments were similar (Fig. 4). Males were not attracted to males. In tests where attraction was observed, on average, 60% of the insects moved from their initial position within the olfactometer. In the control groups, this percentage was only 27%. Attraction was not observed with extracts of one or three equivalents of either sex (data not shown).

The total percentages of copulatory attempts (phases 1 and 2 combined) observed in the bioassays of males with an anesthetized female or with a white knotted string treated with female extract were similar ($P=0.671$; Fig. 5), although in the latter case, most copulatory attempts occurred after contact was induced. The number of males that made contact with an anesthetized female ($N=11$) did not differ significantly from the number that contacted hexane-washed females ($N=7$; $P=0.398$). However, nine of the 11 touches with an anesthetized female were followed by a copulatory attempt. No attempts were observed after contact was made with hexane-washed females. In the bioassays with white knotted string treated with 1 FE, only one contact was made, followed by a copulatory attempt.

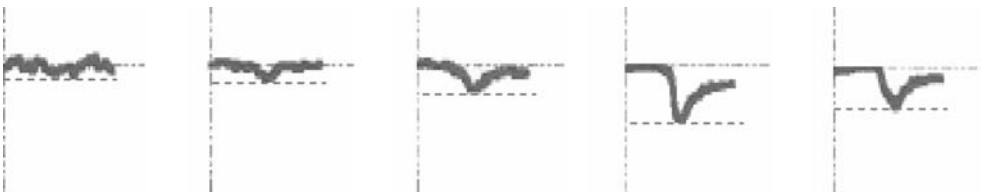

Fig. 2 Example of electroantennographic responses of male *Dermatobia hominis* antennae to hexane cuticle extracts of conspecifics (1 FE = 1 female equivalent; 1 ME = 1 male equivalent). From left to right: baseline, air, hexane, 1FE, 1ME

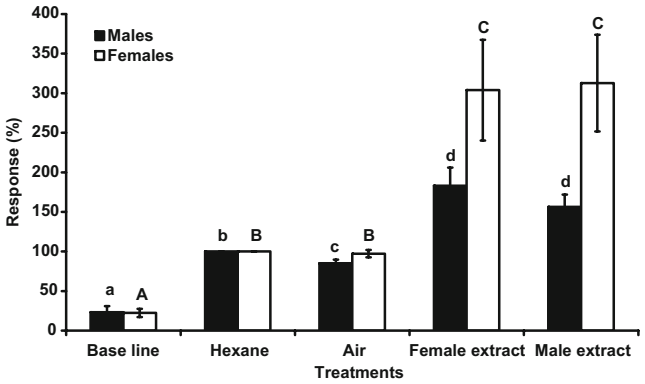

Fig. 3 Electroantennographic responses (mean ± SD) of female and male *Dermatobia hominis* (N=10 for each sex) to hexane cuticle extracts of conspecifics (one insect equivalent). EAG signals were measured as millivolts and normalized by using the response to hexane as a reference (set at 100%). Significant differences among treatment within female and male responses are indicated by *different letters* (lower case letters for males and capital letters in females)

Neither a contact nor a copulatory attempt was observed with untreated white knotted string. In the treatments with anesthetized males or white knotted string treated with 1 ME, no copulatory attempt was observed during phase 1 (Fig. 5). After contact was induced (phase 2), a male tried to copulate with a male control washed with solvent.

Analysis and Identification of Cuticular Hydrocarbons Analysis of the chemical composition of hexane extracts of male and female *D. hominis* revealed the presence of linear and branched hydrocarbons with chains varying from 23 to 41 carbons in males and 21 to 39 carbons in females (Table 1). The alkanes present consisted of linear and mono- or dimethyl-branched chains. The *n*-alkanes were the most abundant type of hydrocarbon, with approximately 43.1% of the total in males and 39.3% in females. Of these percentages, only 3.4% and 5.5%, respectively, were chains with an even number of carbons. Heptacosane (C_{27}) was a major component, representing 20.8% and 18.7% of the total hydrocarbons in males and females, respectively. Tricosane (C_{23}) and pentacosane (C_{25}) were found in relatively greater concentrations in females, as opposed to nonacosane (C_{29}), hentriacontane (C_{31}), and tritriacontane (C_{33}), which were found in relatively larger concentrations in males.

The methyl alkanes consisted of a homologous series of externally and internally branched isomers. The location of the branches in the monomethyl-alkanes varied from position 3 to position 19. Isomers with internal branches beyond position 9 co-eluted, resulting in a KI between XX31, the XX33 (XX are the primary digits of the KI) in chains with an odd number of carbons (from 9-MeC_{xx} to 19-Me C_{xx}, where "$_{xx}$" are the carbon numbers), and XX27 and XX29 in chains with an even number of carbons (from 10-MeC_{xx} to 14-Me C_{xx}, where "$_{xx}$" are the even carbon numbers). Male and female *D. hominis* had relatively similar percentages of monomethyl alkanes, representing 25.7% and 30.9% of total hydrocarbons, respectively. However, the biggest concentration of monomethyl alkanes in females occurred with compounds that had parent chains of 27, 29, and 31 carbons, whereas in males, the biggest concentration occurred with compounds that had parent chains with 35, 37, 39, and 41 carbons.

Dimethyl alkanes represented 25% and 7.4% of the total composition of hydrocarbons in males and females, respectively. Most of these occurred as a homologous

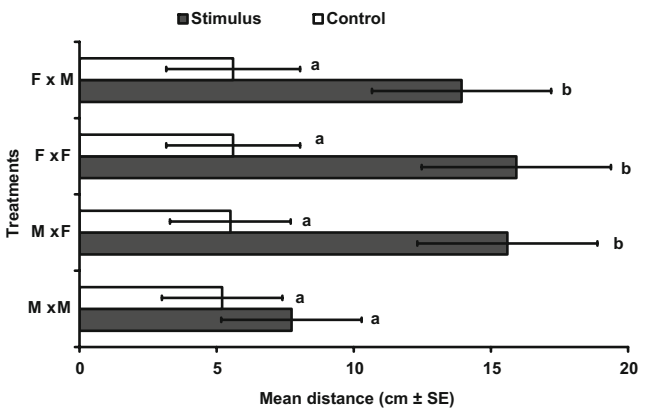

Fig. 4 Maximal distance attained by adult *Dermatobia hominis* (mean ± SD, N=30 for each sex) in a linear olfactometer, as a measure of attraction to conspecifics. Treatments: test insect × stimulus insect, *F* female and *M* male. Significant differences between control (air flow alone) and respective treatments are indicated by *different letters*

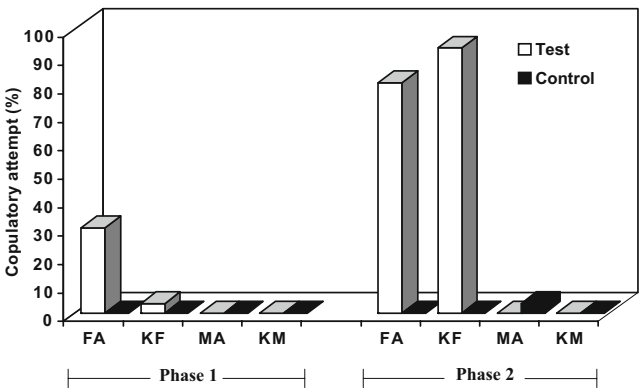

Fig. 5 Behavioral response of male *Dermatobia hominis* (N=30) to dummies in tests carried out in a glass tube. Phase 1: observation of spontaneous contact and copulatory attempt. Phase 2: observation of copulatory attempt after contact between dummy and male test insect was induced (5 min maximum for each phase). *FA* female anesthetized, *KA* knotted string + hexane extract, one female equivalent; *MA* male anesthetized, *KM* knotted string + hexane extract, one male equivalent. Controls: dummies washed with hexane

Table 1 Cuticular hydrocarbons extracted from male and female *Dermatobia hominis*

Compounds[a]	Relative abundance Mean ± SD[b]		CN[c]	KI[d]	Observed diagnostic mass spectral ion fragments[e]
	Males	Females			
7-$C_{21:1}$	–	0.05±0.02	21	2,180	294 (M^+)
C_{21}	–	1.09±0.56	21	2,100	296 (M^+)
3-Me C_{21}	–	0.03±0.01	22	2,171	281
C_{22}	–	0.35±0.15	22	2,200	310 (M^+)
7-$C_{23:1}$	–	11.14±6.28	23	2,282	322 (M^+)
C_{23}	0.37±0.26	1.93±0.89	23	2,300	324 (M^+)
7-Me C_{23}	–	0.10±0.04	24	2,338	112, 253, 323 (M-15)
3-Me C_{23}	–	0.36±0.15	24	2,370	309, 323 (M-15)
C_{24}	0.25±0.17	0.45±0.20	24	2,400	338 (M^+)
9-$C_{25:1}$	–	1.66±0.76	25	2,468	350 (M^+)
7-$C_{25:1}$	–	7.42±4.63	25	2,478	350 (M^+)
C_{25}	2.35±1.26	7.04±1.58	25	2,500	352 (M^+)
13-Me C_{25}^+	–	0.85±0.29	26	2,532	196, 351 (M-15)
11-Me C_{25}^+					168, 224, 351 (M-15)
9-Me C_{25}					140, 252, 351 (M-15)
7-Me C_{25}	–	0.46±0.09	26	2,537	112, 281, 351 (M-15)
5-Me C_{25}	0.11±0.09	0.72±0.10	26	2,549	85, 309, 351 (M-15)
3-Me C_{25}	–	0.79±0.16	26	2,572	337, 351 (M-15)
5,13-DiMe C_{25}^+	–	0.17±0.05	27	2,579	85, 197, 211, 323, 365 (M-15)
5,9-DiMe C_{25}					85, 155, 253, 323, 365 (M-15)
C_{26}	0.52±0.26	0.65±0.08	26	2,600	366 (M^+)
3,9-DiMe C_{25}^+	–	0.12±0.04	27	2,605	155, 253, 351, 365 (M-15)
3,7-DiMe C_{25}					127, 281, 351, 365 (M-15)
13-Me C_{26}^+	–	0.19±0.04	27	2,627	196, 211
12-Me C_{26}^+					182, 224
11-Me C_{26}^+					168, 238
10-Me C_{26}					155, 253
7-$C_{27:1}$	–	0.86±0.37	27	2,673	378 (M^+)
C_{27}	20.76±5.45	18.97±3.61	27	2,700	380 (M^+)
13-Me C_{27}^+	0.85±0.41	5.39±0.97	28	2,732	196, 224, 379 (M-15)
11-Me C_{27}^+					168, 252, 379 (M-15)
9-Me C_{27}					140, 281, 379 (M-15)
7-Me C_{27}	0.47±0.19	1.81±0.61	28	2,738	112, 309, 379 (M-15)
5-Me C_{27}	0.36±0.16	1.32±0.42	28	2,748	85, 337, 379 (M-15)
11,15-DiMe C_{27}^+	–	0.44±0.12	29	2,758	168, 196, 239, 267
9,13-DiMe C_{27}					140, 211, 224, 295
3-Me C_{27}	1.65±0.75	3.62±1.00	28	2,773	365, 379 (M-15)
5,17-DiMe C_{27}^+	0.13±0.06	0.49±0.15	29	2,781	85, 169, 267, 351, 393 (M-15)
5,15-DiMe C_{27}^+					85, 196, 239, 351, 393 (M-15)
5,13-DiMe C_{27}^+					85, 211, 225, 351, 393 (M-15)
5,11-DiMe C_{27}^+					85, 183, 253, 351, 393 (M-15)
5,9-DiMe C_{27}					85, 155, 281, 351, 393 (M-15)
C_{28}	0.71±0.16	0.56±0.12	28	2,800	394 (M^+)
3,11-DiMe C_{27}^+	0.33±0.11	0.55±0.21	29	2,807	183, 253, 379, 393 (M-15)
3,9-DiMe C_{27}^+					155, 281, 379, 393 (M-15)
3,7-DiMe C_{27}					127, 309, 379, 393 (M-15)
7-$C_{29:1}$	–	1.22±0.63	29	2,878	406 (M^+)
C_{29}	13.33±2.63	7.14±1.88	29	2,900	408 (M^+)
15-Me C_{29}^+	0.71±0.20	4.70±1.85	30	2,933	224, 407 (M-15)
13-Me C_{29}^+					196, 252, 407 (M-15)
11-Me C_{29}^+					168, 280, 407 (M-15)
9-Me C_{29}					140, 309, 407 (M-15)
7-Me C_{29}	–	0.53±0.26	30	2,938	112, 337

Table 1 (continued)

Compounds[a]	Relative abundance Mean ± SD[b]		CN[c]	KI[d]	Observed diagnostic mass spectral ion fragments[e]
	Males	Females			
5-Me C_{29}	0.30±0.11	0.58±0.24	30	2,948	85, 365, 407 (M-15)
13,17-DiMe C_{29}^+	–	1.40±0.54	31	2,957	196, 267
11,15-DiMe C_{29}^+					168, 224, 239, 295
9,13-DiMe C_{29}					140, 211, 252, 323
3-Me C_{29}	0.71±0.21	1.10±0.41	30	2,973	393, 407 (M-15)
15-Me C_{30}^+	–	0.39±0.17	31	3,029	224, 238, 421 (M-15)
14-Me C_{30}^+					210, 252, 421 (M-15)
13-Me C_{30}					196, 267, 421 (M-15)
C_{31}	3.83±1.17	0.78±0.16	31	3,100	436 (M^+)
15-Me C_{31}^+	0.81±0.20	3.21±1.55	32	3,133	224, 252
13-Me C_{31}^+					196, 281
11-Me C_{31}^+					168, 308
9-Me C_{31}					140, 337
13,17-DiMe C_{31} +	0.17±0.05	1.36±0.52	33	3,158	196, 224, 267, 295
11,15-DiMe C_{31}^+					168, 239, 252, 323
9,13-DiMe C_{31}					140, 211, 281, 351
C_{32}	–	0.20±0.08	32	3,200	450
C_{33}	1.01±0.37	0.19±0.04	33	3,300	464
17-Me C_{33}^+	1.48±0.29	1.89±0.67	34	3,331	252, 463 (M-15)
15-Me C_{33}^+					224, 280, 463 (M-15)
13-Me C_{33}^+					196, 308, 463 (M-15)
11-Me C_{33}^+					168, 337, 463 (M-15)
9-Me C_{33}					140, 365, 463 (M-15)
15,19-DiMe C_{33} +	0.55±0.11	1.13±0.33	35	3,358	224, 295
13,17-DiMe C_{33} +					196, 252, 267, 323
11,15-DiMe C_{33}					168, 239, 281, 351
Unidentified	0.22±0.04	–	?		–
Unidentified	0.42±0.15	–	?		–
17-Me C_{35}^+	3.01±0.55	1.10±0.28	36	3,531*	252, 280
15-Me C_{35}^+					224, 308
13-Me C_{35}^+					196, 337
11-Me C_{35}					168, 365
15,19-DiMe C_{35}^+	3.04±0.54	1.02±0.21	37	3,558*	225, 252, 295, 323
13,17-DiMe C_{35}^+					196, 267, 280, 351
11,15-DiMe C_{35}					169, 239, 309
Unidentified	0.20±0.08	–	?		
Unidentified	0.69±0.16	–	?		
Unidentified	0.90±0.19	–	?		
19-Me C_{37}^+	7.49±2.50	0.99±0.28	38	3,731*	280
17-Me C_{37}^+					252, 308
15-Me C_{37}^+					224, 337
13-Me C_{37}^+					196, 365
11-Me C_{37}					168, 393
17,21-DiMe C_{37} +	9.17±3.34	0.68±0.18	39	3,758*	252, 323
15,19-DiMe C_{37} +					224, 281, 295, 351
13,17-DiMe C_{37}					196, 267, 308, 379
Unidentified	0.75±0.34	–	?		
Unidentified	1.08±0.28	–	?		
Unidentified	1.95±0.42	–	?		
19-Me C_{39}^+	5.43±1.67	0.79±0.39	40	3,931*	280, 308
17-Me C_{39}^+					252, 336
15-Me C_{39}^+					224, 364
13-Me C_{39}^+					196, 393

Table 1 (continued)

Compounds[a]	Relative abundance Mean ± SD[b]		CN[c]	KI[d]	Observed diagnostic mass spectral ion fragments[e]
	Males	Females			
11-Me C_{39}					168, 421
17,21-DiMe C_{39}^{+}	9.12±1.78	–	41	3,958*	252, 280, 323, 351, 563 (M-15)
15,19-DiMe C_{39}^{+}					224, 295, 308, 379, 563 (M-15)
13,17-DiMe C_{39}					196, 267, 337, 407, 563 (M-15)
15-Me C_{41}^{+}	2.28±0.47	–	42	4,131*	224, 393
13-Me C_{41}^{+}					196, 421
11-Me C_{41}					168
17,21-DiMe C_{41}^{+}	2.52±0.46	–	43	4,158*	253, 308, 323, 379
15,19-DiMe C_{41}^{+}					224, 295, 408
13,17-DiMe C_{41}^{+}					197, 267
11,15-DiMe C_{41}					169, 239

DiMe dimethyl, *Me* methyl, $C_{xx:1}$ unsaturated compound

[a] Abbreviated names of co-eluting compounds are joined by a plus sign. The co-eluting compounds follow the order of elution.

[b] Calculated by gas chromatography–flame ionization detector

[c] Carbon number; *question mark* unknown carbon number

[d] Kovats Index; the asterisk indicates estimated KIs; the compounds were identified by their mass spectrum and by comparison with the corresponding homolog series.

[e] Diagnoses based on comparisons with mass spectral data from Nelson et al. (1981), Pomonis (1989), and Carlson et al. (1998 and 1999). For methyl-branched alkanes, ion clusters occur as even/odd mass pairs depending on the branching point (Nelson et al. 1972). For brevity, only the higher fragment of each pair is listed.

series with internal branches initiated mainly at positions 9, 11, and 13, with three methylene units between the branches; they occurred from C_{27} to C_{41} odd parent chains. A second homologous series, present in relatively low amounts from C_{25} to C_{27} odd parent chains, was composed of 3, *x*-dimethyl alkanes, where the *x* was represented by carbons 7, 9, or 11. 5,*x*-Dimethyl alkanes also occurred with C_{25} and C_{27} parent chains, but in this case, *x* varied between carbons 9, 11, 13, 15, and 17.

Alkenes comprised 22.3% of the total composition of hydrocarbons in females. The double bond was at positions 7 and 9. The compounds 7-tricosene (7-$C_{23:1}$) and 7-pentacosene (7-$C_{25:1}$) were abundant, representing 11% and 7.3% of the total female hydrocarbons. Unidentified peaks comprised only 6.2 % of relative abundance in males, whereas all peaks were identified in females.

Analysis of trapped volatiles revealed that *n*-alkanes and monomethylated alkanes with chain lengths ranging from 23 to 31 carbons (females) and from 25 to 31 carbons (males) could be collected in the headspace (Table 2). All of these compounds were always present in the cuticular extracts of adults in relative concentrations above 1%. Volatiles collected from females were uniquely characterized by 7-tricosene (7-$C_{23:1}$), tricosane (C_{23}), 7-pentacosene (7-$C_{25:1}$), monomethyl pentacosanes, hexacosane (C_{26}), and 5-methyl-heptacosane (5-Me C_{27}). Volatiles collected from males were characterized by several compounds shared with females but also hentriacontane (C_{31}), which was present in extracts of both the sexes, but at a low concentration in the female extract.

Discussion

This study offers the first evidence for sex pheromones in a species in the Oestridae. The bioassays with dummies indicated the occurrence of chemical communication in the copulatory process of *D. hominis* and confirmed the presence of chemical components in the female extract that function as a sexual stimulant. The increase in copulatory attempts observed only after contact of the males was induced with either the anesthetized females or the white knotted string models suggests that chemical stimulation occurs over short distances or by means of contact receptors. Preliminary bioassays demonstrated that white knotted string treated with female extract stimulates males to copulate even after storage for 1 month at −20°C. This indicates that the sexual stimulant is of low volatility and is stable. The presence of a sexual stimulant, however, does not exclude the possibility of an attractive stimulant, as the majority of sex pheromones are multi-component (Wyatt 2003). Olfactometer tests with air flow indicated attractiveness of males to females. This intraspecific attractiveness has been observed in some muscoids, such as *Musca domestica* L. (Murvosh et al. 1965), *Musca autumnalis* Degreer (Chaudhury et al. 1972), *Haematobia irritans* L.,

Table 2 Cuticular hydrocarbons trapped from the volatile headspace above male or female *Dermatobia hominis* by volatile entrapment

Compounds	Carbon number	KI	RA	Males KI	Females RA
7-$C_{23:1}$	23	–		2,284	7.88
C_{23}	23	–		2,300	2.22
7-$C_{25:1}$	25	–		2,482	6.86
C_{25}	25	2,500	10.25	2,500	15.82
7-, 9-, 11-, 13-Me C_{25}[a]	26	–		2,535	8.73
3-Me C_{25}	26	–		2,571	2.51
C_{26}	26	–		2,600	0.83
C_{27}	27	2,700	30.53	2,700	22.10
7-, 9-, 11-, 13-Me C_{27}[a]	28	2,733	7.39	2,731	6.03
5-Me C_{27}	28	–		2,750	1.42
3-Me C_{27}	28	2,773	5.74	2,775	6.18
C_{29}	29	2,900	21.67	2,900	7.06
9-, 11-, 13-, 15-Me C_{29}[a]	30	2,933	5.91	2,933	8.75
C_{31}	31	3,100	13.90	–	–
9-, 11-, 13-, 15-Me C_{31}[a]	32	3,128	4.61	3,133	3.62

Me methyl, $C_{xx:1}$ unsaturated compound, *KI* Kovats Index, *RA* relative abundance
[a] Co-eluting homologous series eluted together.

and *Stomoxys calcitrans* L. (Howard and Blomquist 1982). Most of these studies reported low attractiveness (Murvosh et al. 1965; Howard and Blomquist 1982), although the use of the identified synthetic pheromone, (*Z*)-9-tricosene, against both male and female *M. domestica* in the field has become universal because of its effectiveness when combined with pesticides (Carlson and Beroza 1973). Considering the large variation that we measured in the responses of individual male *D. hominis*, the attraction to the chemical source can also be interpreted as low intensity. Probably for this reason, Banegas and Mourier (1967) concluded that no pheromone was involved or had little importance in the process of orientation by *D. hominis* males. It is also probable that attraction behavior was not observed in the bioassay with glass tube because there was no air flow, which may increase chemical dispersion. On the other hand, in our study, the use of Parafilm® to seal the glass tube or to provide a barrier on the anesthetized dummies could be considered as a potential source of contamination. This material is composed of hydrocarbons (Millar and Sims 1998), and identical compounds were later identified in hexane cuticular extracts from females. A background odor could affect the response of olfactory receptors cells of males. However, if the action of a longer range pheromone was influenced by other molecules present in the glass tube environment, no interference was observed at short range because physical contact with the dummies stimulated courtship.

The absence of attractiveness of male and female *D. hominis* to extracts at one and three insect equivalents could be attributed to an extract concentration that was insufficient to duplicate the odor plume generated by a live insect. The importance of how a stimulus is presented was discussed by Geier and Boeckh (1999) in which they compared the liberation of an extract of human skin odor with a heating system (60°C) or with filter paper. The results demonstrated that, to achieve similar responses, it was necessary to use 300 times the concentration on the filter paper than was used in the heating system. This type of methodology could be used in future studies of *D. hominis* attractiveness, especially because of the difficulty in obtaining large numbers of adults.

The absence of copulatory attempts by males with models treated with extract of conspecifics males indicates the action of a sexual recognition factor in the chemical composition of the species. This type of factor has been demonstrated in other studies of sexual pheromones of flies as the hydrocarbon 19,23-dimethyl-1-tritriacontene, produced by males of *Glossina morsitans* Westwood (Diptera: Glossidae) that inhibits mating activity when applied to the body of conspecifics females (Carlson and Schlein 1991). Previous laboratory studies of courtship behavior of *D. hominis* males (unpublished data) demonstrated reduced number of homosexual copulatory attempts. These data corroborate the presence of a sexual recognition factor and suggest that recognition occurs at a distance and is not limited to physical contact between the insects. The presence of odor receptors on male antennae responsive to the extract of conspecifics males (EAG results) supports the existence of biologically active volatile compounds in the male extract. The presence of a sexual recognition factor in the male cuticle might explain the lack of response of males when conspecifics males were tested in the olfactometer bioassays. In this case, the sexual recognition factor would have an inhibitory function.

Eletroantennographic analyses also demonstrated the presence of odor receptors in female antennae to compounds in extracts of both sexes. Behavioral bioassays have supported these results by demonstrating the attraction of

D. hominis females to both males and females. The intraspecific attraction of females by males has not received much attention previously. Most research has focused on sex pheromones produced by females and behavioral assays that demonstrate the short-range orientation of males. Fletcher et al. (1966) reported a male pheromone of *Cochliomyia hominivorax* (Coquerel; Diptera: Calliphoridae) that elicited "exploratory searching movements" on females. Male aggregation has been observed to *D. hominis* and *C. hominivorax* (Guimarães 1966; Krafsur 1978). If these sites of aggregation work as "mating stations," a pheromone component that elicits "exploratory activity" or signals the meeting site could help females to locate males. Production of a social aggregation pheromone (alcohols, ketones, and alkenes) has been described for several *Drosophila* species (Diptera: Drosophilidae; Symonds and Wertheim 2005). *Cis*-vaccenyl acetate, produced by male *D. melanogaster* Meigen, induces antagonistic behaviors: aggregation of males and females, female sexual receptivity, and inhibition of courtship in the male (Mehren 2007), suggesting that many interesting questions remain to be explored with fly pheromone perception. Another poorly explored area is the attraction between conspecific females. Although the biological significance of this interaction remains obscure, it has been observed in *M. domestica* and *H. irritans* (Murvosh et al. 1965; Howard and Blomquist 1982).

Analysis of cuticular hydrocarbon extracts of male and female *D. hominis* disclosed the chemical nature of the sexual pheromone of the species, and the volatile entrapment technique demonstrated the volatility of some of the cuticular components of adults. It is possible that these blends are involved in the process of attraction as observed in the olfactometer tests. Most of the cuticular hydrocarbons of the American warble fly have been reported for other insects (Lockey 1980; Nelson et al. 1981; Trabalon et al. 1992). Heptacosane characterizes the chemical profile of this species, as it is the major component of the cuticular hydrocarbons and similar in relative concentration in extracts from both of the sexes. On the other hand, sexual dimorphism was evident because of the large concentration of dimethyl alkanes in males and alkenes in females. Alkenes have been identified as sex stimulants in females of various species of flies, such as *H. irritans* (Bolton et al. 1980), *M. autumnalis* (Uebel et al. 1975a), *S. calcitrans* (Sonnet et al. 1979), and three species of the genus *Fannia* (Diptera: Muscidae; Uebel et al. 1975b; 1978a, b). In *M. domestica*, in addition to stimulating copulation, alkenes reportedly had an attractive function as well, based on results of olfactometer and decoy tests (Carlson et al. 1974). The alkenes in *D. hominis* females, therefore, are possible candidates for its sex pheromone, with potential to have a sex stimulant and an attractive function. Identification of *D. hominis* cuticular hydrocarbons in this work serves as the basis for the identification of components with pheromonal activity, which can also include the methyl and dimethyl alkanes. These data could also be used in studies of chemotaxonomy that attempt to characterize populations of different regions or countries.

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References

ANDERSON, J. R., NILSSEN, A. C., and FOLSTAD, I. 1994. Mating behavior and thermoregulation of the reindeer warble fly, *Hypoderma tarandi* L. (Diptera: Oestridae). *J. Insect. Behav.* 7:679–706.

BANEGAS, A. D., and MOURIER, H. 1967. Laboratory observations on the life-history and habits of *Dermatobia hominis* (Diptera, Cuterebridae). I. Mating behavior. *Ann. Entomol. Soc. Am.* 60: 878–881.

BARBOSA, C. G., SANAVRIA, A., PASSOS, M. D., and BARBOSA, R. C. 2000. Período pupal da *Dermatobia hominis* (Diptera: Cuterebridae) em condições de temperatura ambiente. *Parasitol. Dia* 24:63–66.

BOLTON, H. T., BUTLER, J. F., and CARLSON, D. A. 1980. A mating stimulant pheromone of the horn fly, *Haematobia irritans* (L.): demonstration of biological activity in separated cuticular components. *J. Chem. Ecol.* 6:951–964.

CARLSON, D. A., and BEROZA, M. 1973. Field evaluation of (*Z*)-9-tricosene, a sex attractant pheromone of the house fly. *Environ. Entomol.* 2:555–559.

CARLSON, D. A., and SCHLEIN, Y. 1991. Unusual polymethyl alkenes in tsetse flies acting as abstinon in *Glossina morsitans*. *J. Chem. Ecol.* 17:267–284.

CARLSON, D. A., DOOLITTLE, R. E., BEROZA, M., ROGOFF, W. W., and GRETZ, G. 1974. Muscalure and related compounds. I. Response of house flies in olfactometer and pseudofly Petri dish tests. *J. Agric. Food Chem.* 22:194–197.

CARLSON, D. A., OFFOR, I. I., EL MESSOUSSI, S., MATSUYAMA, K., MORI, K., and JALLON, J. M. 1998. Sex pheromone of *Glossina tachinoides*: isolation, identification and synthesis. *J. Chem. Ecol.* 24:1563–1574.

CARLSON, D. A., GEDEN, C. J., and BERNIER, U. R. 1999. Identification of pupal exuviae of *Nasonia vitripennis* and *Muscidifurax raptorellus* parasitoids using cuticular hydrocarbons. *Biol. Control* 15:97–106.

CARLSON, D. A., BERKEBILE, D. R., SKODA, S. R., MORI, K., and MIHOK, S. 2007. Candidate sex pheromones of the New World screwworm *Cochliomyia hominivorax*. *Med. Vet. Entomol.* 21:93–96.

CATTS, E. P., GARCIA, R., and POORBAUGH, J. H. 1965. Aggregation sites of males of the common cattle grub, *Hypoderma lineatum* (De Villers) (Diptera: Oestridae). *J. Med. Entomol.* 1:357–358.

CHAUDHURY, M. F. B., BALL, H. J., and JONES, C. M. 1972. A sex pheromone of the female face fly, *Musca autumnalis,* and its role in sexual behavior. *Ann. Entomol. Soc. Am.* 65:607–612.

EIRAS, A. E. 2001. Mediadores químicos entre hospedeiros e insetos vetores de doenças médico-veterinárias, pp. 99–112, in E. F. Vilela, and T. M. C. Della-Lucia (eds.). Feromônios de Insetos.

Biologia, Química e Emprego no Manejo de PragasEditora Holos, Ribeirão Preto, SP, Brazil.

FLETCHER, L. W., O'GRADY, J. J. Jr., CLABORN, H. V., and GRAHAM, O. H. 1966. A pheromone from male screw-worm flies. *J. Chem. Ecol.* 59:142–143.

FRANCIS, G. W., and VELAND, K. 1981. Alkylthiolation for the determination of double-bond positions in linear alkenes. *J. Chromatogr.* 219:379–384.

GEIER, M., and BOECKH, J. 1999. A new Y-tube olfactometer for mosquitoes to measure the attractiveness of host odors. *Entomol. Exp. Appl.* 92:9–19.

GRISI, L., MASSARD, C. L., MOYA BORJA, G. E., and PEREIRA, J. B. 2002. Impacto econômico das principais ectoparasitoses em bovinos no Brasil. *Hora Vet* 21:8–10.

GUIMARÃES, J. H. 1966. Nota sobre os hábitos dos machos de *Dermatobia hominis* (Linnaeus Jr.) (Diptera: Cuterebridae). *Papéis Avulsos Depto Zool. Sec. Agric. SP.* 18:277–279.

GUIMARÃES, J. H., and PAPAVERO, N. 1999. Myiasis caused by obligatory parasites. VI. *Dermatobia hominis* (Linnaeus Jr.) (Cuterebridae), pp. 257–302, in J. H. Guimarães, and N. Papavero (eds.). Myiasis in Man and Animals in the Neotropical RegionEd Plêiade, São Paulo, SP, Brazil.

HALL, M., and WALL, R. 1995. Myiasis of humans and domestic animals. *Adv. Parasitol.* 35:258–334.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27: 149–172.

KRAFSUR, E. S. 1978. Aggregations of male screwworm flies, *Cochliomyia hominivorax* (Coquerel) in south Texas (Diptera: Calliphoridae). *Proc. Entomol. Soc. Wash* 80:164–170.

LOCKEY, K. H. 1980. Insect cuticular hydrocarbons. *Comp. Biochem. Physiol.* 65B:457–462.

MEHREN, J. E. 2007. Mate recognition: should fly stay or should fly go? *Curr. Biol.* 17:240–242.

MILLAR, J. G., and SIMS, J. J. 1998. Preparation, cleanup, and preliminary fractionation of extracts, p. 1, –, 37, in J.G. Millar, and K. F. Haynes (eds.). Methods in Chemical Ecology. Chemical Methods, vol. 1Kluwer, Norwell, MA.

MURVOSH, C. M., LABRECQUE, G. C., and SMITH, C. N. 1965. Sex attraction in the house fly, *Musca domestica* L. *Ohio J. Sci.* 65:68.

NELSON, D. R., SUKKESTAD, D. R., and ZAYLSKIE, R. G. 1972. Mass spectra of methyl-branched hydrocarbons from eggs of the tobacco hornworm. *J. Lipid Res.* 13:413–421.

NELSON, D. R., DILLWITH, J. W., and BLOMQUIST, G. J. 1981. Cuticular hydrocarbons of the house fly, *Musca domestica*. *Insect Biochem.* 11:187–197.

OLIVEIRA, G. P., MATSUMOTO, T., and OLIVEIRA, M. C. S. (eds). 1999. Importância econômica e controle do berne (*Dermatobia hominis*, Diptera: Cuterebridae) na região de São Carlos, SP. Embrapa Pecuária do Sudeste. Technical Bulletin no. 22.

POMONIS, J. G. 1989. Cuticular hydrocarbons of the screwworm, *Cochliomyia hominivorax* (Diptera: Calliphoridae). Isolation, identification and quantification as a function of age, sex and irradiation. *J. Chem. Ecol.* 15:2301–2317.

SIEGEL, S., and CASTELLAN, N. J. 1988. Nonparametric Statistics for the Behavioral Sciences. McGraw-Hill International Editions, New York, NY.

SONNET, P. E., UEBEL, E. C., LUSBY, W. R., SCHWARZ, M., and MILLER, R. W. 1979. Sex pheromone of the stable fly: identification, synthesis and evaluation of alkenes from female stable flies. *J. Chem. Ecol* 5:353–361.

SYMONDS, M. R. E., and WERTHEIM, B. 2005. The mode of evolution of aggregation pheromones in *Drosophila* species. *J. Evol. Biol.* 18:1253–1263.

TRABALON, M., CAMPAN, M., CLEMENT, J. L., LANGE, C., and MIQUEL, M. T. 1992. Cuticular hydrocarbons of *Calliphora vomitoria* (Diptera: Calliphoridae): relation to age and sex. *Gen. Comp. Endocrinol.* 85:208–216.

UEBEL, E. C., MENZER, R. E., SONNET, P. E., and MILLER, R. W. 1975a. Identification of the copulatory sex pheromone of the little house fly, *Fannia canicularis* (L.) (Diptera: Muscidae). *N. Y. Entomol. Soc.* 83:258–259.

UEBEL, E. C., SONNET, P. E., BIERL, B. A., and MILLER, R. W. 1975b. Sex pheromone of the stable fly: isolation and preliminary identification of compounds that induce mating strike behavior. *J. Chem. Ecol.* 1:377–385.

UEBEL, E. C., SCHWARZ, M., LUSBY, W. R., MILLER, R. W., and SONNET, P. E. 1978a. Cuticular non-hydrocarbons of the female housefly and their evaluation as mating stimulants. *Lloydia* 41: 63–67.

UEBEL, E. C., SCHWARZ, M., MENZER, R. E., and MILLER, R. W. 1978b. Mating-stimulant pheromone and cuticular lipid constituents of *Fannia pusio* (L.). *J. Chem. Ecol.* 4:73–81.

WYATT, T. D. 2003. Pheromones and Animal Behavior. Communication by Smell and Taste. Cambridge University Press, Cambridge, UK.

ZAR, J. H. 1999. Biostatistical Analysis. Prentice-Hall, Upper Saddle River, NJ, USA.

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Behavioral and Olfactory Responses of Female *Salaria pavo* (Pisces: Blenniidae) to a Putative Multi-component Male Pheromone

Rui M. Serrano · Eduardo N. Barata · Michael A. Birkett · Peter C. Hubbard · Patrícia S. Guerreiro · Adelino V. M. Canário

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Abstract The peacock blenny, *Salaria pavo* (Risso 1810), typically breeds in rocky shores of the Mediterranean and adjacent Atlantic coast. Males defend a territory around a hole or cavity wherein females deposit eggs that the male guards until hatching. A pair of exocrine glands on the anal fin (anal glands) of males produces a putative pheromone involved in attraction of reproductively competent females to the nest. We used behavioral assays to assess species-specific attraction of reproductively competent females to putative male pheromones, including the anal gland pheromone. Additionally, chromatographic fractions of anal glands and male-conditioned water were tested for olfactory potency in females by electro-olfactogram analysis (EOG). In a flow-through tank or fluviarium, reproductive females were attracted to male-conditioned water and to the anal gland macerate from conspecifics but not to those of a closely related heterospecific. In addition, attraction of reproductive females to conspecific anal gland macerate occurred only during their initial upstream movement in the fluviarium; this was an ephemeral response when compared with the response to male-conditioned water that attracted females throughout the entire period of observation (5 min). Reproductive females also were attracted during the entire period of observation to water-conditioned by conspecific males whose anal glands had been removed. However, the attraction was more variable than that to water conditioned by intact males. Moreover, females were not attracted to male (without anal glands) odor during their initial upstream movement in the fluviarium. Finally, non-reproductive females were not attracted to the conspecific anal gland macerate. The EOG responses of females to molecular weight fractions and solid-phase extraction and high-performance liquid chromatography fractions of anal gland macerates and male-conditioned water (with and without anal glands) suggest that the anal glands release hydrophilic odorants that consist mainly of molecules smaller than 500 Da. Furthermore, males released potent odorants that do not originate from the anal glands. We hypothesize that females respond to a multi-component male pheromone to find mates. The putative anal gland pheromone is possibly comprised of hydrophilic odorants, whereas the other component(s), presumably of gonadal origin, may be less water-soluble.

Keywords Anal gland · Electro-olfactogram · Fish · HPLC · Olfaction · Peacock blenny · Reproduction · Sex pheromones · Swimming behavior · Video-tracking · Teleost

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R. M. Serrano · E. N. Barata (✉) · P. C. Hubbard · P. S. Guerreiro · A. V. M. Canário
CCMAR-CIMAR Laboratório Associado,
Universidade do Algarve,
Campus de Gambelas,
8005-139 Faro, Portugal
e-mail: ebarata@ualg.pt

R. M. Serrano · E. N. Barata
Departamento de Biologia, Universidade de Évora,
Apartado 94,
7002-554 Évora, Portugal

M. A. Birkett
Biological Chemistry Department, Rothamsted Research,
Harpenden, Hertfordshire AL5 2JQ, UK

Introduction

Hormonal pheromones (steroids, prostaglandins, and/or their metabolites) are generally involved in the reproduction of teleost fishes (reviewed in Stacey and Sorensen 2006), but relatively little is known about either chemical signaling behavior or specialized pheromone glands. In the goldfish, *Carassius auratus* L., which has been extensively studied with respect to reproductive hormonal pheromones, gonadal steroids and prostaglandins released via the urine and gills of females and detected by nearby males, seem to function primarily as timing cues to synchronize spawning (Stacey and Sorensen 2006 and references therein). These cues can be regarded as unspecialized metabolic by-products that are not specifically released to affect the behavior and physiology of the receiver. However, the controlled release of urine by sexually receptive females seems to represent a specialization for delivery of prostaglandin pheromones to mark the spawning site and attract males at short range (Appelt and Sorensen 2007).

More specialized chemical communication seems to exist in species for which the physical environment and/or the reproductive strategy have favored the use of sex pheromones in mate-finding. For example, in the benthopelagic African catfish, *Clarias gariepinus* Burchell 1822, which spawns at night in flooded deltas, the seminal vesicle produces steroid glucuronides that attract females (Resink et al. 1987, 1989; Lambert and Resink 1991). Furthermore, species with mating systems that typically involve male territoriality, nest defense, and paternal investment seem to have evolved specializations for sex pheromone production. In the black goby, *Gobius niger* L., and round goby, *Neogobius melanostomus* Pallas 1814, mesorchial glands in the testis (nonspermatogenic portions with a high density of Leydig cells) produce primarily 5β-reduced androgens that seem to act as hormonal pheromones that attract females (Colombo et al. 1980; Murphy et al. 2001; Belanger et al. 2004; Arbuckle et al. 2005; Gammon et al. 2005). Blennies (Blenniidae) share a similar reproductive strategy with gobies (Gobidae). Steroid glucuronides secreted during the breeding season by the testicular blind pouches (tubular evaginations of the spermatic ducts that do not function as sperm reservoirs) have been hypothesized to act as hormonal pheromones by analogy with the black goby and the African catfish (Lahnsteiner et al. 1993). External exocrine glands in male blennies produce a putative pheromone that attracts females (Laumen et al. 1974; Barata et al. 2008).

Combtooth blennies form the largest family of true blennies (suborder Blennioidei) and include more than 300 species of demersal fishes, living in littoral zones all over the world (Zander 1986). Blennies are polygynandric, and their general breeding pattern consists of males defending a territory wherein females deposit adhesive eggs on the inner surface of cavities (crevices, holes, empty shells, or other nesting cavities), which are guarded by the males until the eggs hatch (Gibson 1969; Almada and Santos 1995). During the spawning season, male peacock blennies, *Salaria pavo* (Risso 1810), and other blennies (e.g., *Salaria* spp. and *Parablennius* spp.) develop a pair of anal glands on the first two rays of the anal fin (Zander 1975; Papaconstantinou 1979). These glands seem involved in the production of a putative pheromone (Laumen et al. 1974) that attracts reproductively competent females and, thereby, increases male reproductive success (Barata et al. 2008). To act as mate-finding sex pheromone, species-specific attraction of reproductive females to the anal gland pheromone, but not of non-reproductive females, would be expected; however, this prediction has not yet been tested. In addition, it remains unclear whether the anal glands are the only source of a sex pheromone involved in female attraction.

In the current study with *S. pavo*, we used behavioral assays to test: (1) whether attraction of reproductively competent females to putative male pheromones is species-specific; (2) whether males release pheromones from locations besides the anal glands; and (3) whether non-reproductive females are attracted to the anal gland pheromone. Initial steps were made to chemically characterize the male-derived odorants by combining chromatographic techniques with assessment of olfactory potency by electro-olfactogram analysis (EOG).

Methods and Materials

Origin and Maintenance of Fish Male and female *S. pavo* were caught in Ria Formosa (southern Portugal) where breeding occurs from May to September (Almada et al. 1994). At this location, holes in bricks that clam culturists use to delimit their fields are used as shelters and as male nests during the breeding season. At low tide, between March and July, *S. pavo* inside the brick holes were caught by shaking the bricks over a plastic container with water. Males and females in the container were caught with a small net and carried inside buckets to the Ramalhete Experimental Station (University of Algarve) in Ria Formosa. At the end of the studies all fish were released at their place of capture.

Hetero-specific stimuli (see below) were derived from *Salaria fluviatilis* (Asso 1801), captured in Lake Banyoles (Catalonia, Spain). Official permission was issued by local authorities to Dr. Joaquim Pou Rovira (Environmental Sciences Department of the University of Girona, Spain) who caught the fish by an electro-fishing technique. The apparatus was custom-made and turned on only when in close proximity to *S. fluviatilis* observed swimming over stones along the shore of the lake. None of the collected

fish died, and the electro-fishing method had no adverse effects on the lake wildlife. The fish were transported from Girona to the Ramalhete Experimental Station by car in appropriate containers with oxygen-saturated water. After the experiments, *S. fluviatilis* were donated to the Vasco da Gama Aquarium (Lisbon, Portugal) for keeping and breeding.

Up to four male and up to 30 female blennies were maintained in aquaria (70 l of aerated seawater for *S. pavo* at 22 to 28°C and aerated freshwater for *S. fluviatilis* at 24± 2°C; 12-hr photoperiod) with sand and stones on the bottom. Gray polyvinyl chloride (PVC) tubes (15 cm length and 3 cm diameter) were provided as nests for the males, and when required, a net partition separated the males to avoid aggression, especially among *S. fluviatilis* males. *Salaria pavo* were fed daily with commercially available frozen mussels, *Mytilus* spp., and *S. fluviatilis* with *Artemia* spp., small shrimp, *Paleomon serratus*, and trout muscle. Female *S. pavo* were acclimated to these conditions for at least 7 d before use in behavioral assays and were kept separate from those males that were used to obtain macerates of anal glands or male-conditioned water (see below). Both species behaved normally, including sexual behavior and spawning.

Behavioral Assays To assess female attraction to male-derived chemical stimuli, behavioral assays were carried out in a flow-through tank or fluviarium made of glass, similar to that described by Bjerselius et al. (1995a,b). The test area (length × width, 450×320 mm) with a 120-mm deep water column was limited upstream and downstream by two plastic nets (5-mm mesh), and the two inner sides were painted with non-toxic grey paint (Arialcin, Corporação Industrial do Norte S.A., Maia, Portugal). At the downstream edge of the test area, a 'fish box' made of transparent acrylic (with holes allowing water passage) was placed where individual females were introduced. The fish box opened to the test area *via* a remotely operated gate. Particle- and charcoal-filtered natural seawater was pumped into the fluviarium forming a continuous laminar flow (12 l/min) through the test area. Tests with a food dye showed that the dye remained in a non-turbulent plume that expanded, largely due to diffusion, as it was carried downstream. Male-related substances and control water were delivered (10 ml/min) into the test area with a peristaltic pump, *via* one of the two gray PVC tubes (7 cm length and 5 mm diameter) on each side of the inflow water. Each tube was placed 3 mm above the bottom of the fluviarium and 1 cm away from the plastic mesh limiting the upstream edge of the test area. Dye delivered into either side (water injected in the opposite side) showed a plume that expanded downstream but remained within the same side of the test area; the dye front reached the fish box at the downstream edge of the test area within 2 min.

The fluvarium was illuminated from below with infrared light. A black and white infrared-sensitive CCD camera (Ikegami ICD 47/47E IR camera) fitted with a 4- to 10-mm zoom lens with an infrared filter was placed 650 mm above the test area. The camera was connected to a black and white video monitor (Panasonic WV-BM1410) and to a computer in an adjacent room for video-tracking of female position and movement. The infrared illumination underneath the test area produced a white background on the video image over which the fish appeared dark gray. Although infrared light was used to track the fish, all experiments took place during the day.

The EthoVision Pro 3.1 system (Noldus Information Technology, Wageningen, The Netherlands) was used for video tracking (25 frames/sec) of females in the test area of the fluviarium. The position of each fish was recorded at a rate of 5/sec. Data filtering was then applied to separate genuine displacement from slight body movement. To calculate mean linear velocity (cm/sec) and mean angular velocity (mean rate of directional change per distance; deg/cm), movement was considered only when the fish traveled at least one third of its length (standard length) between two consecutive data points. To discriminate between the states of motion and no motion (to calculate the time in motion and number of movements), transition from no motion to motion was defined as when linear velocity exceeded one-third body length per second, whereas the reverse transition was defined as when linear velocity decreased to below one-sixth body length per second.

Female attraction to a given chemical stimulus was assessed by calculating a reaction value $(\mathrm{Rv} = [(S - C)/(S + C)] \times 100)$, which represents the difference between the time that each female spent moving in the stimulus side (S) and in the control (C) side (each side was considered as half of the test area along the water flow axis) normalized to the total time in movement. Thus, a positive Rv indicates attraction to a given chemical stimulus. Reaction values were calculated during their initial upstream movement (immediately after leaving the fish box) in the test area and over the total observation period (5 min). Reaction values based on the total time (moving + not moving time) spent in either side of the test area were also calculated. However, these values are not shown, since they all were positively correlated with Rv values based on time moving. To assess whether female locomotory pattern could be affected by the type of chemical stimulus in the fluviarium, female movements were analyzed during their initial upstream swim by the latency to reach the upstream edge of the test area, number of movements, mean linear velocity (cm/sec), and mean angular velocity (deg/cm).

To test whether female attraction to putative male pheromones is species-specific, four groups of reproductive

female *S. pavo* were tested (to male-conditioned water or anal gland macerate from conspecifics, or to equivalent chemical stimuli from heterospecifics, i.e., *S. fluviatilis*). To test whether *S. pavo* males release female-attractive pheromones that do not originate in the anal gland, one group of reproductive females was tested with water conditioned by males after excision of their anal glands. To test whether non-reproductive females are attracted or not to the anal gland pheromone from conspecifics, two groups of females were tested [to anal gland macerate or to a macerate of food (mussels, *Mytilus* spp., positive control)]. Water-only controls were used to test whether females had any preference for either side of the fluvarium.

Selection of Females and Experimental Procedure On each day, females were classified as reproductive or non-reproductive on the basis of abdomen distension (qualitative scale from 0 to 3) and sexual behavior. Up to five females from the stock tanks were placed in another tank with one male and a nesting site (PVC tube) and their behavior observed for 10 min. Females with distended abdomens (scale=2 or 3) that also exhibited conspicuous courtship (i.e., display of nuptial coloration and rapid flickering of the pectoral fins while opening and closing the mouth in synchrony; Patzner et al. 1986; Gonçalves et al. 1996) were considered reproductively competent. Non-courting females with flat abdomens (0 or 1) were considered non-reproductive. Most females with a distended abdomen exhibited courtship behavior toward the male, whereas the females with flat abdomens neither approached the male nor exhibited courtship. Many females with distended abdomens spawned soon after the experiment, and their abdomens became flat; a few females with flat abdomens spawned, and then only several days later after their abdomen had become distended. Previous work (E.N. Barata and R.M. Serrano, unpublished data) has shown that the gonadosomatic index (gonadal weight/fish weight×100) of courting females with a distended (scale=2 or 3) abdomen was greater (mean±SEM=7.1±0.4%, N=12) than that of non-courting females with a flat (scale=0 or 1) abdomen (2.8±0.2%, N=15).

Replicates with individual females (tested only once) started 1–2 hr after lights-on and were carried out for up to 6 hr; female courtship and spawning has been observed during daylight (Patzner et al. 1986; Gonçalves et al. 1996; Barata et al. 2008). In each replicate, one female was placed in the fish box of the fluviarium, and after 5 min of acclimation, the gate was lifted and the fish's swimming behavior recorded for 5 min. Females that did not move within 5 min were excluded from the analysis. Delivery of chemical and control stimuli started 2 min before lifting the gate. Between replicates (15 min), the water in the fluviarium was fully replaced, and the chemical and control stimuli were delivered into opposite sides of the test area; on each day, only one type of treatment was used. After each replicate, the length (standard length, SL) and weight of females were measured, and they were placed in aquaria with males before being released back to their natural environment. The size (SL) of females in different treatments did not differ significantly (overall mean±SD=67.8±8.7 mm; N=246).

Statistical Analysis of Female Behavior One-way analysis of variance (ANOVA) was used to compare time and locomotory parameters (latency to reach the upstream edge of the test area, number of movements, mean linear velocity, and mean angular velocity) of female initial upstream movement among groups of reproductive females tested with water conditioned by intact conspecific males (N=26), water conditioned by conspecific males without anal glands (N=24), water conditioned by intact heterospecific males (N=27), and control water (N=47). The same statistical analysis was used to compare locomotory parameters between reproductive females tested with anal gland macerates from conspecifics (N=37) and heterospecifics (N=29), and between non-reproductive females tested with conspecific anal gland macerate (N=33) and food macerate (N=23). The mean reaction values (during the initial upstream movement and over the total observation period) in each female group were tested for significant difference from zero (P<0.05) by Student's t test.

Male-Conditioned Water and Anal Gland Macerate for Behavioral Assays Six *S. pavo* males [standard length (SL)=100±8 mm (mean ± SD); body weight (BW)=14.4±4.1 g] and six *S. fluviatilis* males [SL=83±5 mm (mean ± SD); BW=10.7±2.6 g] with fully developed anal glands were placed individually in small glass tanks (30×15×19 cm) with aerated artificial seawater (35 ppt, Instant Ocean®, Spectrum Brands, Cincinnati, OH, USA) or dechlorinated tap-water, respectively. The volume of water used was normalized to the fish's weight (5 g/l). Each container was placed next to a similarly sized glass tank with three conspecific females. The water was conditioned for 24 hr at 24°C. Males were anaesthetized by immersion in water containing MS222 (0.3 g/l; 3-aminobenzoic acid ethyl ester, Sigma-Aldrich, Madrid, Spain), and the anal glands of each male were excised with small scissors cutting the base of the two anal-fin rays bearing the glandular tissue. This procedure is almost as simple as fin clipping, and no visible bleeding occurs. An antiseptic cream (Betadine®) was applied to the wound. Anesthetized fish recovered within 5–10 min and were then placed back in the stock tanks where they exhibited normal behavior. For *S. pavo*, 36 hr later, water was conditioned by the same six males (without their anal glands). In both species,

anal gland regeneration was seen after 3–4 wk. Male-conditioned water was filtered (<150 μm), and a similar volume of water from each male (with or without anal glands, respectively) was pooled. Control water was treated in the same way, except that it did not hold any fish. Male-conditioned water and control water were stored at −20°C. Immediately before use, thawed male-conditioned water was diluted in seawater collected at the inflow of the fluviarium and delivered into the test area at 0.05 mg fish/ml (equivalent to 100-fold dilution of the original conditioned water).

The excised anal glands [*S. pavo*, weight=27.2±6.6 mg (mean ± SD), *N*=6; *S. fluviatilis*, weight=9.9±3.7 mg, *N*=6] were pooled and macerated in distilled water (1 mg/ml) followed by filtration (<0.45 μm). Salt (Instant Ocean) was added to the anal gland macerate and control distilled water to obtain a salinity equivalent to that of seawater (35 ppt), and aliquots were stored at −20°C until use. The anal gland macerate was diluted in seawater and delivered into the test area at a concentration of 0.1 μg/ml. Mussels (*Mytilus* spp.) were macerated in the same way and tested at 0.1 mg/ml with non-reproductive females.

Isolation of Male Odorants We assessed the molecular-weight range and relative hydrophilicity of odorants from the anal gland. To estimate the molecular-weight range, fully developed glands [33.5±6.7 mg (mean ± SD), *N*=6] and two portions of skin (35.1±5.9 mg) from the lateral-dorsal area of the body (as control) were removed from anesthetized males as previously described. Tissues were pooled and macerated in distilled water (1 mg/ml) and filtered (<0.45 μm). Part of each pooled macerate (including a water-only control) was frozen at −20°C for later use. The remainder was ultra-filtered by centrifugation at 4°C through 10 kDa, then through 3 kDa filters (Centriprep YM-10, Amicon, Millipore, Madrid, Spain), and finally through a 500 Da filter (Ultrafiltration Membranes 500, Amicon Limited, Stonehouse, UK) according to the manufacturer's instructions. Each fraction was kept at −20°C until use (see below).

To assess the involvement of non-polar, hydrophobic molecules, two different solid-phase extraction (SPE) sorbents were used. Fully developed anal glands [78.4±2.8 mg (mean ± SD), *N*=6] and anal-fin rays (73.1±5.1 mg, *N*=6) were removed and macerated in distilled water (1 mg/ml) as described previously. Half of each filtered (<0.45 μm) macerate was frozen at −20°C, and the other half was passed through two different SPE columns. Part of the anal gland macerate was passed through C18 columns (500 mg packed in 6-ml glass cartridges, IST—Isolute Sorbent Technology, Glamorgan, Hengoed, UK), which are used to separate and concentrate non-polar organic compounds from water (e.g., steroids; see Scott and Ellis 2007 and references therein). The remainder of the macerate was passed through columns with layered phases of C2 over ENV+ (C2/ENV+, 500/200 mg packed in 6-ml glass cartridges, IST), which are used to trap a wider range of compounds from water (Ingvarsdóttir et al. 2002). Both column types were previously activated with HPLC grade methanol (2 ml) followed by washing with distilled water (2 ml). The substances retained on the column were eluted with bi-distilled ethanol (2 ml). The macerate of anal fin rays was passed through the C2/ENV+ columns only. Water-only control separations were also carried out with both types of column. Both filtrate and eluate were kept at −20°C until use (see below).

Extracts of water conditioned by breeding males with fully developed anal glands (before and after excision) were fractionated by HPLC and the olfactory potency of fractions assessed on females. Males with fully developed anal glands [length=116±6 mm (mean ± SD), *N*=6; weight=25.1±2.8 g, gland area=18.6±2.7 mm^2] were captured during the breeding season (late May). Males with partially developed anal glands [length=110±3 mm (mean ± SD); weight=20.8±1.8 g, gland area=12.8±2.4 mm^2] captured during April were used to assess whether excision of their undeveloped anal glands affects the olfactory potency of male-conditioned water. Artificial seawater was conditioned as described previously (5 g fish/l), and C2/ENV+ columns were used to extract male-conditioned water (approximately 1 l of fish-conditioned water or control water per column) before and after anal gland excision (as described above). Male-conditioned water and control water were stored at −20°C until use. Ethanol eluates of male-conditioned water were dried under a stream of nitrogen, re-dissolved in methanol, and aliquots (20 μl of each eluate) were fractionated with a Shimadzu LC-10AD HPLC system equipped with a reverse-phase C18 column (250×4.6 mm, Phenomenex, UK). Eluting compounds were detected by UV (254 nm). Gradient elution (1 ml/min) was used (100% water, 0–3 min; 0–100% methanol, 3–28 min; 100% methanol, 28–60 min), and 5-min fractions were collected. These were then evaporated under high vacuum and kept at −20°C until assessment of their olfactory potency (see below).

Recording of the EOG and Data Analysis To our knowledge, EOGs have not been recorded before in blennies. Perhaps, this is because the vast majority lives in seawater, and nostrils lack a lamellar olfactory rosette at the bottom of the olfactory chamber as typical of many fishes. Instead, female *S. pavo* have two adjacent olfactory lamellae that extend from the dorso-lateral wall of the olfactory chamber (J. Araújo, O. Lopes, and E.N. Barata, unpublished data).

To reduce the shunting effect of seawater, recording of the EOG in females was carried out in 12 ppt seawater (females were pre-adapted to this salinity in two steps over at least 5 d before recording). Females were anesthetized by immersion in water that contained 100 mg/l MS222 followed by subcutaneous injection of 30 μl/10g Saffan™ (Schering-Plough Animal Health, Welwyn Garden City, UK) and placed on a padded V-clamp. Aerated seawater (12 ppt) was pumped over the gills (approximately 100 ml/100 g fish weight per minute) *via* a plastic tube inserted into the mouth. Attempts to record EOGs from exposed olfactory epithelium by cutting the skin and bone surrounding the olfactory chamber were not successful. Recording of EOGs was achieved by positioning the stimulus delivery tube and recording electrode as described by Murphy et al. (2001). L-Cysteine (10^{-5} M) was used as standard stimulus instead of L-alanine. The olfactory chamber was continually irrigated with 12 ppt seawater *via* a gravity-fed system (4 ml/min), which terminated in a small glass tube (stimulus delivery tube) positioned in the exhalent nostril, creating a reversed water flow (into the exhalent nostril and out of inhalant nostril). Odorant-containing water was introduced into this flow *via* a three-way valve for 10 sec. The resulting EOG was recorded differentially with electrodes made from boro-silicate glass micropipettes (tip diameter, 0.2 mm) filled with 3 M NaCl in 1% agar. The recording electrode was placed inside the inhalant nostril at a position that gave the largest response to the standard stimulus (10^{-5} M L-cysteine); the olfactory response to amino acids (data not shown) was similar to that described for other species (Hara 1994). The reference electrode was placed lightly on the skin of the head. The fish was grounded *via* an Ag–AgCl pellet electrode placed under the head. The DC signal was connected to solid-state electronics *via* Ag–AgCl electrodes (in contact with 3 M KCl). The signal was filtered (low-pass 30 Hz), amplified (2,000- to 5,000-fold) with a CP122 AC/DC strain gauge amplifier (Grass Instrument Division, Astro-Med, Inc., West Warwick, RI, USA); see Velez et al. (2005) for further details. Larger females (between 7 and 10 cm SL) were chosen because the nostrils of smaller females were too small. The reproductive condition of the females was not assessed before or after the experiment. Most females survived the experiment, and after 1–2 wk in aquaria at 35 ppt, they were released at their place of capture.

All stimuli were dissolved in diluted seawater (12 ppt). Before dilution, the ethanol of the SPE eluates was evaporated, and the macerated tissues and their respective filtrates were lyophilized. Groups of related stimuli were tested on the same females, e.g., eluates of water conditioned by males before and after anal gland excision. The order in which the stimuli were given varied among fish. Macerates, centrifugal fractions, and SPE fractions from tissue were applied at 0.1 mg/ml; SPE eluates and HPLC fractions of fish-conditioned water were given at 50 mg fish/ml. Concentrations below these did not evoke responses consistently higher than that of blank (stimulus-free background water treated in exactly the same way as stimulus solutions). Between groups of related stimuli, responses to background water 'blank' and to 10^{-5} M L-cysteine were recorded.

In general, the form of the recorded EOG was typical of fish, i.e., a rapid negative deflection at stimulus onset, which was followed by a period of adaptation in the continued presence of the stimulus. However, this adaptation was sometimes rapid, and the signal returned to baseline levels in the presence of the stimulus soon after the initial peak, and sometimes more slowly, only returning to baseline after the cessation of the stimulus. In each case, however, only the amplitude of the initial peak was measured in millivolts. The mean of the preceding and following response to background water 'blank' was subtracted from each response, which was then normalized to the mean of the preceding and following responses to 10^{-5} M L-cysteine (also blank-subtracted); the statistical analysis was done on these normalized EOG responses. Data were tested for normality and homogeneity of variance and, if necessary, were $\log_{10}(n+1.5)$-transformed to reduce variance heterogeneity between groups.

Centrifugal (molecular weight) fractions of macerated skin and anal glands were tested on different groups of five females, and fractions of the same tissue were tested on the same female. Due to heterogeneity of variances among the variables that could not be eliminated through data transformation, non-parametric statistical tests were used. Friedman's ANOVA test was used to compare EOG responses within each group of females to fractions from extracts of the same tissue (skin or anal gland), whereas the Mann–Whitney *U* test was used to compare female EOG responses to equivalent fractions of the two male tissues (skin and anal gland).

One-way repeated measures ANOVA followed by the Tukey Honest Significance Difference test (Tukey HSD) was used to compare (1) the EOG responses of five females to macerated male tissues (fully developed anal glands and fin rays) and corresponding SPE fractions (eluate and filtrate) and (2) the EOG responses of six females to SPE eluates of water conditioned by males with fully developed and partially developed anal glands, before and after the excision of the glands. Student's *t* test for paired samples was used to compare the EOG responses (five females) to equivalent HPLC fractions of eluates of water conditioned by males before and after anal gland excision.

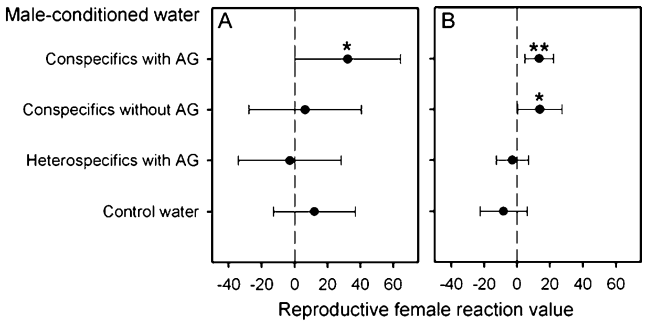

Fig. 1 Reaction values (mean±95% CL) of *Salaria pavo* reproductive females in the fluviarium with water conditioned by conspecific males with anal glands (with AG, N=26); water conditioned by the same males after anal gland excision (without AG, N=24 females); water conditioned by *Salaria fluviatilis* males with anal glands (heterospecifics with AG, N=27); and control water (N=47) during **a** the initial upstream movement (*t_{25}=2.07, P<0.05) and **b** during entire 5-min period of observation (*t_{23}=2.11, P<0.05; **t_{25}=3.13, P<0.01). The variance of female reaction values for conspecific males without anal glands was significantly larger than that of reaction values for intact conspecific males during the entire 5-min period of observation ($F_{24,26}$=2.14, P<0.05)

Results

Behavioral Responses of Females Females in the fluviarium left the fish box and, with successive upstream swimming bouts, reached the edge of the test area close to the stimulus or the control side of the device. After this initial upstream movement, females stayed at the upstream edge for a while (sometimes swimming against the net limiting the test area or across the upstream edge), then swam downstream, eventually moved across the test area, and swam upstream again; females repeated these movements in the test area several times between periods of stillness.

Reproductive females tested with water conditioned by intact conspecific males swam initially upstream along the side, or in the water column, carrying the chemical substances. In contrast, reproductive females tested with water conditioned by conspecific males without anal glands, intact heterospecific males, or control water did not show a preference for either side (Fig. 1a). During the entire 5-min observation, females swam preferentially along the side of the fluviarium containing either water conditioned by conspecific males with or without the anal glands, whereas no preference was shown with water conditioned by intact heterospecific males or control water (Fig. 1b). However, the variability of female reaction values in response to water conditioned by conspecific males without anal glands was greater than that to water conditioned by intact conspecific males (Fig. 1b). During the initial upstream movement, the locomotion parameters were not different among the four groups of reproductive females (Table 1). Furthermore, the time that females spent moving in the test area over the entire observation period was not different [conspecific males with anal glands, 166.7± 9.0 sec (mean ± SEM); conspecific males without anal glands, 163.4±9.5 sec; intact heterospecific males, 153.5±11.4; control water, 140.9±10.3 sec; $F_{3,120}$=1.25, P=0.295].

For the anal gland macerates from conspecifics, reproductive females initially swam upstream along the stimulus side of the fluviarium but did not show a preference for either side when the stimulus was from heterospecific males (Fig. 2a). During the total observation period, however, females showed no preference for either side in response to either treatment (Fig. 2b). Non-reproductive females tested for the macerate of anal glands from conspecific males did not show a preference during the initial upstream movement or throughout the total observation period. However, non-reproductive females swam preferentially along the stimulus side of the fluviarium in response to the mussel positive control during both initial upstream movement and the total observation period (Fig. 2a, b). The locomotory parameters of initial upstream movement (Table 2) and time

Table 1 Parameters (mean ± SEM) that characterize the initial upstream movement of *Salaria pavo* females in the fluviarium (male-conditioned water)

Parameter[a]	Control[b]	Conspecifics with anal glands[c]	Conspecifics without anal glands[d]	Heterospecifics with anal glands[e]
Latency to up-stream edge (sec)	15.2±2.2	22.9±4.8	22.3±5.1	15.2±2.3
Linear velocity (cm/sec)	8.7±0.5	7.7±0.6	7.4±0.7	7.4±0.5
Angular velocity (deg/cm)	5.6±0.5	5.2±0.7	5.5±0.7	4.8±0.4
Number of movements	4.7±0.5	7.0±1.2	7.7±1.6	5.6±0.7

[a] The locomotion parameters were not significantly different among the four groups of females (one-way ANOVA): latency to up-stream edge of the test area, $F_{3,120}$=1.50, P=0.219; mean linear velocity, $F_{3,120}$=1.43, P=0.238; mean angular velocity, $F_{3,120}$=0.42, P=0.737; number of movements, $F_{3,120}$=2.15, P=0.100.
[b] Reproductive females without any male-related stimulus (control) (N=47 females).
[c] Reproductive females tested to water conditioned by conspecific males with anal glands (N=26 females).
[d] Reproductive females tested to water conditioned by conspecific males without anal glands (N=24 females).
[e] Reproductive females tested to water conditioned by heterospecific males (*Salaria fluviatilis*) with anal glands (N=27 females).

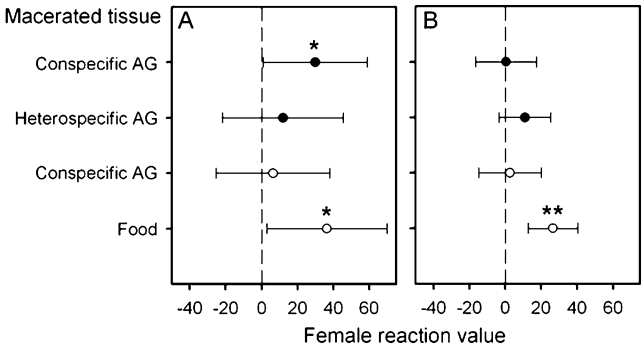

Fig. 2 Reaction values (mean±95% CL) of reproductive (*filled circles*) and non-reproductive (*open circles*) *Salaria pavo* females in the fluviarium with an anal gland macerate from conspecific males (reproductive, N=37; non-reproductive, N=33), an anal gland macerate from *Salaria fluviatilis* (reproductive, N=29), and a macerate of food (mussels), *Mytilus* spp. (non-reproductive, N=23) during **a** the initial upstream movement (*reproductive, t_{36}=2.17, P<0.05; non reproductive, t_{22}=2.78, P<0.05) and **b** the entire 5-min period of observation (**non-reproductive, t_{22}=4.02, P<0.01)

spent moving during the total observation period were not different among the four groups of females [reproductive females tested with conspecific anal glands, 129.1±6.9 sec (mean ± SEM); reproductive females tested with heterospecific anal glands, 127.6±6.6 sec; non-reproductive females tested with conspecific anal glands, 140.2±7.4; non-reproductive females tested with food-odor, 129.6± 5.7 sec; $F_{3,118}$=0.73, P=0.537].

EOG Responses to Anal Gland Odorants Females gave different amplitude responses to different molecular-weight fractions from the anal gland macerate (Friedman ANOVA, N=5, df=3, χ^2=14.76, P<0.005) and the skin macerate (Friedman ANOVA, N=5, df=3, χ^2=13.70, P<0.005; Fig. 3). The majority of the anal gland olfactory potency resided in the less than 500 Da fraction of the macerate. However, odorants with a molecular weight greater than 500 Da were also present. In the skin macerate, all olfactory activity was found in the <500 Da fraction. The anal gland macerate evoked larger EOG amplitudes than the skin macerate (Mann–Whitney U test, Z=2.40, P<0.05). Although not significantly different (Mann–Whitney U test, Z=1.78, P=0.07), the fraction containing substances less than 500 Da from the anal gland macerate evoked larger EOG amplitudes than that of skin macerate.

The olfactory potency of the C2/ENV+ eluate was not different from that of the original anal gland macerate. Both the macerate and the eluate evoked larger EOG amplitudes than the filtrate (Fig. 4). The olfactory potency of the fin-ray macerate was weaker than that of the anal gland, and the C2/ENV+ sorbent only extracted a fraction of the olfactory activity; olfactory potency of the eluate was less than that of the macerate. The sum of the EOG amplitudes elicited by either fraction was approximately equal to that of the untreated macerate (Fig. 4). The EOG responses to the anal gland macerate and corresponding C18 filtrate and eluate showed that the potency of the filtrate was stronger than that of the eluate and not significantly different from the original macerate (data not shown). This type of sorbent is, therefore, ineffective in extracting the odorants from the anal gland.

EOG Responses to Eluates of Male-Conditioned Water and their HPLC Fractions The eluate of water conditioned by males with fully developed anal glands elicited the largest EOG responses, and subsequent excision of the glands caused a decrease in olfactory potency (Fig. 5). In contrast, the olfactory potency of water conditioned by males with partially developed anal glands was not affected by their excision (Fig. 5). The eluate of water-only control elicited lower responses than the eluates of male-conditioned water (data not shown).

Table 2 Parameters (mean ± SEM) that characterize the initial upstream movement of *Salaria pavo* females in the fluviarium (macerated tissues)

Parameter[a]	Reproductive females		Non-reproductive females	
	Conspecific anal glands[b]	Heterospecific anal glands[c]	Conspecific anal glands[d]	Food macerate[e]
Latency to up-current edge (sec)	16.7±3.3	16.6±3.8	16.7±3.1	21.0±2.9
Linear velocity (cm/sec)	9.0±0.3	9.2±0.7	9.0±0.5	8.0±0.4
Angular velocity (deg/cm)	5.5±0.6	4.5±0.4	5.0±0.5	5.2±0.6
Number of movements	5.8±1.1	5.1±0.6	4.5±0.5	6.3±0.9

[a] The locomotion parameters were not significantly different among the four groups of females (one-way ANOVA): latency to up-stream edge of the test area, $F_{3,118}$=0.35, P=0.793; mean linear velocity, $F_{3,118}$=0.97, P=0.411; mean angular velocity, $F_{3,118}$=0.86, P=0.463; number of movements, $F_{3,118}$=0.90, P=0.445.
[b] Reproductive females tested to water with macerated anal glands from conspecific males (N=37 females).
[c] Reproductive females tested to water with macerated anal glands from heterospecific (*Salaria fluviatilis*) males (N=29 females).
[d] Non-reproductive females tested to water with macerated anal glands from conspecific males (N=33 females).
[e] Non-reproductive females tested to water with macerated mussels, *Mytilus* spp. (N=23 females).

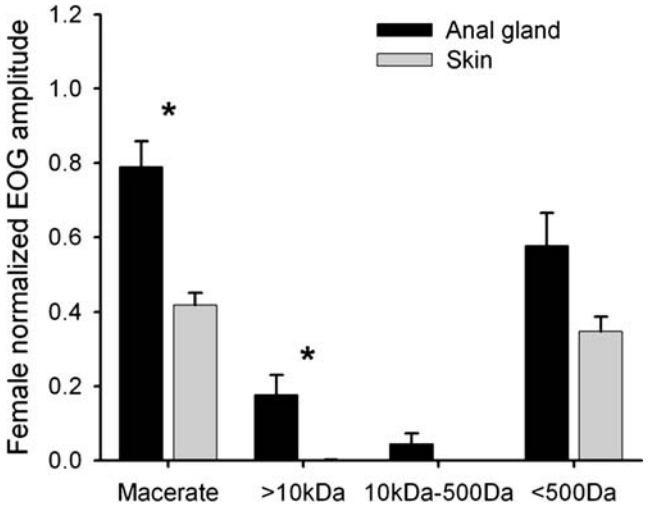

Fig. 3 Normalized EOG amplitudes (mean + SEM) of *Salaria pavo* females (N=5) to macerates and corresponding molecular weight fractions (>10 kDa, between 10 kDa and 500 Da, and <500 Da) of anal gland (*solid bar*) and skin (*gray bar*) extracts. Concentration of macerates and fractions: 0.1 mg/ml. Significant differences (Mann–Whitney U test) between equivalent fractions of the two tissues are indicated, *P<0.05)

The EOG responses to HPLC fractions of water conditioned by males with fully developed anal glands (Fig. 6) showed that the three 5-min fractions eluting between 0–15 min (F1–F3) elicited significantly higher EOG amplitudes than the same fractions after anal gland excision (t_4=7.43, P<0.01 for F1; t_4=5.18, P<0.01 for F2 and t_4=8.69, P<0.001 for F3). The percentage of methanol in the mobile phase increased from zero to about 48%

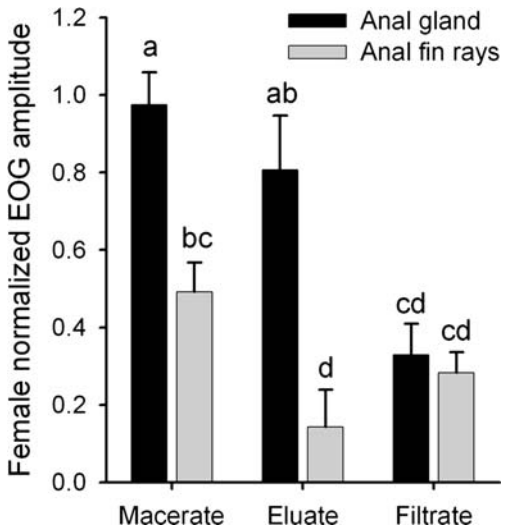

Fig. 4 Normalized EOG amplitudes (mean + SEM) of *Salaria pavo* females (N=5) to macerates and corresponding solid phase extraction fractions (eluate and filtrate) of male anal gland (*black bar*) and anal fin ray (*gray bar*) tissues. Concentration of macerates and fractions was 0.1 mg/ml. *Different letters above the bars* indicate significant differences (repeated-measures ANOVA, $F_{5,20}$=12.98, P<0.001, followed by Tukey HSD test, P<0.05). Solid phase extraction was performed with a 6-ml glass cartridge packed with C2/ENV+

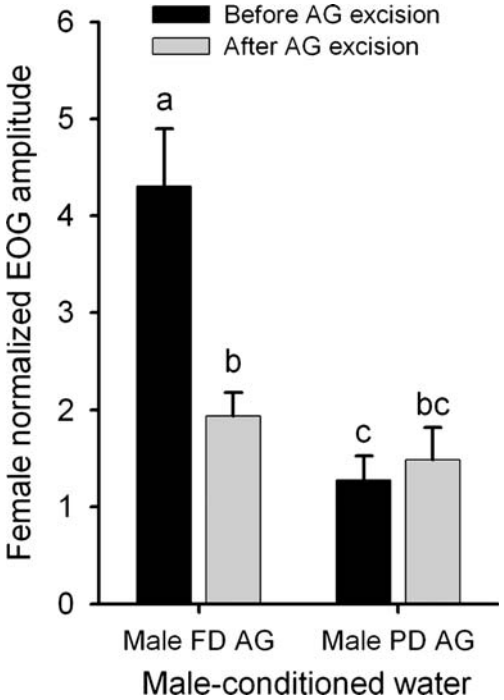

Fig. 5 Normalized EOG amplitudes (mean + SEM) of *Salaria pavo* females (N=6) to SPE eluates of male-conditioned water at 50 mg fish-weight/ml. Male-conditioned water before (*black bar*) and after (*gray bar*) removal of fully developed (*FD*) or partially developed (*PD*) anal gland (*AG*). *Different letters above the bars* indicate significant differences (repeated-measures ANOVA, $F_{3,15}$=185.88, P<0.001, followed by Tukey HSD test, P<0.01). Solid phase extraction was performed with a 6-ml glass cartridge packed with C2/ENV+

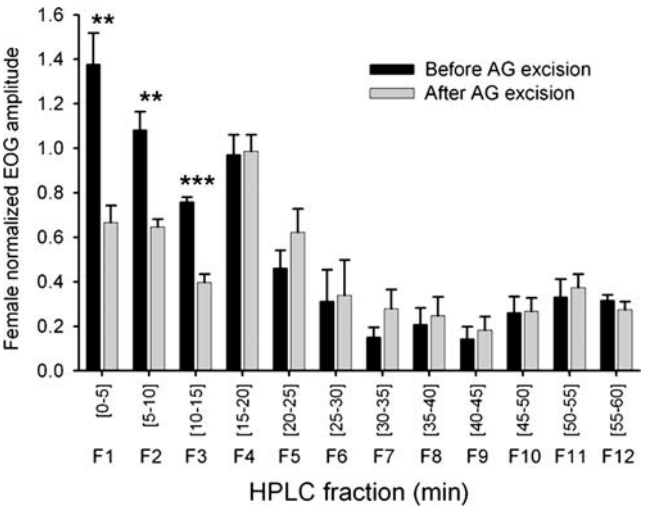

Fig. 6 Normalized EOG amplitudes (mean + SEM) of *Salaria pavo* females (N=5) to 5 min HPLC fractions (50 mg fish-weight/ml) of SPE eluates (extraction with 6-ml glass cartridge packed with C2/ENV+) of water conditioned by breeding males before (*solid bar*) and after (*gray bar*) excision of fully developed anal glands (*AG*). Significant differences (Student's t test for paired samples) are indicated,**P<0.01 or ***P<0.001)

during the elution of these fractions (F1, from 0% to 8%; F2, up to 28%; F3, up to 48%). The fraction eluting between 15 and 20 min (F4; up to 68% methanol in the mobile phase) also elicited relatively large EOG amplitudes, but no significant differences were found between before and after anal gland excision (t_4=0.24, P=0.82). The eight fractions eluting later than 20 min (F5 to F12) elicited relatively small EOG amplitudes without any differences (P>0.05) between males with or without anal glands. The chromatograms obtained at 254 nm could not be related to the pattern of EOG responses and, therefore, are not shown.

Discussion

We present new evidence on the putative pheromonal system of the peacock blenny and possibly other blenniids. First, reproductive females were attracted to male-conditioned water (whole male odor) and to anal gland macerates (anal gland odor) from conspecifics but not to those of heterospecifics, suggesting that attraction to putative male pheromones is species-specific. Second, although reproductive females were initially attracted to conspecific anal gland odor, this response was ephemeral compared with that to whole male odor. Furthermore, whole odor from males without anal glands attracted reproductive females over 5 min; however, female attraction was significantly more variable than that to whole odor from intact males, and during initial upstream movement, females were not attracted to odor of males without anal glands. These observations suggest that attraction of reproductive *S. pavo* females to conspecific males possibly involves a multicomponent pheromone that consists of the putative anal gland pheromone and another components not originating from the anal glands. Finally, non-reproductive females were not attracted to conspecific anal gland odor, thus supporting a role for the putative anal gland pheromone in mate-location and mate-choice as previously suggested (Barata et al. 2008).

Previous assays (Barata et al. 2008) carried out in Y-maze flow-through tanks (about tenfold larger than the fluviarium) showed that reproductively competent females are attracted to odor of intact males and anal gland odor but not to odor of males without anal glands or female odor. These results apparently contradict the current study in which reproductive females were attracted to odor of males without anal glands and initially attracted to anal gland odor. With respect to the anal gland, the difference could be due to different concentrations used in the two assays (at least 50-fold higher in the Y-maze than the fluviarium). However, differences in concentration cannot account for the different responses to male-conditioned water without anal glands (at least 16-fold lower in the fluviarium than the Y-maze).

Common to both assays is the preference of females to swim initially upstream along the side containing odor of intact males or anal gland odor, showing no preference for the side containing odor of males without anal glands. In the fluviarium, as in the Y-maze, females were not attracted to whole odor of males without anal glands during their initial upstream movement. However, in contrast to the Y-maze, females in the fluviarium could move freely between the two odor plumes, and therefore, attraction to odor from males without anal glands during the entire observation period could be assessed. The comparison of these two results suggests that the anal gland odor especially affects female spatial orientation when they initially swim towards the odor source, whereas whole male odor has the additional effect of arresting females within it. Possibly, in the natural environment, the anal gland odor initially attracts females to closer proximity where other components of male odor may be important in the mate-choice and/or to entice females into the nest. Intact male blennies get more visits from reproductive females and receive more eggs in their nests than males without anal glands (Barata et al. 2008).

The current study also shows that the majority of odorants released by the anal glands of breeding males are hydrophilic molecules smaller than 500 Da. In addition, breeding males release other, less hydrophilic odorants (especially olfactory-active in HPLC fraction F4) that do not originate from the anal gland. Possibly, the highly hydrophilic odorants in male water constitute the putative anal gland pheromone, whereas the less hydrophilic potent odorants constitute another male pheromone.

The anal glands of *S. pavo* consist of extensive integumental folds in the first two anal fin rays, with numerous clusters of secretory cells that differentiate during the breeding season. The ultra-structure of the secretory cells indicates intense glycoprotein synthesis, especially of neutral mucins (Serrano et al., unpublished data). Most of the proteinaceous material in the anal glands is hydrophilic, whereas only a relatively small amount is hydrophobic (Giacomello et al. 2006). Therefore, further studies should address whether proteins and/or enzyme products of such proteins (such as small peptides and/or amino acids) constitute the putative anal-gland pheromone. In some newts, male abdominal glands produce and release protein-derived peptides that are female-attractive pheromones (Toyoda et al. 2004; Kikuyama et al. 2005). Furthermore, skin glands of anuran males secrete peptide pheromones that attract conspecific females (Wabnitz et al. 2000; Kikuyama et al. 2005). In crustaceans and other marine invertebrates, there is increasing evidence for peptide-like pheromones and kairomones and substituted amino sugar kairomones derived from enzyme action on glycoproteins and proteoglycans (Rittschof and Cohen 2004). Whether

the anal gland pheromone of male blennies consists of peptides, peptide-derivatives of larger protein precursors synthesized by the anal gland secretory cells, or another type of hydrophilic molecule, its release together with mucus would suggest a sustained, slow release by males in their nest.

The less-hydrophilic potent odorants found in odor of breeding males with and without anal glands (HPLC fraction F4) may derive from the testes. Blennies have two testicular accessory organs that produce steroids, the testicular glands and the testicular blind pouches. In each testis, the testicular gland consists of irregularly arranged tubules formed by a monolayered epithelium of cells that secrete glycoproteins (acidic mucins—sialomucins) and steroid-producing cells (do not seem to produce steroid glucuronides) in the *interstitium* (Seiwald and Patzner 1989) that are the major source of 11-ketotestosterone (Oliveira et al. 2001). However, 11-ketotestosterone did not elicit significant EOG responses in females (data not shown). In contrast, secretory cells lining the epithelium of the blind pouches seem to produce mainly steroid glucuronides (Lahnsteiner et al. 1993). Although the blind pouch fluid is a potent odorant (data not shown), it is not known whether the odorants are steroid glucuronides that could act as sex pheromones by analogy with marine and freshwater gobies (Colombo et al. 1980; Murphy et al. 2001; Belanger et al. 2004; Arbuckle et al. 2005; Gammon et al. 2005).

Taken together, the results show that *S. pavo* males release a species-specific odor that attracts reproductively competent females. This odor consists of hydrophilic odorants from the anal gland and less hydrophilic odorants that possibly originate from the testes or blind pouches. Therefore, we suggest that male blennies release a complex multicomponent pheromone to attract reproductively competent females.

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References

ALMADA, V. C., and SANTOS, R. S. 1995. Parental care in the rocky intertidal: A case study of adaptation and exaptation in Mediterranean and Atlantic blennies. *Rev. Fish Biol. Fish.* 5:23–37.

ALMADA, V. C., GONÇALVES, E. J., SANTOS, A. J., and BAPTISTA, C. 1994. Breeding ecology and nest aggregations in a population of *Salaria pavo* (Pisces: Blenniidae) in an area where nest sites are very scarce. *J. Fish Biol.* 45:819–830.

APPELT, C. W., and SORENSEN, P. W. 2007. Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Anim. Behav.* 74:1329–1338.

ARBUCKLE, W. J., BELANGER, A. J., CORKUM, L. D., ZIELINSKI, B. S., LI, W. M., YUN, S. S., BACHYNSKI, S., and SCOTT, A. P. 2005. In vitro biosynthesis of novel 5 b-reduced steroids by the testis of the round goby, *Neogobius melanostomus*. *Gen. Comp. Endocrinol.* 140:1–13.

BARATA, E. N., SERRANO, R. M., MIRANDA, A., NOGUEIRA, R., HUBBARD, P. C., and CANÁRIO, A. V. M. 2008. Putative pheromones from the anal glands of male blennies attract females and enhance male reproductive success. *Anim. Behav.* 75:379–389.

BELANGER, A. J., ARBUCKLE, W. J., CORKUM, L. D., GAMMON, D. B., LI, W., SCOTT, A. P., and ZIELINSKI, B. S. 2004. Behavioural and electrophysiological responses by reproductive female *Neogobius melanostomus* to odours released by conspecific males. *J. Fish Biol.* 65:933–946.

BJERSELIUS, R., OLSÉN, K. H., and ZHENG, W. 1995a. Behavioural and endocrinological responses of mature male goldfish to the sex pheromone 17a,20b-dihydroxy-4-pregnen-3-one in the water. *J. Exp. Biol.* 198:747–754.

BJERSELIUS, R., OLSÉN, K. H., and ZHENG, W. 1995b. Endocrine, gonadal and behavioral responses of male crucian carp to the hormonal pheromone 17a,20b-dihydroxy-4-pregnen-3-one. *Chem. Senses* 20:221–230.

COLOMBO, L., MARCONATO, A., and FRISCO, C. 1980. Endocrinology of teleost reproduction. A testicular steroid pheromone in the black goby, *Gobius jozo* L. *Boll. Zool.* 47:355–364.

GAMMON, D. B., LI, W., SCOTT, A. P., ZIELINSKI, B. S., and CORKUM, L. D. 2005. Behavioural responses of female *Neogobius melanostomus* to odours of conspecifics. *J. Fish Biol.* 67:615–626.

GIACOMELLO, E., MARCHINI, D., and RASOTTO, M. 2006. A male sexually dimorphic trait provides antimicrobials to eggs in blenny fish. *Biology Lett.* 2:330–333.

GIBSON, R. N. 1969. The biology and behavior of littoral fish. *Oceanogr. Mar. Biol. Ann. Rev.* 7:367–410.

GONÇALVES, E. J., ALMADA, V. C., OLIVEIRA, R. F., and SANTOS, E. J. 1996. Female mimicry as a mating tactic in males of the blenniid fish *Salaria pavo*. *J. Mar. Biol. Ass. U. K.* 76:529–538.

HARA, T. J. 1994. The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* 4:1–35.

INGVARSDÓTTIR, A., BIRKETT, M. A., DUCE, I., GENNA, R. L., MORDUE, W., PICKETT, J. A., WADHAMS, L. J., and MORDUE, A. J. 2002. Semiochemical strategies for sea louse control: Host location cues. *Pest Manag. Sci.* 58:537–545.

KIKUYAMA, S., NAKADA, T., TOYODA, F., IWATA, T., YAMAMOTO, K., and CONLON, J. M. 2005. Amphibian pheromones and endocrine control of their secretion. *Ann. N. Y. Acad. Sci.* 1040:123–130.

LAHNSTEINER, F., NUSSBAUMER, B., and PATZNER, R. A. 1993. Unusual testicular accessory-organs, the testicular blind pouches of blennies (Teleostei, Blenniidae)—fine-structure, (enzyme) histochemistry and possible functions. *J. Fish Biol.* 42:227–241.

LAMBERT, J. G. D., and RESINK, J. W. 1991. Steroid glucuronides as male pheromones in the reproduction of the African catfish *Clarias gariepinus*—a brief review. *J. Steroid Biochem. Mol. Biol.* 40:549–556.

LAUMEN, J., PERN, U., and BLUM, V. 1974. Investigations on the function and hormonal regulation of the anal appendices in *Blennius pavo* (Risso). *J. Exp. Zool.* 190:47–56.

MURPHY, C. A., STACEY, N. E., and CORKUM, L. D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: Olfactory and behavioral responses. *J. Chem. Ecol.* 27:443–470.

OLIVEIRA, R. F., ALMADA, V., GONÇALVES, E. J., FORSGREN, E., and CANÁRIO, A. V. M. 2001. Androgen levels and social interactions in breeding males of the peacock blenny. *J. Fish Biol.* 58:897–908.

PAPACONSTANTINOU, C. A. 1979. Secondary sex characters of Blennioid fishes (Pisces: Blenniidae). *Thalassographica* 1:57–75.

PATZNER, R. A., SEIWALD, M., ADLGASSER, M., and KAURIN, G. 1986. The reproduction of *Blennius pavo*. 5. Reproductive behavior in natural environment. *Zool. Anz.* 216:338–350.

RESINK, J. W., VAN DEN HURK, R., GROENINX, R. F. O., and HUISMAN, E. A. 1987. The seminal vesicle as source of sex attracting substances in the African catfish, *Clarias gariepinus*. *Aquaculture* 63:115–127.

RESINK, J. W., VOORTHUIS, P. K., VAN DEN HURK, R., PETERS, R. C., and VANOORDT, P. G. W. J. 1989. Steroid glucuronides of the seminal vesicle as olfactory stimuli in African catfish, *Clarias gariepinus*. *Aquaculture* 83:153–166.

RITTSCHOF, D., and COHEN, J. H. 2004. Crustacean peptide and peptide-like pheromones and kairomones. *Peptides* 25:1503–1516.

SCOTT, A. P., and ELLIS, T. 2007. Measurement of fish steroids in water—a review. *Gen. Comp. Endocrinol.* 153:392–400.

SEIWALD, M., and PATZNER, R. A. 1989. Histological, fine-structural and histochemical differences in the testicular glands of gobiid and blenniid fishes. *J. Fish Biol.* 35:631–640.

STACEY, N., and SORENSEN, P. 2006. Reproductive pheromones, pp. 359–412, in K. A. Sloman, R. W. Wilson, and S. Balshine (eds.). Behaviour and Physiology of Fish. Academic, London.

TOYODA, F., YAMAMOTO, K., IWATA, T., HASUNUMA, I., CARDINALI, M., MOSCONI, G., POLZONETTI-MAGNI, A. M., and KIKUYAMA, S. 2004. Peptide pheromones in newts. *Peptides* 25:1531–1536.

VELEZ, Z., HUBBARD, P. C., BARATA, E. N., and CANÁRIO, A. V. M. 2005. Evidence for functional asymmetry in the olfactory system of the Senegalese sole (*Solea senegalensis*). *Physiol. Biochem. Zool.* 78:756–765.

WABNITZ, P. A., BOWIE, J. H., TYLER, M. J., WALLACE, J. C., and SMITH, B. P. 2000. Differences in the skin peptides of the male and female Australian tree frog *Litoria splendida*: The discovery of the aquatic male sex pheromone splendipherin, together with Phe8 caerulein and a new antibiotic peptide caerin 1.10. *Eur. J. Biochem.* 267:269–275.

ZANDER, C. D. 1975. Secondary sex characteristics of blennioid fishes (Perciformes). *Publ. Staz. Zool. Napoli* 39:717–727.

ZANDER, C. D. 1986. Blenniidae, pp. 1096–1112, in P. J. P. Whitehead, M. L. Bauchot, J. C. Hureau, J. Nielsen, and E. Tortonese (eds.). Fishes of the North-eastern Atlantic and the Mediterranean. UNESCO, Paris.

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Chemical Characterization of Territorial Marking Fluid of Male Bengal Tiger, *Panthera tigris*

B. V. Burger · M. Z. Viviers · J. P. I. Bekker · M. le Roux · N. Fish · W. B. Fourie · G. Weibchen

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Abstract The territorial marking fluid of the male Bengal tiger, *Panthera tigris*, consists of a mixture of urine and a small quantity of lipid material that may act as a controlled-release carrier for the volatile constituents of the fluid. Using gas chromatography and gas chromatography–mass spectrometry, 98 volatile compounds and elemental sulfur were identified in the marking fluid. Another 16 volatiles were tentatively identified. The majority of these compounds were alkanols, alkanals, 2-alkanones, branched and unbranched alkanoic acids, dimethyl esters of dicarboxylic acids, γ- and δ-lactones, and compounds containing nitrogen or sulfur. Several samples of the marking fluid contained pure (*R*)-3-methyl-2-octanone, (*R*)-3-methyl-2-nonanone, and (*R*)-3-methyl-2-decanone, but these ketones were partly or completely racemized in other samples. The γ-lactone (*S*)-(+)-(*Z*)-6-dodecen-4-olide and the C_8 to C_{16} saturated (*R*)-γ-lactones and (*S*)-δ-lactones were present in high enantiomeric purities. The chiral carboxylic acids, 2-methylnonanoic acid, 2-methyldecanoic acid, 2-methylundecanoic acid, and 2-ethylhexanoic acid were racemates. Cadaverine, putrescine, and 2-acetylpyrroline, previously reported as constituents of tiger urine, were not detected. The dominant contribution of some ketones, fatty acids, and lactones to the composition of the headspace of the marking fluid suggests that these compounds may be important constituents of the pheromone. Although it constitutes only a small proportion, the lipid fraction of the fluid contained larger quantities of the volatile organic compounds than the aqueous fraction (urine). The lipid derives its role as controlled-release carrier of the chemical message left by the tiger, from its affinity for the volatiles of the marking fluid. Six proteins with masses ranging from 16 to 69 kDa, *inter alia*, the carboxylesterase-like urinary protein known as cauxin, previously identified in the urine of the domestic cat and other felid species, were identified in the urine fraction of the marking fluid.

Keywords Bengal tiger · *Panthera tigris* · Semiochemical · Pheromone · Territorial marking · Mass spectrum · Chiral gas chromatography · Urinary protein · Cauxin · Lactone

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B. V. Burger (✉) · M. Z. Viviers · M. le Roux · N. Fish
Laboratory for Ecological Chemistry, University of Stellenbosch,
Private Bag X1,
Matieland 7602, South Africa
e-mail: LECUS@sun.ac.za

J. P. I. Bekker
Institute for Plantbiotechnology,
Private Bag X1,
Matieland 7602, South Africa

W. B. Fourie
Tygerberg Zoo,
P.O. Box 524, Kraaifontein 7569, South Africa

G. Weibchen
Institut für Organische Chemie, Universität Hamburg,
Martin-Luther-King-Platz 6,
20146 Hamburg, Germany

Introduction

The tiger, *Panthera tigris*, is the largest existing member of the Felidae. Of the six subspecies of tiger, the Bengal tiger, *P. tigris tigris*, occurs throughout India and Indochina (Ellerman and Morrison-Scott 1951). The wide geographical distribution implies a great adaptability to different environmental conditions; the only requisites for its

survival are some vegetative cover, a supply of water, and sufficient prey (Schaller 1967). Tigers are normally solitary animals but occasionally travel in groups of four to eight (Schaller 1967). They use different sounds, postures, and gestures to communicate with each other when they are together (Leyhausen 1960). Although vocal communication is the main form of communication, they vocalize relatively infrequently (Schaller 1967). Because of their nocturnal and solitary habits, they make extensive use of semiochemical communication, in which urine and feces appear to be the main vectors of the semiochemicals. Urine mixed with scent gland secretions is sprayed as a territorial marking fluid on conspicuous objects. Apparently, it is a common way of signaling that another tiger is in the vicinity, providing a visitor the opportunity to avoid conflict (Leyhausen and Wolff 1959). It is thought that these scent marks are a major source of information, revealing how recently a tiger has passed by, its gender, and in the case of a female, whether she is sexually receptive. Both sexes mark territories, but a male's territory may overlap several females' territories (Schaller 1967). Females scent mark intensively just prior to estrus. This behavior is reduced during estrus. Males, on the other hand, mark more frequently when females are in estrus than during other stages of the females' cycle (Smith et al. 1989). Another source of semiochemicals is the strong-smelling secretion produced by the anal glands, which, when mixed with the feces, is also used to mark territory. Because there is no connecting channel between the urinary tract and the anal gland (Hashimoto et al. 1963), it has been concluded that the marking fluid does not contain anal gland secretion (Brahmachary and Dutta 1987).

Brahmachary and Dutta (1979) described some of the chemical and physical properties of the animal's marking fluid. 2-Phenylethylamine (Brahmachary and Dutta 1979) and 2-acetylpyrroline (Brahmachary 1996) were identified as two of the compounds responsible for the characteristic odor of the animal's urine. This odor was less persistent in the absence of the lipid fraction of the fluid (Brahmachary et al. 1990). The lipid material consists of a complex mixture of, *inter alia*, long-chain free and esterified saturated and unsaturated fatty acids and alcohols with carbon chains of 14 to 22 carbon atoms (Poddar-Sarkar 1996).

In bioassays carried out during investigation into the semiochemical role of the urine of the cheetah, *Acinonyx jubatus* (Burger et al. 2006b), it was observed that whereas a male Bengal tiger showed minimal interest in the cheetah's urine and in various other odorants used as controls, it immediately attempted to overmark a mixture of a few of the synthetic constituents of its own marking fluid. These constituents have not been reported in earlier studies on the tiger's marking fluid, suggesting that some constituents of the marking fluid were not detected in previous studies. In this paper, we report our attempts at a comprehensive chemical characterization of the volatile organic fraction of the tiger's marking fluid and results of behavioral experiments with synthetic constituents of the marking fluid, as far as this was feasible with one pair of tigers in captivity.

In what follows, the lipid and aqueous fractions of the marking fluid will be referred to as the lipid fraction and urine, respectively.

Methods and Materials

General All Pyrex glassware used in the handling of biological material and extracts and in the preparation of reference compounds was heated to 500°C in an annealing oven to remove traces of organic material. Dichloromethane (Pestanal Grade, Riedel-de Haën, Seelze, Germany) was analyzed by gas chromatography (GC) and found to be pure enough for extraction purposes when used in small quantities. Syringes, stainless steel needles, and other tools were cleaned by rinsing with this solvent.

Collection of Marking Fluid A pair of Bengal tigers was available for the present study at the Tygerberg Zoo near Cape Town, South Africa. Shortly before completion of our research, the female had a cub, and although she must have been in estrus during the period the work was carried out, it is not known whether the intermittent collection of marking fluid from the male on occasion coincided with estrus in the female. Spraying behavior could not be induced in the female, but marking fluid was obtained from the 5-year-old male as follows. One edge of an aluminum sheet (100×500×2 mm) was folded over to form a small gutter on that side. A hole was made at one end in the bottom of the gutter while pressing the aluminum outward to form a funnel through which the marking fluid could flow into a 100-ml glass bottle. The collection device was thoroughly cleaned with soap and water, rinsed with methanol, and heated with an industrial paint stripper. The device was suspended on the outside of the steel mesh of the tiger's cage with the fluid-collecting channel facing toward the cage and at an angle that allowed the fluid to flow into the bottle. To induce the tiger to spray its marking fluid toward the collection device, various odorants (see "Results and Discussion") were applied to the back of the aluminum. Thus, a filter paper loaded with a dilute solution of a compound (1 µl in 1 ml of dichloromethane) or a mixture of compounds (10 µl in 2 ml of dichloromethane) was taped to the back of the aluminum. The collected marking fluid was stored at −20°C until it was used for the experiments.

Analytical Methods GC analyses were carried out with Carlo Erba 4200 and 5300 gas chromatographs equipped with flame ionization detectors and split/splitless injectors, operated at 220°C and 280°C, respectively, and with cool on-column injectors. Hydrogen was used as the carrier gas (50 cm/sec at 40°C).

The capillary columns used in this project were manufactured in the Laboratory for Ecological Chemistry (LECUS, Stellenbosch University) and were provided with integrated retention gaps of 1 to 2 m. Column A (glass, 40 m×0.25 mm inner diameter [i.d.]) was coated with 0.25 μm of the polar stationary phase AT-1000 (FFAP equivalent), column B (glass, 40 m×0.25 mm i.d.) coated with 0.25 μm of PS-089-OH (DB-5 equivalent), the enantioselective columns C (glass, 30 m×0.3 mm i.d.) with 0.25 μm of OV-1701-OH containing 10% heptakis(2,3-di-*O*-methyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin), D (glass, 30 m×0.3 mm i.d.) with 0.25 μm of PS-089-OH containing 10% heptakis(2,3-di-*O*-acetyl-6-*O*-*tert*-butyldimethylsilyl)-β-cyclodextrin, E (glass, 30 m×0.3 mm i.d.) with 0.25 μm of OV-1701-OH containing 10% of heptakis (2,3,6-tri-*O*-methyl)-β-cyclodextrin, and the preparative column F (fused silica, 32 m×0.53 mm i.d.) with 2.0 μm of PS-255 (DB-1 equivalent).

Extracts of the urine and solutions of reference compounds were injected in split mode at a column temperature of 30°C to cold trap the volatiles entering the column. Column A was then programmed at 2°C from 40°C to 250°C (hold 10 min) and column B at 2°C from 40°C to 280°C (hold 10 min). Enantioselective columns C and D were programmed at 1°C from 40°C to 200°C and to 220°C, respectively, and column E at 1°C from 40°C to 140°C. The preparative column F was programmed with an isothermal period during which the particular target analyte was collected.

Synthetic 3-methyl-2-alkanones were purified by preparative GC on column F, collecting fractions manually (Burger et al. 2008). Preparative chromatography on enantioselective column C gave the pure enantiomers of synthetic 3-methyl-2-octanone (**10**). A sample enriched 2:1 with respect to the (*R*)-enantiomer with the shorter retention time on column C was used for the synthesis of 2-methylheptanoic acid enriched with respect to the second-eluting (*R*)-enantiomer.

Electron impact mass spectra (EIMS) were obtained at 70 eV on a Carlo Erba QMD 1000 GC-mass spectrometry (MS) instrument by using columns A to E with helium as the carrier gas (28.6 cm/sec at 40°C). The GC-MS interface and ion source temperatures were 250°C and 180°C, respectively. Methane was used as reactant gas at a source-chamber pressure of about 2×10^{-4} torr to obtain low-resolution chemical ionization spectra.

Quantitative analyses were done with the above GC and GC-MS instruments with on-column sample introduction, using the 11 homologous ketones from 2-heptanone to 2-heptadecanone as external standards.

^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ on Varian VNMR-300 or ANOVA-400 spectrometers at 25°C, or on a Bruker AMX-400 instrument at 27°C. Chemical shifts are given in parts per million (δ) relative to $CDCl_3$ (7.26 and 77.04 ppm for ^{1}H and ^{13}C NMR, respectively).

Solvent Extraction of the Volatile Organic Constituents The urine and lipid fractions of the marking fluid were separated by centrifuging 58.47 g of the collected marking fluid for 15 min at 3,000 rpm. The aqueous layer (urine) was removed with a syringe. During further prolonged centrifugation of the lipid fraction, the temperature of the material increased to about 30°C, and a supernatant layer of clear, almost colorless oil was formed. This was separated from a further small volume of urine, which was combined with the previous urine fraction. The supernatant oil (0.45 g) and the combined urine fractions (58 g) were stored at −20°C until analyzed.

Using centrifugation to facilitate separation of the phases, the organic material was repeatedly extracted from a sample (33.8 g) of the urine with 3-ml aliquots of dichloromethane, monitoring the removal of the organic material by GC-MS analysis. The extraction was terminated after the 15th extraction cycle. The combined extracts (approximately 40 ml) were concentrated to about 60 μl by slow evaporation of the solvent under nitrogen atmosphere, without blowing nitrogen directly into the vial containing the extract (Reiter et al. 2003). As the solvent was evaporated, the extract was transferred to increasingly smaller vials. Aliquots of 1.0 μl of the concentrated solution were analyzed by GC and GC-MS.

A second sample of marking fluid was centrifuged to separate the lipid and urine fractions. The urine was subjected to five extraction cycles with methyl *tert*-butyl ether (Extra pure, Merck, Darmstadt, Germany). The combined extracts were concentrated under argon atmosphere to 50 μl. Aliquots were used for the determination of double-bond positions in the unsaturated constituents by dimethyl disulfide (DMDS) derivatization and GC-MS analysis (Vincenti et al. 1987).

A further urine sample (8.87 ml) was partitioned twice with methyl *tert*-butyl ether, and the combined extracts were concentrated to 7 μl and used for the quantitative determination of the sulfur content of the urine fraction by using elemental sulfur as the external standard.

Headspace Analysis A sample enrichment probe (SEP; MasChrom Analisetegniek, Stellenbosch, South Africa) was used to collect volatile organic constituents from the

headspace of both the unprocessed marking fluid and the urine fraction for GC and GC-MS analyses. This was done by exposing the sorptive phase of the SEP (28 mg of polydimethylsiloxane rubber) to the headspace of, for example, a fresh sample of the urine (e.g., 46.67 g) at 40°C for periods of 15 to 48 hr. The volatile compounds were desorbed at 220°C in the GC-MS injector, leaving the probe in the injector until completion of the analysis (Burger et al. 2006a).

Solventless Sample Introduction of the Lipid Fraction The lipid phase of the marking fluid was analyzed by GC and GC-MS for its volatile organic content using the injector-internal thermal desorption technique (Biedermann et al. 2005). Aliquots of approximately 3 to 4 mg of the lipid material were applied to the inside wall of an injector liner containing just enough silylated glass wool to prevent the lipid sample from running down the liner to the bottom of the injector. The liner was installed in the injector at 220°C, the carrier gas was turned on, and the analysis was completed in the usual manner.

Protein Analysis The urine fraction of the marking fluid used for protein analysis was kept frozen in an Eppendorf tube at −80°C until analyzed. The protein content of the urine was estimated according to Bradford (1976) using bovine serum albumin as the standard. Prior to gel electrophoresis, proteins from lipid-free urine were concentrated, and the proteins were precipitated by adding an equal volume of 20% trichloroacetic acid to each sample. The samples were left on ice for 1 hr and then centrifuged for 15 min at 4°C and 16,000×*g*. The supernatant was removed, the pellet was washed with 300 µl of ice-cold acetone, and the suspension was centrifuged as before. The supernatant was removed, and the pellet was dried, resuspended in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) loading buffer, denatured by heating at 65°C for 3 min, and loaded onto the gel. The proteins were analyzed by denaturing PAGE (12% SDS PAGE; Laemmli 1970) and stained with Coomassie blue according to Sambrook et al. (1989). In-gel digestion with trypsin was performed according to published methods (Shevchenko et al. 1996). The resulting partially digested peptides were separated on a Waters CapLC instrument fitted with an Atlantis dC18 column (150 mm×100 µm, bead size 3 µm). The peptides were loaded in 2% acetonitrile/0.2% formic acid (solvent A), and the column was eluted by using a linear gradient from 3% of solvent B (98% acetonitrile/0.2% formic acid) to 100% of solvent B in 50 min at a flow rate of 1.5 µl/min. MS/MS data of the eluting peptides were acquired from *m/z* 400 to 1,995 at 0.5 sec per scan and an interscan delay of 0.1 sec by nano-electrospray quadrupole time-of-flight MS (Waters API Q-TOF Ultima). The resulting data were analyzed by the Matrix software. Fragments were sequence identified by database searches against publicly available mammalian databases using the Mascot search engine (Perkins et al. 1999) and confirmed using the Basic Local Alignment Search Tool (BLAST; Altschul et al. 1990). Probability-based MOWSE (Perkins et al. 1999) scores were estimated by comparing searched results against an estimated random match population and were reported as $-10\times\log_{10}(P)$, with P as the absolute probability.

Reference Compounds Most of the compounds required for comparison with constituents of the marking fluid were purchased from Sigma-Aldrich (Cape Town) or other chemical suppliers or were synthesized during previous research projects. The syntheses of 3-methyl-2-octanone (**10**), 3-methyl-2-nonanone (**14**), (*R*)-2-methylheptanoic acid, 2-methylnonanoic acid (**77**), 2-methyldecanoic acid (**84**), 2-methylundecanoic acid (**89**), dimethyl succinate (**33**), dimethyl glutarate (**43**), dimethyl adipate (**55**), *N*-benzylidenepentylamine (**51**), *N*-pentylurea (**104**), *N*-benzylidene-2-phenylethylamine (**117**), and (*S*)-(+)-(*Z*)-6-dodecen-4-olide (**86**) are described in the Supplementary Information for this article.

Results and Discussion

In a project aimed at finding ways to count cheetah in the wild, Balme (2005) found that the animals were attracted to various perfumes, notably to Obsession for Men by Calvin Klein. To obtain territorial marking fluid for the present research, attempts were made to elicit territorial marking behavior by both the male and the female tigers, by applying different odorants to the back of the aluminum sheet that was used to collect the marking fluid. The male tiger reacted to dodecan-4-olide (**85**), (*Z*)-6-dodecen-4-olide (**86**), the perfume Elle (Paco Rabanne), and a synthetic mixture of the compounds that were identified early in the investigation (mixture A, Table 1) by spraying urine/marking fluid in the direction of the collection device, but it ignored DMDS, musk ambrette (Haarman & Reimer, Johannesburg), and the perfume Romance (Ralph Lauren). No clear relationship between the unpleasantness of an odor (to the human nose) and the reaction of the tiger was observed. As could be expected, the tiger marked more readily just after his cage had been cleaned. Marking behavior was stimulated most by the synthetic mixture and by the perfume Elle. The major constituents of this perfume were not detected in the marking fluid that was collected when this perfume had been used to induce spraying behavior, proving that the collected fluid was not contam-

inated by the stimulant. Generally, relatively small volumes of the marking fluid were recovered, largely because the tiger usually started spraying before having fully turned his back on the collection device. Nevertheless, on two occasions, it was possible to collect 100-ml quantities of fresh fluid. In contrast to the cheetah, *A. jubatus*, which avoided turning its back on its reflection in the shiny aluminum surface, the tiger sprayed more readily after having inspected the shiny surface than when it was presented with a surface sprayed with green lacquer.

When left standing, the marking fluid separated into two layers: a bottom urine layer and a small upper layer of off-white material. Centrifugation and separation of the urine from the upper layer produced a supernatant layer of clear, viscous oil.

Two sample preparation and introduction techniques were employed for GC and GC-MS analyses. The first of these, a simplified version of the recently developed high-capacity SEP (Burger et al. 2006a), was used to collect the volatile constituents from the headspace of the urine fraction and to introduce the resulting sample into the injector of the GC or GC-MS instrument. The implementation of the simplified version of the SEP technique does not require modification of the injector of the GC. In general practice and depending on the application, this technique could be more efficient than collection by solid-phase microextraction (SPME) by about two orders of magnitude (Burger and Herbert, unpublished results). The total ion chromatogram (TIC) obtained in a typical SEP analysis of the urine headspace is depicted in Fig. 1. The prominent peaks in the lower retention time range were from alcohols, aldehydes, ketones, and fatty acids, whereas γ-lactones (alkan-4-olides) and δ-lactones (alkan-5-olides) constitute some of the most prominent peaks at higher retention times. A comparison of the headspace analyses of the urine and of the whole marking fluid revealed that these liquids gave TICs with similar constituent profiles. For conventional GC and GC-MS analyses and for retention time comparison, the organic material was extracted from the urine fraction of the marking fluid with dichloromethane.

Biedermann et al. (2005) recently introduced a GC sample introduction technique, described as injector-internal thermal desorption, in which volatile organic analytes present in heavy, relatively nonvolatile oils are vaporized in the injector of the gas chromatograph, cold-trapped on the column, and analyzed in the usual manner. In this technique, the heavy oil serves as a nonvolatile solvent. If samples exceeding a critical volume are injected, the oil can run down the injector liner, and some volatile material could thus be lost. This could be a problem in quantitative analysis. However, this problem can be minimized by using a small plug of silylated glass wool in the liner to support the oil at that point. By using injector-internal thermal desorption as a sample introduction technique, excellent qualitative and quantitative results were obtained in GC and GC-MS analyses of the neat lipid fraction of the marking fluid.

The territorial marking fluid of the male Bengal tiger contained a complex mixture of compounds, distributed between the aqueous (urine) and lipid phases of the fluid. The analytical problems resulting from the presence of these two phases and particularly the coelution of a large percentage of the volatile organic constituents of the marking fluid were compounded by the presence of a number of fatty acids that eluted as broad distorted peaks from the apolar capillary columns. Therefore, most of the GC and GC-MS analytical work was done on a capillary column coated with the polar phase AT-1000 (FFAP equivalent) from which the carboxylic acids eluted as sharp peaks, minimizing overlap of the carboxylic acid peaks with those of other constituents. Polar compounds (e.g., amides) eluted later than related but less polar compounds with higher molecular masses (e.g., certain N- or C-alkyl-substituted amides) from this phase. AT-1000 is also slightly acidic, resulting in some of the nitrogen-containing compounds eluting as broad, fronting peaks or, in some cases, not eluting at all. Thus, although *N*-benzylidenepentylamine (**51**) was present in TICs obtained with this column and with column C, this compound and *N*-benzilidene-2-phenylethylamine (**117**) were identified by using the apolar column B.

Samples of the marking fluid collected over a period of 3 years ranged in size from a few milliliters to 100 ml. The fluid contained varying proportions of the lipid phase, and little or no lipid was present in the smaller samples that were collected, probably because some of the viscous lipid material adhered to the collection apparatus. Analytical techniques were developed that would give a reliable picture of the qualitative and quantitative composition of the animal's marking fluid. These techniques were then used to analyze fresh marking fluid that was free from contaminants and which contained a relatively high proportion of the lipid material to obtain a comprehensive picture of a typical sample of the fluid. Thus, crude samples were subjected to SEP-GC and -GC-MS analyses, after which the lipid material was separated from the urine and analyzed by GC and GC-MS by using solventless sample introduction. The organic material was exhaustively extracted from samples of the urine fraction with dichloromethane or methyl *tert*-butyl ether, and the extracts were analyzed by using split injection (GC and GC-MS) or on-column sample introduction (quantitative GC). The relevant qualitative and quantitative data are given in Table 1.

We tried to use DMDS derivatization of unsaturated compounds and GC-MS analysis of the derivatives (Vincenti et al. 1987) to determine the positions of double bonds in

Table 1 Compounds identified and quantified in the urine fraction, the headspace of the urine fraction, and the lipid fraction of the territorial marking fluid of the male Bengal tiger

Number in Fig. 1[a]	Compound[b]	Identification[c]	Quantitative data			Compounds in Mixture A	Compounds in Mixture B
			Collected from urine headspace (Relative %)[d]	Extracted from urine fraction (ng)[e]	Desorbed from lipid fraction (ng)[f]		
1	Decane	a, b	15.7	c	c		
2	Hexanal	a, b	c	c	1.5		
3	Undecane	a, b	2.8	c	c		
4	3-Heptanone	a, b	0.3	0.03	c		
5	Heptanal	a, b	c	c	4.7		✓
6	2-Heptanone	a, b	12.9	2.2	c	✓	✓
7	3-Methyl-1-butanol	a, b	1.7	3.5	c		✓
8	2-Octanone	a, b	0.3	c	c		
9	Octanal	a, b	0.6	c	0.9		
10	3-Methyl-2-octanone	a, b	0.8	c	c		
11	2-Nonanone	a, b	77.8	0.3	1.3	✓	✓
12	1-Hexanol	a, b	c	c	0.5		
13	Nonanal	a, b	0.3	0.1	12.5		✓
14	3-Methyl-2-nonanone	a, b	0.3	c	c		
15	(*Z*)-2-Octenal	a, b	c	c	0.6		
16	(*E*)-2-Octenal	a, b	c	c	0.2		✓
17	Unidentified	a	c	c	0.2		
18	Tetradecane	a, b	0.1	c	c	✓	
19	1-Heptanol	a, b		0.2	0.3		
20	Acetic acid	a, b	0.7	0.1	5.4		
21	2-Decanone	a, b	1.6	c	0.2	✓	
22	Benzaldehyde	a, b	6	1.4	0.6		✓
23	Decanal	a, d, e	5.2	c	c		
24	Unidentified	a	c	c	0.3		
26	3-Methyl-2-decanone	a, d, e	1.8	c	0.1		
28	Propanoic acid	a, b	c	c	3.7		
30	1-Octanol	a, b	1.8	c	2.3	✓	
31	Pentan-4-olide	a, b	c	0.1	c		
32	2-Undecanone	a, b	65.8	c	2.5	✓	✓
33	Dimethyl succinate	a, b	0.7	c	c		
35	Acetophenone	a, b	3.5	0.1	0.3		✓
36	Butanoic acid	a, b	c	c	0.3		
37	Propenoic acid	a, b	c	c	0.7		
38	1-Methyl-2-pyrrolidone	a, b	c	0.1	0.8		
39	1-Nonanol	a, b	1.3	c	0.7		
40	Furfuryl alcohol	a, b	c	c	0.2		
41	Ethyl decanoate	a, b	0.5	c	c		
42	Hexan-4-olide	a, b	0.5	0.13	0.1		
43	Dimethyl glutarate	a, b	3.1	c	c		
45	(*Z*)-2-Undecenal	a, b	c	c	1		
46	But-2-enolide	a, b	c	c	0.7		
47	Pentanoic acid	a, b	c	c	1.1		✓
48	3-Tridecanone	a, d	1.4	c	0.8		
49	Pentan-5-olide	a, b	0.1	0.1	1.3		✓
50	Acetamide	a, b	0.2	c	4.4		
51	*N*-Benzylidenepentylamine	a, b	1.7	c	c		
52	Formamide	a, b	c	c	9.3		
53	2-Tridecanone	a, b	1.1	c	1.1	✓	
54	Propionamide	a, b	c	c	0.7		
55	Dimethyl adipate	a, b	2.2	c	c		
56	Geranylacetone	a, b	1.1	c	0.9	✓	

Table 1 (continued)

Number in Fig. 1[a]	Compound[b]	Identification[c]	Quantitative data			Compounds in Mixture A	Compounds in Mixture B
			Collected from urine headspace (Relative %)[d]	Extracted from urine fraction (ng)[e]	Desorbed from lipid fraction (ng)[f]		
57	Hexanoic acid	a, b	0.2	0.2	1		✓
58	Dimethylsulphone	a, b, f	0.7	75.6	3.3		✓
59	Octan-4-olide	a, b	0.4	c	c		
60	2-Phenylethanol	a, b	4.1	5.7	c	✓	✓
61	Octan-5-olide	a, b, f	6.4	2.8	c		
62	2-Ethylhexanoic acid	a, b	7.6	c	c		
63	Heptanoic acid	a, b	c	0.9	11		✓
64	Phenol	a, b	0.4	0.2	1		✓
65	Nonan-4-olide	a, b	0.7	0.1	c		
66	Tridecane-2,4-dione	a, d	0.7	c	c		
67	2-Pentadecanone	a, b	0.1	c	3		✓
68	Nonan-5-olide	a, b	c	0.1	c		
69	Nerolidol	a, b	1.6	c	0.3		
70	Octanoic acid	a, b, f	8.3	21.1	5.7		✓
71	1,1-Dimethylurea	a, b	c	c	50		
72	2-Piperidone (δ-valerolactam)	a, b, f	1.6	5.4	12.4		✓
73	Decan-4-olide	a, d, e	8.8	1	0.2		
74	Hexahydrofarnesylacetone	a, b	c	c	1		
75	Decan-5-olide	a, b	42	1.3	6.5		✓
76	Nonanoic acid	a, b	3.1	0.4	16.5		✓
77	2-Methylnonanoic acid	a, b	0.8	1.1	0.2		✓
78	1-Tetradecanol	a, b	2.6	c	7.5		✓
79	Undecan-4-olide	a, b	0.7	c	c		
80	2-Heptadecanone	a, b	c	c	15.7		✓
81	Cycloheptadecanone	a, d	c	c	43.2		
82	Undecan-5-olide	a, b	0.4	0.05	0.05		
83	Decanoic acid	a, b	15	22	28.5		✓
84	2-Methyldecanoic acid	a, b	2.1	3	5.1		✓
85	Dodecan-4-olide	a, b, f	100	1.5	31.2	✓	✓
86	(*Z*)-6-Dodecen-4-olide	a, b, g	39.9	0.5	4.8	✓	✓
87	Undecanoic acid	a, b	0.1	0.2	c		
88	Dodecan-5-olide	a, b, f	60.9	1.9	27.9	✓	✓
89	2-Methylundecanoic acid	a, b	c	2.3	1.4		✓
90	1-Hexadecanol	a, b	1.3	c	58.1	✓	✓
91	(*Z*)-9-Hexadecen-1-ol	a, b, d	c	c	2.6		✓
92	Unidentified, branched fatty acid	a	c	c	48.8		
93	Tridecan-4-olide	a, d, e	0.2	0.3	c		
94	Dodecanoic acid	a, b	2.6	3.7	15.9	✓	✓
95	Tridecan-5-olide	a, d, e	0.2	0.3	1.3		
96	(*Z*)-9-Dodecenoic acid	a, b, d, f	21	14.2	63.5		✓
97	Phenylacetic acid	a, b	0.3	c	1.5		
98	Tetradecan-4-olide	a, d	0.9	0.8	8		✓
99	1-Octadecanol	a, b	c	c	10		✓
100	Tetradecan-5-olide	a, b, e	2.4	1.9	14.1		✓
101	(*Z*)-9-Octadecen-1-ol	a, b, d	c	c	105.9		✓
102	(*Z,Z*)-9,12-Octadecadien-1-ol	a, b, d	c	c	31.1		✓
103	1-Methylhydantoin	a, b, f	0.7	7.5	34.9		✓
104	*N*-Pentylurea	a, b	6.9	c	82.5		
105	Urea	a, b	1.4	c	100		
106	Tetradecanoic acid	a, b	0.2	3.9	66.4	✓	✓
107	Hexadecan-4-olide	a, b	c	c	1.7		
108	Pentadecanoic acid	a, b	0.5	0.3	4		

Table 1 (continued)

Number in Fig. 1[a]	Compound[b]	Identification[c]	Quantitative data			Compounds in Mixture A	Compounds in Mixture B
			Collected from urine headspace (Relative %)[d]	Extracted from urine fraction (ng)[e]	Desorbed from lipid fraction (ng)[f]		
109	3-Phenyl-2-propenoic acid[g]	a, d	0.1	c	6.2		
110	Hexadecan-5-olide	a, d, e	0.1	c	12.9		
111	Hexadecanoic acid	a, b	10	3.8	99.4	✓	✓
112	(*Z*)-9-Hexadecenoic acid	a, b, g	3.3	3.1	60.8		✓
113	Octadecanoic acid	a, b	0.9	1.5	18.5		✓
114	(*Z*)-9-Octadecenoic acid	a, b, d	1.2	1.3	4.5		
115	Squalene	a, b	c	3.04	2897		
116	2-Phenylethylamine	a, d, h				✓	
51	*N*-Benzylidenepentylamine[g]	a, b, h					
117	*N*-Benzylidene-2-phenylethylamine[g]	a, b, h					
118	Elemental sulfur (S_8)	a, b, i					

[a] Compounds are listed in order of elution from column A (Fig. 1), although all compounds were not detected with each of the three analytical methods used.
[b] Stereochemical information on chiral compounds is given in Table 2.
[c] a GC-EIMS data, b retention time comparison with synthetic compounds, c not detected or values lower than 0.03 ng, d tentative identification, e retention time increment comparison, f GC-CI(CH_4)MS data, g double bond location by DMDS derivatization and GC-MS analysis, h nitrogen-containing compounds were collected by SEP from the headspace of the urine fraction and analyzed using the apolar capillary column B, i identified in the material collected by SEP from the headspace of several samples of the marking fluid. Extraction with methyl *tert*-butyl ether from a urine fraction (8.87 ml) and GC-MS analysis using sulfur as external standard gave a sulfur concentration of 80 ng/ml urine
[d] GC and GC-MS analyses of volatile organic constituents collected from the headspace of 46.67 g of urine for 20 hr at 40°C using a SEP. Quantitative data were normalized with respect to dodecan-4-olide as 100%.
[e] GC and GC-MS analyses of the volatile organic material extracted with dichloromethane from 33.8 g of the urine fraction of the marking fluid. Extract concentrated to 60 μl and 1 μl injected on-column. The 11 homologous ketones from 2-heptanone to 2-heptadecanone were used as external standards. Quantitative data in nanograms per 33.8 g of urine
[f] GC and GC-MS analyses using sample introduction by injector-internal thermal desorption of 3.28 mg of the 262.2 mg of lipid fraction separated from the above urine fraction. Alkanones were used as external quantitative standards. Quantitative data in nanograms per 262.2 mg of lipid material
[g] Stereochemistry not determined

the unsaturated constituents of the fluid. However, the major unsaturated compounds were not quantitatively derivatized and appeared unchanged in the TIC of the reaction product, and the minor unsaturated constituents were not detected, either as the unchanged compounds or as their derivatives. Using a larger sample of the extract and a larger excess of the reagents, the derivatives of the major unsaturated compounds were still formed in very low yields. No unchanged compounds were detected in the TIC, even when a large sample (6 μl) of the final product was analyzed. Fortunately, information on the positions of the double bonds in the unsaturated γ-lactone (**86**) and 9-hexadecenoic acid (**112**) was obtained in both of these experiments. In a repetition of the experiment with a sample of the lipid fraction, the iodine and probably also the DMDS were rapidly consumed and had to be replenished several times. Only the DMDS derivative of 9-hexadecenoic acid was detected in the product. Thus, some of the other compounds extracted from the urine or present in the lipid appear to interfere with the reaction. The double bond positional and geometric (*E/Z*) isomers of long-chain unsaturated compounds are not always completely resolved in capillary GC-MS analyses. Thus, although these compounds coeluted with authentic synthetic reference compounds, some of them are listed as tentatively identified in Table 1 if the positions of their double bonds could not be conclusively established.

A larger number of compounds were identified in the urine fraction by using the SEP sample introduction technique than by conventional injection of an extract of the urine. These differences are the result of the differences in sensitivity of these methods for compounds that differ in volatility and polarity. The SEP technique particularly facilitated the analysis of highly volatile constituents, and the profile of the volatiles obtained with this method probably gives a more accurate picture of the composition of the semiochemical message released by the tiger's marking fluid than information obtained by other methods. Regardless of the sampling technique used, GC-MS analysis showed the volatile fraction of the marking fluid to be complex, with a coelution of many constituents. Thus, although constituents were quantified by using GC data,

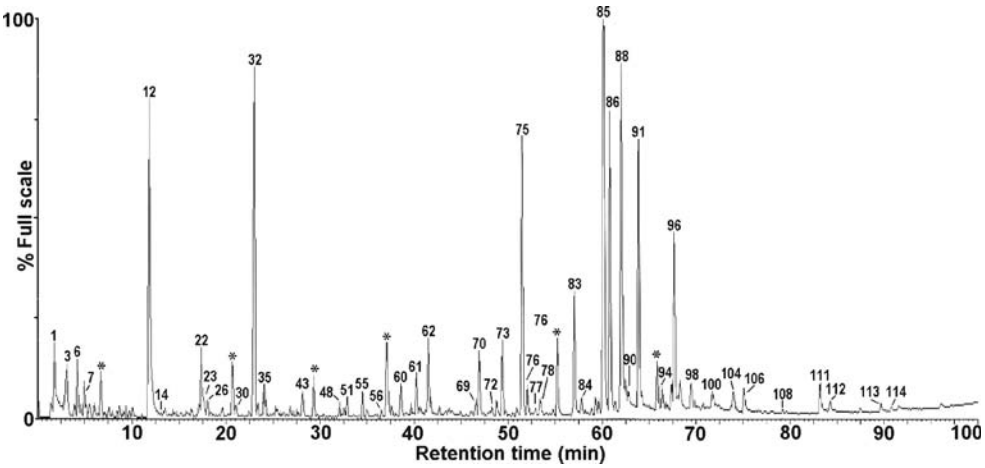

Fig. 1 TIC obtained by GC-EIMS analysis of the volatile organic compounds collected with a SEP at 40°C from 46.67 g of the urine fraction of the territorial marking fluid of the Bengal tiger on the glass capillary column A (40 m× 0.25 mm i.d.) coated with 0.25 μm of the polar stationary phase AT-1000 (FFAP equivalent). The column was programmed at 2°C from 40°C to 280°C. *Asterisk*, Unidentified or contaminants

qualitative and quantitative GC-MS data were considered to avoid incorrect assignment of peaks and to quantify overlapping or coeluting peaks. GC-MS data were also used to determine the enantiomeric composition of chiral compounds. Because samples collected on different occasions were normally not pooled for analysis and different sample preparations, different sample introduction techniques, and different columns were employed in this research, the quantitative results reported in Table 1 have to be considered with some circumspection. The different ratios in which some compounds, belonging to the same compound class, were present in the urine and lipid fractions have not yet been investigated. It is nonetheless clear that the overall qualitative and quantitative picture differs substantially from the published information on the composition of the marking fluid of the tiger discussed in the introduction.

A comparison of the results of the quantitative analyses of the urine and lipid fractions of one typical sample of the marking fluid reveals that if squalene is excluded from the equation, 0.45 g of the lipid fraction contains about seven times the amount of volatile organic material extracted from the associated urine fraction (58 g), illustrating the high affinity of the lipid material for the volatile organic constituents of the marking fluid, which probably enhances the lifetime of a territorial mark.

Perhaps the most remarkable feature of the results reported here is the large number of lactones present in the marking fluid. Some of these lactones have relatively low vapor pressures and will persist in territorial marks, although they may not be the major active constituents of the territorial marking pheromone. The major constituents of the headspace of the urine fraction and of the whole marking fluid were 2-heptanone (**6**), 2-nonanone (**11**), 2-undecanone (**32**), decanoic acid (**83**), (*Z*)-9-dodecenoic acid (**96**), hexadecanoic acid (**111**), dodecan-4-olide (**85**), (*Z*)-6-dodecen-4-olide (**86**), decan-5-olide (**75**), dodecan-5-olide (**88**) and, in some analyses, the nitrogen compounds *N*-benzylidenepentylamine (**51**), 2-phenylethylamine (**116**), and *N*-benzylidene-2-phenylethylamine (**117**). Cadaverine and putrescine, reported as constituents of tiger urine (Brahmachary 1986), were not detected in our study, possibly because animals in the wild will occasionally eat putrid meat containing these compounds, whereas the animals used in this project were only fed fresh chicken. 2-Acetylpyrroline, mentioned as a constituent of tiger urine in several publications (e.g., Brahmachary 1996), also was not detected in our study. However, Brahmachary (1996) suggested that this compound could possibly be an artifact formed by a Maillard reaction during sample preparation. The two imines, *N*-benzylidenepentylamine (**51**) and *N*-benzylidene-2-phenylethylamine (**117**), and *N*-pentylurea (**104**) identified in the present study might also be artifacts formed at the high temperature in the GC injector from amines and carbonyl compounds present in the tiger's marking fluid. This possibility was not investigated, but it could be responsible for the variable quantitative results obtained for these compounds under different analytical conditions. Elemental sulfur was not found in the sample of marking fluid under discussion, but it was present in most of the analyses of SEP-collected material. In a methyl *tert*-butyl ether extract of the urine fraction, it was present in a concentration of 80 ng/ml of urine. Elemental sulfur eluted from column A as a broad and fronting peak, extending over a retention time range of about 5 min, at about the retention time of tetradecan-5-olide (**100**). Sulfur has previously been identified in the urine of the cheetah, *Acynonix jubatus* (Burger et al. 2006b).

Except for a few minor terpenoids, the 3-methyl-2-alkanones and 2-methylcarboxylic acids, 2-ethylhexanoic acid, and a series of γ-alkylsubstituted γ-lactones and δ-alkylsubstituted δ-lactones were the only chiral constituents identified in the marking fluid. No information is available on the order of elution of the enantiomers of 3-methyl-2-alkanones from chiral columns. However, it is known that (*S*)-2-methylheptanoic acid, for example, elutes before the

(*R*)-enantiomer from the phase used in column C (Maas et al. 1994a). Thus, iodoform oxidation of the first-eluting enantiomer of 3-methyl-2-octanone produced the second-eluting (*R*)-enantiomer of 2-methylheptanoic acid. The first-eluting enantiomer of the ketone therefore has (*R*) configuration. In a repetition of this experiment with a 2:1 mixture of the ketone enantiomers, the presence of the other enantiomer of the ketone in the product served as retention time references and ruled out any possibility of incorrect interpretation of the results. By using GC-MS analysis of the SEP-collected volatiles on the enantioselective column C, in conjunction with cochromatography of the enriched material and racemic synthetic samples, and by using the base peak at *m/z* 72 in the mass spectra of these compounds for quantification, it was found that in some samples, these ketones had racemized, but that pure (*R*)-3-methyl-2-octanone (**10**), (*R*)-3-methyl-2-nonanone (**14**), and (*R*)-3-methyl-2-decanone (**26**) were present in most samples (Table 2).

In GC-MS analyses that used column C, the chiral carboxylic acids 2-methylnonanoic acid (**77**), 2-methyldecanoic acid (**84**), and 2-methylundecanoic acid (**89**) were all found to be racemates. 2-Ethylhexanoic acid (**62**) eluted from column E as the racemate. The enantiomeric 2-methylcarboxylic acids were separated on column C with resolutions (R_S) varying from 2.7 for 2-methylnonanoic acid to 2.5 for 2-methylundecanoic acid. These acids were eluted from the nonpolar enantioselective column D as broad and barely resolved peaks. It is unlikely that these acids were racemized during handling because they are expected to be less susceptible to racemization at pH 6.5 than the ketones discussed above.

The enantiomers of the saturated γ- and δ-lactones were resolved on column D with relatively high R_S values, ranging from 4.9 for the C_8 to 2.4 for the C_{16} γ-lactones and from 1.9 for the C_9 to 1.1 for the C_{16} δ-lactones. According to Maas et al. (1994b), the (*R*)-γ-lactones and (*S*)-δ-lactones under discussion elute before the respective (*S*)- and (*R*)-enantiomers from this phase. The elution order of the lactone enantiomers was confirmed by comparing published data (e.g., Mosandl et al. 1989) with analyses on column D of the headspace volatiles of strawberries and raspberries, which contain the enantiomers of some of the γ- and δ-lactones present in the marking fluid. The results of the enantioselective analyses of the lactones are given in Table 2. The enantiomeric ratios of the C_5 and C_6 lactones were not determined because they were present in insufficient concentrations. The majority of five marking fluid samples and two mixtures of, respectively, three and eight smaller samples contained the (*R*)-γ- and (*S*)-δ- lactones (enantiomeric ratios are given in order of elution) in such high enantiomeric purities that it can be assumed that these lactones are excreted as the pure enantiomers (see Table 2).

Table 2 Enantiomeric composition[a] of chiral constituents of the marking fluid determined by GC-MS using columns C, D, and E

Compounds	
γ-Lactones (*R*:*S*)	
C_8	72:28 to 58:42 (*n*=3, C[b])
C_9	100:0 to 63:37 (*n*=4, C); 100:0 to 76:24 (*n*=4, D[c])
C_{10}	100:0 to 89:11 (*n*=5, C); 97:3 to 96:4 (*n*=6, D)
C_{11}	96:4 to 91:9 (*n*=2, C); 100:0 to 93:7 (*n*=5, D)
C_{12}	100:0 to 51:49 (*n*=6, C); 100:0 to 53:47 (*n*=7, D)
C_{13}	51:49 (*n*=1, C); 100:0 (*n*=2, D)
C_{14}	100:0 to 79:21 (*n*=6, C); 100:0 to 86:14 (*n*=4, D)
C_{16}	60:40 to 51:49 (*n*=2, C)
(*Z*)-6-dodecen-4-olide (*S*:*R*)	
C_{12}	95:5 to 64:36 (*n*=6, C); 93:7 to 87:13 (*n*=6, D)
2-Methylcarboxylic acids (column C)	
C_{10}	Racemic (*n*=2)
C_{11}	Racemic (*n*=3)
C_{12}	Racemic (*n*=3)
δ-Lactones (*S*:*R*)	
C_8	0:100 (*n*=5, C); 0:100 to 25:75 (*n*=3, D)
C_9	0:100 (*n*=1, C); 0:100 (*n*=1, D)
C_{10}	0:100 to 1:99 (*n*=6, C); 0:100 (*n*=6, D)
C_{11}	0:100 to 4:96 (*n*=2, C); 0:100 (*n*=5, D)
C_{12}	5:95 to 10:90 (*n*=6, C); 0:100 (*n*=7, D)
C_{13}	0:100 (*n*=5, D)
C_{14}	0:100 to 8:92 (*n*=6, C); 5:95 to 6:94 (*n*=4)
C_{16}	0:100 to 12:88 (*n*=2, C)
3-Methyl-2-alkanones (*R*:*S*) (column C)	
C_9	100:0 (*n*=4)
C_{10}	100:0 to 47:53 (*n*=5)
C_{11}	100:0 (*n*=4)
2-Ethylhexanoic acid (column E)	
C_8	Racemic (*n*=3)

[a] The ratios were determined by integration of single ion plots of the base peaks of the individual compounds. The quantitative data are given in the order of elution of the enantiomers from column C or D. In some samples, one or both of the enantiomers coeluted with other constituents and could thus not be quantified, which accounts for the different numbers of samples (*n*) shown in the table.
[b] Enantioselective column C
[c] Enantioselective column D

The racemization of the chiral constituents of the marking fluid has not yet been investigated. However, some of these samples had spent 15 hr or more at 40°C for the SEP collection of volatiles for qualitative and/or quantitative analyses before the enantioselective analyses were done, and it is possible that the variation in enantiomeric ratios could be explained in terms of some racemization having taken place under these conditions.

The position of the double bond in the unsaturated γ-lactone, (*Z*)-6-dodecen-4-olide (**86**), was determined by MS analysis of its DMDS derivative. The (*S*)- and (*R*)-enantiomers (in the order of elution) were separated (R_S 1.6) on column D. In contrast to the saturated γ-lactones, the (*R*)-enantiomer eluted first from this column. (*S*)-(+)-

Table 3 Identification of proteins in the urine fraction of the marking fluid of the Bengal tiger

Bands from 1D SDS-PAGE (kDa)	MASCOT/BLAST result	Accession number	Theoretical molecular mass (kDa)	MASCOT MOWSE score[a]	Percentage coverage (number peptides identified)
68.29	Carboxylesterase-like urinary excreted protein (*F. silvestris catus*)	Q8I034_FELCA	60.467	270	20 (6)
56.74	Serum albumin precursor (*F. silvestris catus*)	S57632	68.615	176	16 (8)
31.55	N-Acylsphingosine amidohydrolase (acid ceramidase)-like isoform CRA_d (*Homo sapiens*)	EAX05759	43.440	116	15 (4)
23.31	Immunoglobulin lambda chain (*Mus musculus*)	AAA37484	25.387	72	7 (1)
14.57	Immunoglobulin kappa light chain (*F. silvestris catus*)	AAF09245	26.694	45	4 (1)
13.61	Transthyretin precursor (*H. sapiens*)	VBHU	15.877	53	8 (1)

[a] Probability-based MOWSE score: Ions score is $-10\times\mathrm{Log}(P)$, where P is the probability that the observed match is a random event. Protein scores are derived from ions scores as a nonprobabilistic basis for ranking protein hits. Individual ions scores indicating identity or extensive homology ($p<0.05$) for each of the proteins were as follows: Q81034_FELCA≥27; S57632≥27; EAX05759≥38; AAA37484≥37; AAF09245≥28; VBHU≥37.

(*Z*)-6-Dodecen-4-olide has recently been characterized in the interdigital secretion of the bontebok, *Damaliscus pygargus pygargus* (=*D. dorcas dorcas*; Burger et al., unpublished). The (*R*)-and (*S*)-enantiomers of this lactone are also present in the tarsal tuft of the black-tailed deer in a ratio of 89:11, respectively (Müller-Schwarze et al. 1978). In this animal, the lactone does not originate in the tarsal structure itself but is extracted from the animal's urine by the tarsal tuft (Müller-Schwarze et al. 1977).

Because only one pair of captive Bengal tigers was available for this research, little time was devoted to bioassays of the identified compounds. Nevertheless, we tested the major volatile constituent of the marking fluid, dodecan-4-olide, which elicited overmarking behavior, provided that bioassays were not done more often than about once per day. A small sample of synthetic marking fluid, the composition of which is given in Table 1 (mixture B), can be made available to researchers for field tests with tigers living in the wild.

The extraction of volatiles from the urine was complicated by the formation of a thin layer of semisolid material, probably denatured proteins, between the solvent and the urine. This solid material could not be fully characterized, but positive- and negative-ion electrospray time-of-flight MS revealed the presence of scores of compounds with molecular weights ranging from 80 to 750 Da. Although our primary goal was the identification of the volatile constituents of the marking fluid, we undertook an exploratory investigation into the possibility that proteins in the marking fluid could be involved in territorial marking in the tiger. The urine fraction of a sample of marking fluid contained proteins (0.318 mg/ml) with molecular weights ranging from 16 to 180 kDa. Nine electrophoretically separable protein bands were selected for peptide analyses, from which six proteins were identified (Table 3).

Eight peptides representing 16% coverage of the serum albumin precursor of *Felis silvestris catus* (Miyazaki et al. 2003) were identified in the urine of *P. tigris*, with a high degree of sequence conservation for albumin between these species. Serum albumin is the most abundant plasma protein in mammals. Lazar et al. (2004) identified urinary albumin in the Asian elephant (*Elephas maximus*), where it binds the pheromone component (*Z*)-7-dodecenyl acetate and releases it *via* a pH change.

Carboxylesterase-like urinary excreted protein, also known as cauxin, is secreted in the urine of the domestic cat (*F. silvestris catus*), bobcat (*Lynx rufus*), and the lynx (*L. lynx*; Miyazaki et al. 2003, 2006a), and according to Miyazaki et al. (2006a), this protein is not present in the urine of *Panthera* species. However, in the present study,

Fig. 2 Protein sequence of carboxylesterase-like urinary protein (*F. silvestris catus*). Peptides identified in the urine of *P. tigris* are *underlined*

MSGMWVHPGRTLIWALWVLAAVIKGPAADAPVRSTRLGWVRGKQTTVLGSTVPVNMFLGIPYAAPPLGPL
RFKQPKPALPGNDFRNATSYPKLCFQDLEWLVSYQHVLKVRYPKLEASEDCLYLNIYAPAHADNGSNLPV
MVWFPGGAFKMGSASSFDGSALAAYEDVLIVTTQYRLGIFGFFDTGDEHARGNWALLDQVAALTWVRDNI
EFFGGDPRSVTIFGESAGAISVSSLILSPIANGLFHKAIMESGVAILPLLMRPPGDERKKDLQVLARICG
CHASDSAALLQCLRAKPSEELMDISKKLTFSIPVIDDFFFPDEPVALLTQKAFNSVPSIIGVNNHECAFL
LSTEFSEILGGSNRSLALYLVHTFLNIPTQYLHLVADHYFYNKHSPVEIRDSFLDLLGDVLFVVPGVVTA
RYHRDAGAPVYFYEFQHPPQCLNDTRPAFVKADHSDEIRFVFGGAFLKGDIVMFEGATEEEKLLSRKMMR
YWANFARTGDPNGEGVPLWPAYTQSEQYLKLDLSVSVGQKLKEQEVEFWMNTIVP

cauxin was identified as one of the component proteins in the urine fraction of the Bengal tiger. Six peptides representing 20% coverage of the cauxin sequence were identified. All peptides fell within the esterase-, lipase-, and coesterase-conserved domains of cauxin (covering 23% and 22% of these domains, respectively) and showed 100% homology to the published *F. silvestris catus* sequence (Fig. 2). Highly conserved amino acids in the substrate-binding pocket and the catalytic triad were also represented, further confirming the identity of cauxin. The function of cauxin is not known, but Miyazaki et al. (2006b) suggested that it might regulate the production of felinine (2-amino-7-hydroxy-5,5-dimethyl-4-thiaheptanoic acid), a putative pheromone precursor in the domestic cat. Carboxylesterase and *N*-acylsphingosine amidohydrolase (acid ceramidase) catalyze reactions that form long-chain carboxylic acids. Acid ceramidase has previously been identified in human urine (Bernardo et al. 1995). The presence of these enzymes possibly could explain the high fatty acid composition of the lipid fraction of the marking fluid. Two immunoglobulin peptides, (λ)- and (κ)-chain, were identified. These small polypeptide subunits are components of immunoglobulins, T cell receptors, CD1 cell surface glycoproteins, secretory glycoproteins, and major histocompatibility complex class I/II molecules and have previously been identified in the urine of rodents (Hurst et al. 2005). Transthyretin is known to act as a carrier protein for various molecules, including retinol (Monaco 2000), and has been identified in rat urine (Wait et al. 2001).

Healthy mammals excrete small amounts of urinary proteins, and usually proteinuria is an indicator of renal abnormality (Raila et al. 2005). However, several studies have proposed that urinary proteins are involved in chemical communication in mammals. The role of the major urinary proteins (MUPs) in the semiochemical communication of the house mouse, *Mus domesticus*, has been studied in great detail during the past decade (e.g., Novotny 2003). For example, it was found that MUPs bind dehydro-*exo*-brevicomin and 2-(*sec*-butyl)-4,5-dihydrothiazole (Bacchini et al. 1992), which elicits male aggression in the house mouse (Novotny et al. 1985). It has also been suggested that the pattern of MUPs present in male urine may act as a type of individuality barcode that signals the identity of the owner of a particular scent mark (Beynon and Hurst 2003). On the other hand, Miyazaki et al. (2006b) suggested that the excreted MUPs of the domestic cat act as an enzyme in the synthesis of putative pheromone precursors and not as carrier proteins.

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References

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W., and LIPMAN, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

BACCHINI, A., GAETANI, E., and CAVAGGIONI, A. 1992. Pheromone binding proteins of the mouse, *Mus musculus*. *Experientia* 48:419–421.

BALME, G. 2005. Counting cats. *Afr. Geogr.* 13:37–43.

BERNARDO, K., HURWITZ, R., ZENK, T., DESNICK, R. J., FERLINZ, K., SCHUCHMAN, E. H., and SANDHOFF, K. 1995. Purification, characterization, and biosynthesis of human acid ceramidase. *J. Biol. Chem.* 270:11098–11102.

BEYNON, R. J., and HURST, J. L. 2003. Multiple roles of major urinary proteins in the house mouse, *Mus domesticus*. *Biochem. Soc. Trans.* 31:142–146.

BIEDERMANN, M., FISELIER, K., and GROB, K. 2005. Injector-internal thermal desorption from edible oils. Part 1: visual experiments on sample desorption on the liner wall. *J. Sep. Sci.* 28:1550–1557.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

BRAHMACHARY, R. L. 1986. Ecology and chemistry of mammalian pheromones. *Endeavour, New Ser.* 10:65–68.

BRAHMACHARY, R. L. 1996. The expanding world of 2-acetyl-1-pyrroline. *Curr. Sci.* 71:257–258.

BRAHMACHARY, R. L., and DUTTA, J. 1979. Phenylethylamine as a biochemical marker of tiger. *Z. Naturforsch.* 34c:632–633.

BRAHMACHARY, R. L., and DUTTA, J. 1987. Chemical communication in the tiger and leopard, pp. 296–302, in R. L. Tilson and U.S. Seal (eds.). Tigers of the World: The Biology, Biopolitics, Management and Conservation. Noyes, Park Ridge, NJ.

BRAHMACHARY, R. L., PODDAR-SARKAR, M., and DUTTA, J. 1990. The aroma of rice... and tiger. *Nature* 344:26.

BURGER, B. V., MARX, B., LE ROUX, M., and BURGER, W. J. G. 2006a. Simplified analysis of organic compounds in headspace and aqueous samples by high-capacity sample enrichment probe. *J. Chromatogr. A* 1121:259–267.

BURGER, B. V., PETERSEN, W. G. B., EWIG, B. T., NEUHAUS, J., TRIBE, G. D., SPIES, H. S. C., and BURGER, W. J. G. 2008. Semiochemicals of the Scarabaeinae. VIII. Identification of the active constituents of the abdominal sex-attracting secretion of the male dung beetle, *Kheper bonellii*, using gas chromatography with flame ionization and electroantennographic detection in parallel. *J. Chromatogr. A.* 1186:254–253.

BURGER, B. V., VISSER, R., MOSES, A., and LE ROUX, M. 2006b. Elemental sulfur identified in urine of cheetah, *Acinonyx jubatus*. *J. Chem. Ecol.* 32:1347–1352.

ELLERMAN, J., and MORRISON-SCOTT, T. 1951. Checklist of Palaearctic and Indian Mammals, 1758 to 1946. British Museum, London (reference in G.B. Schaller. 1967. The Deer and the Tiger. University of Chicago Press, Chicago).

HASHIMOTO, Y., EGUCHI, Y., and ARAKAWA, A. 1963. Historical observation of the anal sac and its glands of a tiger. *Jpn. J. Vet. Sci.* 25:29–32 (reference in K. F. Andersen and T. Vulpius. 1999. Urinary volatile constituents of the lion, *Panthera leo*. *Chem. Senses* 24:179–189).

HURST, J. L., THOM, M. D., NEVISON, C. M., HUMPHRIES, R. E., and BEYNON, R. J. 2005. MHC odours are not required or sufficient for recognition of individual scent owners. *Proc. R. Lond. Soc. B* 272:715–724.

LAEMMLI, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227:680–685.

LAZAR, J., RASMUSSEN, L. E., GREENWOOD, D. R., BANG, I. S., and PRESTWICH, G. D. 2004. Elephant albumin: a multipurpose pheromone shuttle. *Chem. Biol.* 11:1093–1100.

LEYHAUSEN, P. 1960. Verhaltenstudien an Katzen, in G. B. Schaller. The Deer and the Tiger. University of Chicago Press, Chicago.

LEYHAUSEN, P., and WOLFF, R. 1959. Das Revier einer Hauskatze. *Z. Tierpsych.* 16:666–670.

MAAS, B., DIETRICH, A., and MOSANDL, A. 1994a. Collection of enantiomer separation factors obtained by capillary gas chromatography on chiral stationary phases. *J. High Resolut. Chrom.* 17:109–115.

MAAS, B., DIETRICH, A., and MOSANDL, A. 1994b. Collection of enantiomer separation factors obtained by capillary gas chromatography on chiral stationary phases. *J. High Resolut. Chrom.* 17:169–173.

MIYAZAKI, M., KAMIIE, K., SOETA, S., TIARA, H., and YAMASHITA, T. 2003. Molecular cloning and characterization of a novel carboxylesterase-like protein that is physiologically present at high concentrations in the urine of domestic cats (*Felis catus*). *Biochem. J.* 370:101–110.

MIYAZAKI, M., YAMASHITA, T., HOSOKAWA, M., TIARA, H., and SUZUKI, A. 2006a. Species-, sex-, and age-dependent urinary excretion of cauxin, a mammalian carboxylesterase. *Comp. Biochem. Physiol. Part B* 145:270–277.

MIYAZAKI, M., YAMASHITA, T., SUZUKI, Y., SAITO, Y., SOETA, S., TIARA, H., and SUZUKI, A. 2006b. Major urinary protein of the domestic cat regulates the production of felinine, a putative pheromone precursor. *Chem. Biol.* 13:1071–1079.

MONACO, H. L. 2000. The transthyretin-retinol-binding protein complex. *Biochim. Biophys. Acta* 1482:65–72.

MOSANDL, A., HENER, U., HAGENAUER-HENER, U., and KUSTERMANN, A. 1989. Direct enantiomer separation of chiral γ-lactones from food and beverages by multidimensional gas chromatography. *J. High Resolut Chromatogr.* 12:532–536.

MÜLLER-SCHWARZE, D., RAVID, U., CLAESSON, A., SINGER, A. G., SILVERSTEIN, R. M., MÜLLER-SCHWARZE, C., VOLKMAN, N. J., ZEMANEK, K. F., and BUTLER, R. G. 1978. The deer lactone: Source, chiral properties, and responses by black-tailed deer. *J. Chem. Ecol.* 4:247–256.

MÜLLER-SCHWARZE, D., VOLKMAN, N. J., and ZEMANEK, K. F. 1977. Osmetrichia: specialized scent hair in black-tailed deer. *J. Ultrastruct. Res.* 59:223–230.

NOVOTNY, M., HARVEY, S., JEMIOLO, B., and ALBERTS, J. 1985. Synthetic pheromones that promote inter-male aggression in mice. *Proc. Natl. Acad. Sci. USA* 82:2059–2061.

NOVOTNY, M. V. 2003. Pheromones, binding proteins and receptor responses in rodents. *Biochem. Soc. Trans.* 31:117–122.

PERKINS, D. N., PAPPIN, D. J., CREASY, D. M., and COTTRELL, J. S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20:3551–3567.

PODDAR-SARKAR, M. 1996. The fixative lipid of tiger pheromone. *J. Lipid Mediat. Cell Signal.* 15:89–101.

RAILA, J., FORTERRE, S., and SCHWEIGERT, F. J. 2005. Physiologic and pathophysiologic fundamentals of proteinuria—a review. *Berl. Münch. Tierärztl. Wochenschr.* 118:229–239.

REITER, B., BURGER, B. V., and DRY, J. 2003. Mammalian exocrine secretions. XVIII: Chemical characterization of interdigital secretion of red hartebeest, *Alcelaphus buselaphus caama*. *J. Chem. Ecol.* 29:2235–2252.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. 1989. Molecular Cloning: A Laboratory Manual. Cold Spring Harbour Laboratory Press, Cold Spring Harbor, NY.

SCHALLER, G. B. 1967. The Deer and the Tiger. University of Chicago Press, Chicago.

SHEVCHENKO, A., WILM, M., VORM, O., and MANN, M. 1996. Mass spectrometric sequencing of proteins from silver-stained polyacrylamide gels. *Anal. Chem.* 68:850–858.

SMITH, J. L. D., MCDOUGAL, C., and MIQUELLE, D. 1989. Scent marking in free-ranging tigers, *Panthera tigris*. *Anim. Behav.* 37:1–10.

VINCENTI, M., GUGLIELMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double-bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. *Anal. Chem.* 59:694–699.

WAIT, R., GIANAZZA, E., EBERINI, I., SIRONI, L., DUNN, M. J., GERMEINER, M., and MILLER, I. 2001. Proteins of rat serum, urine, and cerebrospinal fluid: VI. Further protein identifications and interstrain comparison. *Electrophoresis* 22:3043–3052.

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Development of Tolerance to the Dietary Plant Secondary Metabolite 1,8-cineole by the Brushtail Possum (*Trichosurus vulpecula*)

Stuart McLean · Sue Brandon · Rebecca R. Boyle · Natasha L. Wiggins

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Abstract The common brushtail possum (*Trichosurus vulpecula*) is a generalist herbivore whose diet includes *Eucalyptus* leaves that are well defended by plant secondary metabolites (PSM) such as the terpene 1,8-cineole (cineole). We accustomed possums to a terpene-free diet, then challenged them with the addition of 2% cineole to the diet. Initially, there was a 50% reduction in total overnight food consumption associated with a marked decrease in the mass of the major feeding bout. After nine nights, however, cineole tolerance had developed as total food consumption had returned to the control amount. Compared to the control diet, the cineole diet was eaten in a larger number of smaller bouts, which were also eaten at a slower rate. The experiment was repeated with animals that had been accustomed to day-time feeding to take blood samples during feeding sessions. Feeding variables and blood concentration data for cineole were compared on the first and seventh day of the cineole diet. Although the total food consumed increased 2.5-fold after 7 days of the cineole diet, there was no increase in average blood cineole concentration, measured as the area under the concentration–time curve. This indicates that induction of liver enzymes resulted in greater pre-systemic metabolism of cineole and reduced bioavailability. The maximum tolerated blood concentration of cineole also increased, suggesting some adaptation of the central nervous system to the cineole aversive effects. This appears to be the first report in a vertebrate herbivore that consumption of a dietary PSM leads to metabolism induction and that this contributes to development of tolerance to the PSM. Overall, herbivores adapt to newly encountered dietary PSMs by immediate changes in feeding behavior followed by development of increased metabolism of PSM and probably diminished cellular responsiveness to effects.

Keywords 1 · 8-Cineole · Herbivory · Plant secondary metabolite · Brushtail possum · Enzyme induction · Tolerance · Blood concentrations · Pharmacokinetics

S. McLean (✉) · S. Brandon · R. R. Boyle
School of Pharmacy, University of Tasmania,
Private Bag 26,
Hobart, Tas 7001, Australia
e-mail: stuart.mclean@utas.edu.au

N. L. Wiggins
School of Plant Science, University of Tasmania,
Private Bag 55,
Hobart, Tas 7001, Australia

Introduction

Woody plants produce a great variety of chemicals that appear to have no further role in plant metabolism but serve a number of external functions, including defense against attack by pathogens or herbivores (Fraenkel 1959; Bryant et al. 1991; Foley et al. 1999). These plant secondary metabolites (PSMs) act through a number of mechanisms to produce toxicity or other aversive effects on herbivores (Iason 2005). Mammalian herbivores must eat to live, and they have developed countermeasures that enable them to subsist on a diet of chemically defended plants. The countermeasures include regulation of intake to avoid consuming a quantity of PSM that would be damaging and biotransformation of absorbed PSMs into metabolites that are usually less toxic and more readily excreted (Dearing et al. 2005). The behavioral and physiological responses of mammalian herbivores can be represented as a continuum from avoidance to tolerance, with most animals lying between the extremes (Iason and Villalba 2006). The

coevolution of plant defenses and herbivore countermeasures has resulted in a balanced coexistence of plants and animals in wild communities, although poisonings can occur when stock animals eat plants that are novel (Provenza 1995; Pfister et al. 1997).

The enzymes in animals that metabolize PSMs are principally the cytochrome P450 oxidases (CYPs) and conjugating enzymes such as glucuronosyltransferase (Dearing et al. 2005). Although considered to have evolved in part as a response to plant toxins (Gonzalez and Nebert 1990), these enzymes are remarkably nonspecific and metabolize other non-nutrient compounds (xenobiotics), including drugs and industrial chemicals. A search of the literature shows that most studies of the enzymes that metabolize xenobiotics have been undertaken in the fields of pharmacology and toxicology.

Genetic and environmental factors determine the capacity of an individual animal to metabolize xenobiotics. For example, CYP genes differ widely in allelic forms and expression that leads to marked differences among and within species in enzyme activity (Gonzalez and Kimura 2003; Wilkinson 2004). In addition to this inherited variation, which is probably driven by evolved species foraging characteristics, a temporary change in enzyme activity can be produced by exposure to certain xenobiotics that can inhibit or induce various CYP enzymes (Hollenberg 2002). The activity of CYPs and other enzymes of xenobiotic metabolism are regulated by a complex system of nuclear receptors that bind to certain xenobiotics resulting in increased transcription and synthesis of xenobiotic metabolizing enzymes (Pascussi et al. 2004). Many PSMs are inducing agents (Zhou et al. 2003), and it is generally considered that the ability to increase xenobiotic metabolizing activity evolved in response to challenges from dietary chemicals (Ioannides 2002). The consequences of induction have been most extensively studied for drug metabolism. In particular, induction of metabolism of a drug, whether by the drug itself or another drug (Hollenberg 2002; Wang and LeCluyse 2003) or a herbal product (Ioannides 2002; Zhou et al. 2003), results in decreased blood levels of the drug and diminished therapeutic effect. The effects of dietary PSMs on drug metabolism have also been studied as food–drug interactions (Harris et al. 2003).

The hypothesis that continued feeding on a diet containing PSMs that can induce their own metabolism will enable increased consumption of the diet has been supported by a study of the larval tobacco hornworm (*Manduca sexta*). This insect herbivore increased its consumption of a nicotine diet over 36 hr, associated with induction of CYP metabolism of nicotine. Both effects were reversed by the CYP-inhibitor, piperonyl butoxide (Snyder and Glendinning 1996). There have been no direct studies in mammalian herbivores, however, to show whether induction of the metabolism of dietary PSM increases consumption of the PSM diet.

Feeding studies have shown that mammalian herbivores can develop a limited tolerance to the presence of a deterrent PSM in their diet. For example, the *Eucalyptus* terpene 1,8-cineole (cineole) acts as a dose-dependent deterrent when added to the diet of the common ringtail possum (*Pseudocheirus peregrinus*) and the common brushtail possum (*Trichosurus vulpecula*), but both animals increase their intake of cineole over several days until it reaches a maximum tolerable amount (Lawler et al. 1999; Boyle and McLean 2004). When brushtail possums were fed for 10 days a diet containing a mixture of four terpenes, including cineole, the hepatic CYPs were induced (Pass et al. 1999) and the *in vitro* microsomal metabolism of cineole was increased (Pass et al. 2001). This suggests that enhanced metabolism contributes to the development of tolerance to a cineole diet (pharmacokinetic tolerance), but it is also possible that animals become tolerant to the pharmacological effects of cineole (pharmacodyamic tolerance). Cineole has a depressant effect on the central nervous system (CNS; Dziba et al. 2006; McLean et al. 2007), and the CNS usually adapts to the presence of various types of drugs (e.g., alcohol, nicotine, and morphine; O'Brien 2006). Therefore, it also appears likely that CNS adaptation contributes to the development of tolerance to cineole.

The aim of this study was to examine the development of tolerance to cineole by the common brushtail possum and to determine whether this can be attributed to induction of metabolism or CNS adaptation, or both. This was approached by measuring and subsequently linking the common brushtail possum's behavioral and physiological responses to a cineole-containing diet.

Methods and Materials

Animals Approval for animal experimentation was granted by the University of Tasmania's Animal Experimentation Ethics Committee. Brushtail possums were trapped in the wild and housed in individual pens (2×2×2.5 m) with wooden nest boxes (28×28×26 cm) hung from the wire mesh walls. Possums were maintained on an artificial terpene-free basal diet of 46% apple, 35% silver beet, 10% carrot, 5% lucerne (ground to pass a 1-mm sieve), and 4% raw sugar (Wiggins et al. 2006). The constituents were chopped and mixed in a food processor, and the final diet was 20% dry matter. Food weights are given as wet matter. Food and water were provided each evening as possums are nocturnal. Animals were tested with the experimental feeding setups, and only those that became acclimated were used in the study. 1,8-Cineole (99%, Sigma, Castle

Hill, NSW, Australia) was mixed into the basal diet as required immediately before being offered to possums.

Experimental Design The study design was to monitor feeding and blood cineole concentrations in possums before and after tolerance had developed to a cineole diet. We have found previously that the antifeedant effect of cineole in the brushtail possum is associated with the concentration of cineole in the blood reaching a critical value. This indicates that it may be the aversive signal detected by the animal (Boyle et al. 2005). An increase in this critical concentration would be evidence of CNS adaptation. The relationship between cineole ingested and its blood concentration was used as a measure of the relative rate of cineole metabolism. The study first established the time-course of the development of tolerance to a diet containing added cineole. As possums are nocturnal, this was done by offering food at night with the animal freely moving around its pen. The development of tolerance was taken to be when total overnight food consumption returned to the pre-cineole level. The next study required blood sampling during feeding, which was done with animals that had been catheterized to enable blood sampling and that were acclimated to eating from their nest box during the day. Possums readily adjust to diurnal feeding but remain less active than at night, when it would be difficult to manage the blood sampling. After a control period of 3 d, cineole was added to the basal diet. Blood was taken on the first day of the cineole diet and again 1 wk later after tolerance had developed.

Night Feeding Possums (seven male, four female; body weight, 3.45±0.11 kg) were offered the basal diet (500 g wet weight) and water overnight. The stainless steel food dish was placed on a balance and housed in a plywood box. The box had a hinged lid with an opening in the top through which the food was presented. The box protected the dish and balance from being knocked over by the possum. The balance was connected to a laptop computer outside the pen, and the weight of food remaining was recorded every minute throughout the night. This method of monitoring feeding has been described previously (Boyle et al. 2005). Night-feeding experiments were commenced late afternoon (4–5 pm) and stopped at about 9 am the following morning (i.e., after about 16 hr). The time and weight recordings were used to monitor feeding throughout the night. Animals were offered the basal diet for at least three nights, then the same diet containing 2% cineole (wet weight) for nine nights. The possums had been maintained on a terpene-free diet for a minimum of 2 wk (median, 7 wk) before being offered the cineole diet.

Day Feeding Possums (four male, three female; body weight, 3.47±0.07 kg) were fasted overnight and taken in the morning in their nest box from their pen to the laboratory. The front opening of the nest box was fitted with a gate through which the possum could reach into a food dish but not leave the box, as described in Boyle et al. (2005). The weight of the food dish was recorded every minute on a laptop computer. Feeding experiments were commenced at 9 am and continued for 3 hr. After the experiment, the animal was returned to its pen in its nest box.

Based on the findings of the night-feeding experiments, 7 d were allowed for the development of tolerance to the cineole diet. The experiment was commenced with three control days, then 2% cineole was introduced into the diet, and blood samples were taken on the first and seventh cineole days. Because, at first, little of the cineole diet was eaten during the 3-hr experiment, the uneaten food was left overnight in the pen for the animal to continue eating. The night-feeding experiments had shown that, after a few days, most of the cineole diet was eaten by morning. The offering of uneaten food ceased on days 5 and 6 to re-establish daytime feeding before the second blood sampling experiment on day 7.

Blood Sampling and Analysis These methods have been described previously in detail in Boyle et al. (2005). At least 1 wk before experiments started, a vascular access port was implanted between the scapulae, and its catheter was placed in the jugular vein. On the day of an experiment, an infusion set needle was inserted into the port and the other end of the catheter left outside the closed lid of the nest box. Blood samples (0.1 ml) were taken when feeding commenced and continued every 5 min until 10 min after the bout finished. Between bouts, blood samples were taken every 15–30 min. Blood samples were stored in heparinized tubes at −18°C until analysis of cineole by gas chromatography (Boyle et al. 2002).

Data Analysis The time–weight data were used to calculate total food intake, the number and size of feeding bouts, and their duration. A feeding bout was arbitrarily defined by a consumption rate of more than 10 g in 1 min, although rests of up to 2 min were accepted as part of a bout. We had previously defined a bout as >3 g/min (Boyle et al. 2005), but this included intakes that were subsequently considered to be too small to be significant for the analysis of bouts. Since some food was consumed at slower rates, the total food intake was greater than the sum of the bouts, and the percentage eaten in bouts was calculated. The rate of food intake was calculated by dividing the amount eaten in a bout by the time taken.

The blood concentration of cineole (C) is expressed in nanogram per milliliter (1 ng/ml=6.494 nM). The concentration–time data were used to determine the maximum

concentration (C_{max}) and its corresponding time (T_{max}), and the area under the curve (AUC) during the 3-hr daytime-feeding experiment. In one experiment, the catheter failed after 85 min, so the AUC and total food intake for this and the other blood-sampling day for the same animal were calculated for this time. The relative bioavailability of cineole was estimated from the ratio AUC/amount of cineole ingested.

Data are expressed as mean±SE. Means were compared by one-way analysis of variance (ANOVA) or, when only two means were compared, Student's paired *t* test. When the ANOVA *F* test was significant ($P<0.05$), post-tests were conducted for linear trend or to compare pairs of means (Tukey's multiple comparison test), as appropriate. T_{max} data were extremely skewed and were compared as medians using Wilcoxon's matched pairs test. All comparisons were made with matched (repeated measures) tests since there was considerable individual variability in feeding behavior. Night-time and daytime experimental findings were compared in the six possums that underwent both procedures. GraphPad Prism software version 4 (San Diego, CA, USA) was used for calculating AUC, statistical analyses, and graphing.

Results

Night Feeding Seven possums were offered the basal diet (500 g) in the night feeding setup for seven nights. There was a significant increase in total food consumption over time (ANOVA, $F_{6,6}=6.59$, $P<0.001$), which post-testing showed was due to the increase after the first night and that, thereafter, food consumption was constant for each possum. For these seven possums, the last three nights before 2% cineole was added to the diet were used as the control period. An additional four possums were fed the control diet for just the three nights before the cineole diet. The total time spent feeding varied greatly between individual possums: On the last control night, the median was 7.9 hr and range 0.9 to 10.5 hr ($N=11$).

Figure 1 shows the total food eaten overnight for three control nights and nine nights of cineole diet, and Table 1 summarizes the analysis of feeding variables. There was no difference between the three control nights, but introduction of 2% cineole halved total food intake on the first night. Total intake of the cineole diet increased progressively, and by the third night, it was not significantly different from either the control or the cineole diet intake on nights 4–9. However, because the upward trend continued as shown in Fig. 1, data from cineole night 9 (the last night) were used to measure the adjustments associated with tolerance.

The number of feeding bouts did not change from control night 3 to cineole night 1, but nearly doubled after adaptation to the cineole diet (Table 1). The mass of the major feeding bout, which was usually the first bout, decreased about threefold when cineole was added to the diet. The mass eaten during the major bout had doubled by the third cineole night, and thereafter, it remained constant and was still only 60% of the amount on control night 3. The rate of eating also decreased from control night 3 to cineole night 1 and remained slower on subsequent cineole nights. Nearly all (96%) of the control diet was eaten in bouts of >10 g. This proportion fell to 70% when cineole was first introduced but returned to control levels by cineole night 9.

At the end of the night-feeding experiments, mean possum weights (3.52±0.13 kg) had not significantly changed ($t=1.83$, $df=10$, $P=0.097$).

Day Feeding In preliminary trials for 3–5 days, animals became used to daytime feeding from their nest box by the second day and, on subsequent days, did not increase total food consumption during the 3-hr feeding session. Therefore, a 3-d control period was used for the seven possums in the day-feeding experiments. During this control period, there was a non-significant increase in food consumption after the first day to a constant amount on days 2 and 3 (ANOVA, $F_{2,6}=2.12$, $P=0.149$).

Day and night feeding on the basal diet were compared in six possums that had been fed under both conditions. Total food intake during control day 3 (280±12 g) was about three quarters of that during control night 3 (404±39 g; $t=3.56$, $df=5$, $P=0.016$). However, as the time available for feeding was much less during the day (3 h) than the night (about 16 hr), the overall rate of feeding was greater during the daytime sessions. Moreover, the major feeding bout was larger during the day (183±11 g) than the night (128±22 g; $t=2.86$, $df=5$, $P=0.035$).

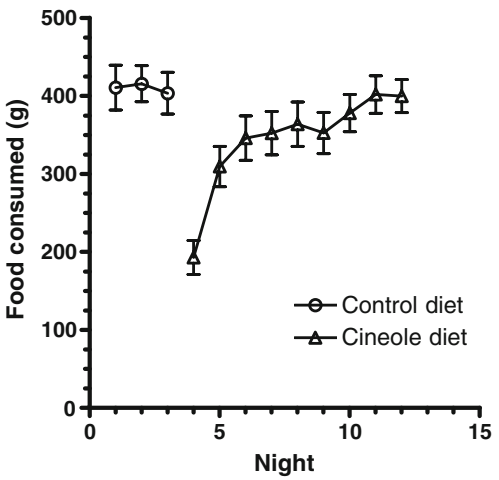

Fig. 1 Night feeding on basal diet (control) and 2% cineole in basal diet (cineole). Data are shown as mean±SE ($N=11$). Statistical analyses are given in Table 1

Table 1 Night-feeding variables

	Control night 3	Cineole night 1	Cineole night 9	Cineole nights 1 to 9; ANOVA F, P[a]
Total food eaten (g)	414±25	193±22	400±21	28.47, <0.001
P vs. control[b]		<0.001	0.526	
Number of feeding bouts	5.6±0.8	5.6±0.6	10.1±1.5	3.984, <0.001
P vs. control[b]		0.939	<0.023	
Amount consumed during major feeding bout (g)	152±26	45±7	93±17	6.593, <0.001
P vs. control[b]		0.001	0.013	
Rate of eating major bout (g/min)	8.93±0.98	6.05±0.99	5.59±0.63	1.634, 0.128
P vs. control[b]		0.028	0.003	
Percentage eaten in bouts >10 g	96.3±1.6	70.9±6.4	92.7±1.9	6.443, <0.001
P vs. control[b]		0.004	0.176	

Data shown as mean ± SE (N=11)

[a] Repeated measures ANOVA for cineole nights 1–9, $F_{8,10}$, P of post-test for linear trend

[b] P values calculated by t test, df=10

Seven animals were used to study the development of tolerance to cineole in day-feeding experiments. When cineole was added to the basal diet, total food consumption initially fell to about one fifth of the control amount (Table 2). The daytime consumption remained constant for the following 4 days, while the residual cineole diet was given to the animals for overnight consumption. Most of the diet was eaten overnight, and the total food consumption (day+night) was about 400 g, equivalent to that of the night feeding experiments.

Then, food was withheld on nights 5 and 6 to re-establish day-feeding behavior before the blood-sampling experiment on day 7. The amount eaten during the day remained similar from days 1 (64±5 g) to 5 (60±12 g), but after the overnight fast, it increased on day 6 to 134±25 g (t=4.93, df=6, P=0.003) and was not significantly greater on day 7 (157±18 g; t=1.28, df=6, P=0.248). After 7 days of cineole diet, the total amount eaten during the day had increased 2.5-fold to about half the control values (Table 2).

Although the number of bouts did not change significantly after cineole commenced, the major bout was smaller on cineole day 1 than on control day 3, but this increased to about half the control value on cineole day 7 (Table 2). The rate of consumption during the major bout also fell when cineole was introduced but rose to about the control level by day 7. The percentage of the total food intake that was eaten in bouts >10 g dropped by a third on cineole day 1 but had recovered to the control value by the seventh day of cineole.

At the end of the day-feeding experiments, possum weights had slightly increased to 3.72±0.13 kg (t=3.38, df=6, P =0.015).

Pharmacokinetics Figure 2 shows an example of blood concentration and feeding data for one possum on cineole days 1 and 7. The rate of feeding was usually constant during a bout, although sometimes it slowed, as can be seen in the first bout on day 1. The blood cineole concentration rose rapidly when feeding commenced and fell promptly

Table 2 Day feeding variables

	Control day 3	Cineole day 1	Cineole day 7	P cineole days 1 vs. 7
Total food eaten (g)	308±29	64±5	157±18	0.003
P vs. control		<0.001	<0.001	
Number of feeding bouts	3.6±0.4	2.6±0.5	3.7±0.8	0.066
P vs. control		0.134	0.853	
Amount consumed during major feeding bout (g)	181±9	29±4	96±13	0.001
P vs. control		<0.001	<0.001	
Rate of eating major bout (g/min)	7.01±0.73	3.38±0.77	6.17±0.61	0.002
P vs. control		0.004	0.297	
Percentage eaten in bouts >10 g	98.1±0.6	67.3±5.2	96.2±1.7	0.003
P vs. control		0.001	0.295	

Data shown as mean ± SE (N=7). P values from paired t tests (df=6)

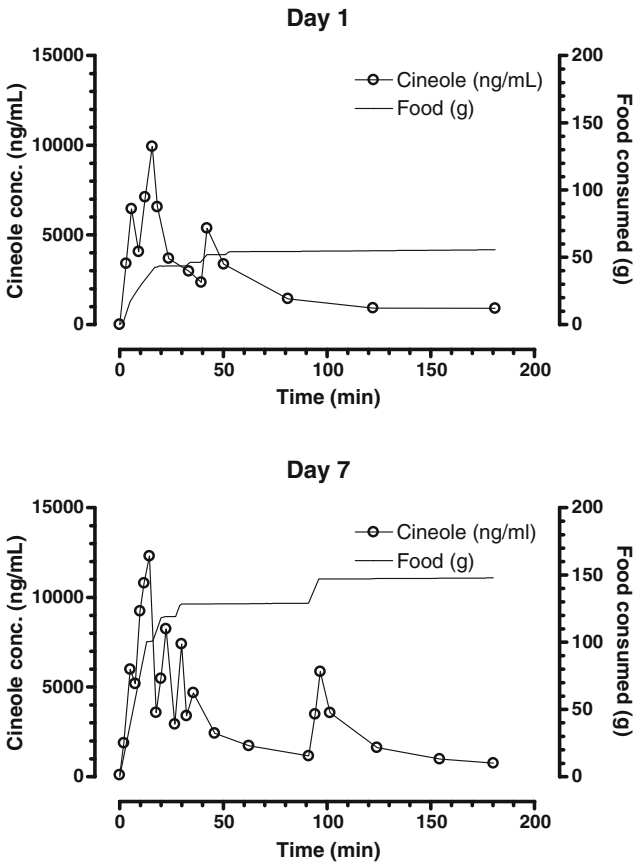

Fig. 2 Food consumption and blood concentrations of cineole on days 1 and 7 of the 2% cineole diet. Data are shown for a single possum

when feeding slowed or ceased. Table 3 compares the mean pharmacokinetic and feeding data for all possums. The total intake of food and, therefore, cineole increased from cineole day 1 to 7 (the slight discrepancy from food intake values in Table 2 is due to the adjustment required for one animal whose catheter blocked, ending the blood sampling after 85 min instead of 3 hr).

There was an increase in C_{max} on day 7 but no significant change in AUC. T_{max} was highly variable, but comparison of the median values showed that it occurred earlier on day 7 (15.8; range, 12–160 min) than day 1 (53.9; range, 3–43 min; Wilcoxon's matched pairs test, P=0.016). The ratio of AUC/cineole ingested decreased after 7 days, indicating that the bioavailability of cineole also fell.

Discussion

Night Feeding The night-feeding experiments confirmed the previously reported antifeedant effect of 2% cineole (Lawler et al. 1999; Boyle and McLean 2004). Full tolerance to cineole, defined by food intake returning to the pre-cineole amount, was evident by the ninth cineole night. Although total food intake was not significantly different from the control value on the third cineole night, the trend continued upwards until the eighth night (Fig. 1 and Table 1) indicating that about a week was required for full tolerance.

Breakdown of the feeding variables (Table 1) showed that the initial response to cineole was to reduce greatly the amount consumed in the major bout and to eat it more slowly, and to eat about half the total control amount of food and about a third of this in small quantities (<10 g). Similar changes to feeding behavior of brushtail possums have been found when increasing levels of cineole were added to a basal diet (Wiggins et al. 2003; Boyle and McLean 2004). Overall, after tolerance had developed, the total pre-cineole food intake was achieved by eating a larger number of smaller bouts and at a slower rate.

Day Feeding The day-feeding situation was quite different from night feeding, as it took place during the normal rest period for possums, the animals were confined in their nest boxes, and the time available for feeding was reduced to 3 hr. Nevertheless, feeding was active under these conditions, as two thirds of the overnight (16 hr) control diet intake was consumed, and the major bout was 40% greater than at night.

The addition of cineole to the diet resulted in a greater initial decrease in total food consumption than in the night experiments, most likely due to the shorter available feeding time. As with the night experiments, there was a commensurate decrease in the mass of the major bout and its rate of consumption and an increase in the amount of

Table 3 Pharmacokinetic data from day feeding on a 2% cineole diet

	Cineole day 1	Cineole day 7	P value
Total food eaten (g)	60±5	153±18	0.003
Total cineole ingested (mg)	1,202±97	3,061±358	0.003
C_{max} (ng/ml)	6,653±1,309	10,588±1,595	0.001
T_{max} (min)	61±20	20±5	0.067
AUC (min.ng/ml)	442,961±103,865	480,477±49,006	0.726
AUC/cineole ingested	345±52	160±11	0.008

Data shown as mean±SE (N=7). P values from paired t tests (df=6)

food eaten in quantities less than the defined bout size of 10 g. By the seventh day of the cineole diet, possums showed evidence of adaptation similar to that seen in the night experiments, i.e., increases in total food consumption and amounts eaten in the major bout and the percentage of food eaten in bouts. However, there were some differences from the night-feeding experiments, which were possibly due to the shorter feeding time: Total food consumption did not reach the control amount, and there was no increase in the number of bouts, but their rate of consumption did increase to about the control value.

Food consumption depends on the balance between positive drives (especially hunger) and the negative influences of satiety and the aversive effects of PSMs (Provenza 1995). The night-feeding experiments indicated that 7 days of the cineole diet would be sufficient for tolerance to develop. However, the development of pharmacological tolerance (whether pharmacokinetic or pharmacodynamic) depends on the amount of the drug ingested and the period of exposure (Hollenberg 2002; O'Brien 2006), and the lower daytime food consumption would also result in a lower daily cineole dose and a possible failure to develop tolerance.

The dilemma was to ensure that possums not only ate a sufficient quantity of the cineole diet over 7 d to maintain adequate nutrition and develop tolerance to cineole, as occurred with night feeding, but also were fasted long enough to develop a degree of hunger on cineole day 7 comparable to that on cineole day 1. This was achieved in two stages. For the first 4 days, possums were offered the remaining uneaten cineole diet for overnight consumption. Although daytime feeding remained low, total 24-hr food consumption equaled that of the night-feeding experiments, indicating that nutrition and cineole tolerance would also be similar. Then, food was withheld on nights 5 and 6 to re-establish day-feeding behavior before the blood-sampling experiment on day 7. The amount eaten during the day increased from days 5 to 6 but was not significantly greater on day 7. Thus, as for the control diet, it took 2 days to establish constant daytime feeding.

While it is not possible to compare directly the levels of hunger on days 1 and 7 of the cineole diet, the feeding behavior was in some respects (mass of major bout and percentage eaten in bouts) similar to that on cineole nights 1 and 9, respectively. This suggests that the conditions of the possums when first exposed to cineole and after tolerance had developed were comparable in the day and night experiments. Although total food consumption was less during the day than at night, there was no loss of body weight during the day-feeding experiments. Possibly, animals compensated by being less active overall during the periods of the daytime-feeding regime.

The fall in food consumption when cineole was introduced may have been an example of the general cautionary response of herbivores to a new diet (Provenza 1995) as much as to any direct aversive effect of cineole. However, the continuance of the low daily intake suggests that aversion was more important than unfamiliarity.

Pharmacokinetics Blood analyses offer insights into the mechanisms that underlie the development of tolerance to dietary cineole. The maximum blood concentration of cineole followed the largest feeding bout and indicated the highest concentration that would be tolerated to continue feeding. On the first day of cineole, C_{max} was 6,653±3,462 ng/ml or 43±22 μM, similar to the 52±14 μM found previously to be the critical concentration that caused immediate cessation of feeding (Boyle et al. 2005). After 7 d of the cineole diet, this critical concentration had increased by 60% to 10,588±4,220 ng/ml (69±27 μM). This may indicate that significant tolerance had developed to the aversive effects of cineole, although an increase in hunger cannot be discounted. However, on cineole day 7 and cineole night 9, the amount of cineole diet consumed in the major bout and its rate of consumption were similar, indicating that both the level of hunger and the critical aversive blood concentration of cineole would have been similar on both occasions, despite the differences in the feeding regimens. This supports the view that the increase in C_{max} associated with adaptation to a cineole diet is due at least in part to tolerance to its aversive effects.

There are two possible antifeedant effects of cineole for which pharmacodynamic tolerance could occur. Tolerance would be expected to develop to the CNS depressant effect of cineole, as mentioned in "Introduction." Another possible aversive effect is nausea (Provenza 1995), which is mediated at least partly via $5HT_3$ serotonergic receptors (Lawler et al. 1998). $5HT_3$ receptors characteristically show rapid desensitization with continued stimulation (Spiller 2002).

By day 7 of the cineole diet, the total cineole intake had increased 2.5-fold, but the AUC had not changed. The AUC is a measure of the completeness of absorption into the systemic circulation, and it increases in proportion to dose, provided that absorption is unchanged (Rowland and Tozer 1995). The fraction of a dose that reaches the general circulation, termed the bioavailability, is calculated from the ratio of AUCs after oral and intravenous administration, corrected for any differences in dose. We have found previously that cineole is absorbed rapidly and completely from the gut in the brushtail possum and that only a small fraction of an oral dose reaches the general circulation (McLean et al. 2007). This phenomenon is due to extensive pre-systemic metabolism of cineole by enzymes in the gut wall and liver during the absorption process (the 'first-pass effect'). In the present experiment, the AUC corrected for the amount of cineole ingested (AUC/cineole ingested) fell

by one half after 7 d of the cineole diet, indicating that there had been an increase in enzyme activity and pre-systemic metabolism of cineole resulting in a decrease in its bioavailability. Induction of liver enzymes is known to decrease the oral bioavailability of any drug that, like cineole, is rapidly metabolized in the liver (Rowland and Tozer 1995).

The increase in size of the major bout supports this view. The maximum tolerable dose of cineole, estimated from the major bout, was 163±22 mg/kg on cineole day 1 and 527± 80 mg/kg on day 7. This suggests that pre-systemic metabolism had greatly increased after 7 days, since there was not a proportionate (i.e., threefold) increase in blood cineole concentration (C_{max}). In fact, the expected increase in C_{max} is greater than threefold, since the bioavailability of cineole is increased after higher doses due to saturation of the enzymes responsible for pre-systemic metabolism (McLean et al. 2007).

A potential complication arises because ingestion of cineole through feeding takes longer than its administration as an oral dose, effectively prolonging the absorption time of dietary cineole. Slowing the absorption of a drug gives lower blood levels even if bioavailability is unchanged (Rowland and Tozer 1995). In addition, for a drug like cineole that has low bioavailability due to rapid first-pass metabolism, slow absorption would be expected to decrease further its bioavailability and therefore its blood concentrations. This is because slow absorption results in a lower concentration of cineole being presented to the enzymes in the gut wall and liver, resulting in less saturation of enzymes and more complete metabolism of the ingested cineole (effectively a consequence of the dose-dependent kinetics mentioned above).

Consistent with this, when possums were first presented with cineole, they ate the major bout more slowly, which would be expected to produce lower blood levels for the same amount of cineole ingested. This slower rate continued after tolerance had developed in the night-feeding experiments; that is, tolerance was in part due to a behavioral change in the rate of feeding. However, in the time-limited day-feeding experiments, the feeding rate on the major bout was decreased on day 1 but had returned to the pre-cineole level on day 7. As this change would tend to increase blood cineole levels, it is further evidence that the relatively low cineole concentrations were due to induction of the enzymes of first-pass metabolism.

We have demonstrated previously that a diet containing cineole and other terpenes induces the liver enzymes that metabolize cineole (Pass et al. 1999, 2001). There is a large literature on the induction of drug metabolism by herbal preparations and other drugs, and it is generally considered that the induction mechanism evolved in herbivores as a response to chemical defenses of plants. However, this is the first demonstration in a vertebrate herbivore that consumption of a dietary PSM leads to induction of its metabolism and a consequent increase in feeding.

Linking Behavioral and Physiological Data The behavioral feeding patterns demonstrated by brushtail possums offer support for our hypothesis that possums are able to develop a level of tolerance to a cineole diet. Their initial reduction in total intake, feeding bout mass, and intake rate when first exposed to a 2% cineole diet had, after 7 days, largely returned to the behavioral feeding patterns displayed on the control diet. Our findings also support the avoidance-tolerance model of Iason and Villalba (2006), as the increase in detoxification capacity and probably pharmacodynamic tolerance leads to a reduction in the behavioral strategy of avoidance.

We have also demonstrated that intake appears to be correlated highly with blood cineole concentrations – where large bouts of eating are mirrored by sharp peaks in blood cineole concentrations. The alteration of intake, intake rate, and bout mass should thus be a direct response to blood cineole levels, used as a behavioral regulatory mechanism. The evidence presented in this study indicates that brushtail possums can adapt to a dietary PSM initially by altering feeding behavior and subsequently by induction of the enzymes responsible for its metabolism, probably coupled with some decreased CNS sensitivity.

The unique combination of physiological and behavioral data measured in this experiment has not only enabled us to test the hypothesis of tolerance and metabolic induction in the brushtail possum, but it also provides support for the hypothesis proposed by Wiggins et al (2006) that the feeding decisions of generalist mammalian herbivores, specifically in relation to diet mixing, may largely be driven over a short-term, possibly minute-by-minute basis, in direct response to their exposure to blood concentrations of ingested dietary PSMs.

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References

BOYLE, R. R., and MCLEAN, S. 2004. Constraint of feeding by chronic ingestion of 1,8-cineole in the brushtail possum (*Trichosurus vulpecula*). *J. Chem. Ecol.* 30:757–775.

BOYLE, R. R., MCLEAN, S., BRANDON, S., PASS, G. J., and DAVIES, N. W. 2002. Application of solid-phase microextraction to the quantitative analysis of 1,8-cineole in blood and expired air in a

Eucalyptus herbivore, the brushtail possum (*Trichosurus vulpecula*). *J. Chromatogr. B.* 780:397–406.

BOYLE, R. R., MCLEAN, S., BRANDON, S., and WIGGINS, N. 2005. Rapid absorption of dietary 1,8-cineole results in critical blood concentration of cineole and immediate cessation of eating in the common brushtail possum (*Trichosurus vulpecula*). *J. Chem. Ecol.* 31:2775–2790.

BRYANT, J. P., PROVENZA, F. D., PASTOR, J., REICHARDT, P. B., CLAUSEN, T. P., and DUTOIT, J. T. 1991. Interactions between woody-plants and browsing mammals mediated by secondary metabolites. *Annu. Rev. Ecol. Syst.* 22:431–446.

DEARING, M. D., FOLEY, W. J., and MCLEAN, S. 2005. The influence of plant secondary metabolites on the nutritional ecology of herbivorous terrestrial vertebrates. *Annu. Rev. Ecol. Evol. Syst.* 36:169–189.

DZIBA, L. E., HALL, J. O., and PROVENZA, F. D. 2006. Feeding behavior of lambs in relation to kinetics of 1,8-cineole dosed intravenously or into the rumen. *J. Chem. Ecol.* 32:2391–408.

FOLEY, W. J., IASON, G. R., and MCARTHUR, C. 1999. Role of plant secondary metabolites in the nutritional ecology of mammalian herbivores: How far have we come in 25 years? pp. 130–209, in H.-J. G Jung and G. C. Fahey (eds.). San Antonio, Texas, USA. American Society of Animal Science, Savoy, Illinois.

FRAENKEL, G. S. 1959. Raison d'etre of secondary plant substances. *Science* 129:1466–1470.

GONZALEZ, F. J., and KIMURA, S. 2003. Study of P450 function using gene knockout and transgenic mice. *Arch. Biochem. Biophys.* 409:153–158.

GONZALEZ, F. J., and NEBERT, D. W. 1990. Evolution of the P450 gene superfamily: animal-plant 'warfare', molecular drive and human genetic differences in drug oxidation. *Trends Genet.* 6:182–186.

HARRIS, R. Z., JANG, G. R., and TSUNODA, S. 2003. Dietary effects on drug metabolism and transport. *Clin. Pharmacokinet.* 42:1071–1088.

HOLLENBERG, P. F. 2002. Characteristics and common properties of inhibitors, inducers, and activators of CYP enzymes. *Drug Metab. Rev.* 34:17–35.

IASON, G. 2005. The role of plant secondary metabolites in mammalian herbivory: ecological perspectives. *Proc. Nutr. Soc.* 64:123–131.

IASON, G. R., and VILLALBA, J. J. 2006. Behavioral strategies of mammal herbivores against plant secondary metabolites: The avoidance-tolerance continuum. *J. Chem. Ecol.* 32:1115–1132.

IOANNIDES, C. 2002. Topics in xenobiochemistry - pharmacokinetic interactions between herbal remedies and medicinal drugs. *Xenobiotica* 32:451–478.

LAWLER, I. R., FOLEY, W. J., PASS, G. J., and ESCHLER, B. M. 1998. Administration of a 5HT(3) receptor antagonist increases the intake of diets containing *Eucalyptus* secondary metabolites by marsupials. *J. Comp. Physiol. B* 168:611–618.

LAWLER, I. R., STAPLEY, J., FOLEY, W. J., and ESCHLER, B. M. 1999. Ecological example of conditioned flavor aversion in plant-herbivore interactions: Effect of terpenes of *Eucalyptus* leaves on feeding by common ringtail and brushtail possums. *J. Chem. Ecol.* 25:401–415.

MCLEAN, S., BOYLE, R. R., BRANDON, S., DAVIES, N. W., and SORENSEN, J. S. 2007. Pharmacokinetics of 1,8-cineole, a dietary toxin, in the brushtail possum (*Trichosurus vulpecula*): Significance for feeding. *Xenobiotica* 37:903–922.

O'BRIEN, C. P. 2006. Drug addiction and drug abuse, pp. 607–627, in L. L., Brunton, J. S., Lazo, K. L., and Parker (eds.). Goodman and Gilman's The Pharmacological Basis of Therapeutics11th edn.McGraw Hill, New York.

PASCUSSI, J. M., GERBAL-CHALOIN, S., DROCOURT, L., ASSENAT, E., LARREY, D., PICHARD-GARCIA, L., VILAREM, M. J., and MAUREL, P. 2004. Cross-talk between xenobiotic detoxication and other signalling pathways: Clinical and toxicological consequences. *Xenobiotica* 34:633–664.

PASS, G. J., MCLEAN, S., and STUPANS, I. 1999. Induction of xenobiotic metabolising enzymes in the common brushtail possum, *Trichosurus vulpecula*, by *Eucalyptus terpenes*. *J. Comp. Biochem. Physiol.* 124:239–246.

PASS, G. J., MCLEAN, S., STUPANS, I., and DAVIES, N. 2001. Microsomal metabolism of the terpene 1,8-cineole in the common brushtail possum (*Trichosurus vulpecula*), koala (*Phascolarctos cinereus*), rat and human. *Xenobiotica* 31:205–221.

PFISTER, J. A., PROVENZA, F. D., MANNERS, G. D., GARDNER, D. R., and RALPHS, M. H. 1997. Tall larkspur ingestion: Can cattle regulate intake below toxic levels? *J. Chem. Ecol.* 23:759–777.

PROVENZA, F. D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. *J. Range Manag.* 48:2–17.

ROWLAND, M., and TOZER, T. N. 1995. Clinical Pharmacokinetics - Concepts and Applications. p. 601. Lea & Febiger, Philadelphia.

SNYDER, M. J., and GLENDINNING, J. I. 1996. Causal connection between detoxification enzyme activity and consumption of a toxic plant compound. *J. Comp. Physiol. A Sens. Neural Behav. Physiol.* 179:255–261.

SPILLER, R. 2002. Serotonergic modulating drugs for functional gastrointestinal diseases. *Br. J. Clin. Pharmacol.* 54:11–20.

WANG, H. B., and LECLUYSE, E. L. 2003. Role of orphan nuclear receptors in the regulation of drug-metabolising enzymes. *Clin. Pharmacokinet.* 42:1331–1357.

WIGGINS, N. L., MCARTHUR, C., MCLEAN, S., and BOYLE, R. 2003. Effects of two plant secondary metabolites, cineole and gallic acid, on nightly feeding patterns of the common brushtail possum. *J. Chem. Ecol.* 29:1447–1464.

WIGGINS, N. L., MCARTHUR, C., DAVIES, N. W., and MCLEAN, S. 2006. Spatial scale of the patchiness of plant poisons: A critical influence on foraging efficiency. *Ecology* 87:2236–2243.

WILKINSON, G. R. 2004. Genetic variability in cytochrome P450 3A5 and in vivo cytochrome P450 3A activity: Some answers but still questions. *Clin. Pharmacol. Ther.* 76:99–103.

ZHOU, S. F., GAO, Y. H., JIANG, W. Q., HUANG, M., XU, A. L., and PAXTON, J. W. 2003. Interactions of herbs with cytochrome P450. *Drug Metab. Rev.* 35:35–98.

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Phytotoxic Catechin Leached by Seeds of the Tropical Weed *Sesbania virgata*

Kelly Simões · Jiang Du · Fernanda S. Kretzschmar · Corey D. Broeckling · Frank S. Stermitz · Jorge M. Vivanco · Marcia R. Braga

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Abstract *Sesbania virgata* (Cav.) Pers (wand riverhemp) is a fast-growing tropical legume species that has been used for revegetation of riparian forests and rehabilitation of degraded areas and that exhibits an invasive behavior in certain regions of Brazil. Preliminary studies have shown that seed leachates inhibit the germination and development of seedlings of some crop species. In this study, we report that the seed leachates of *S. virgata* inhibit the growth of *Arabidopsis thaliana* and rice. The flavonoid (+)-catechin is found in high amounts in these leachates. It was active at concentrations of 50 μg ml^{-1}, and its effect was not distinguishable from the (+)-catechin obtained from a commercial source. We found that (+)-catechin is located in the seed coat and is rapidly released in high concentrations (235 μg per seed) at the beginning of imbibition. Quercetin was also detected in the seed coat of *S. virgata*, but it was not released from the seeds. Other phytotoxic compounds in the seed leachates were also detected. The fact that *S. virgata* releases high amounts of (+)-catechin, which also has antimicrobial activity, and other phytotoxins from its seeds at the earliest stages of its development might represent some adaptative advantage to the seedling that contributes to its invasive behavior and successful establishment in different soils.

Keywords *Sesbania virgata* · Seed leachate · (+)-Catechin · Allelopathy · Allelochemicals · Quercetin

K. Simões · F. S. Kretzschmar
Departamento de Biologia Celular e Estrutural,
Universidade Estadual de Campinas,
C.P. 6109, Campinas 13083-863 São Paulo, Brazil

K. Simões · F. S. Kretzschmar · M. R. Braga (✉)
Seção de Fisiologia e Bioquímica de Plantas,
Instituto de Botânica,
C.P. 3005, São Paulo 01061-970 São Paulo, Brazil
e-mail: bragamr@ig.com.br

J. Du · F. S. Stermitz
Department of Chemistry, Colorado State University,
Fort Collins, CO 80523, USA

J. Du · C. D. Broeckling · J. M. Vivanco
Center for Rhizosphere Biology, Colorado State University,
Fort Collins, CO 80523, USA

Introduction

The seed stage is a crucial phase of plant development that allows plants to effectively establish in the natural environment. One strategy that enables the success of the early establishment of plants is the release of secondary metabolites and other low-molecular-weight compounds at germination, which can inhibit the proliferation of soil pathogens and the germination and growth of competing plants (Nelson 2004).

Some studies have examined how phytotoxic secondary metabolites exuded by the roots can influence the ability of a plant to become invasive in a new environment, such as the enantiomers (±)-catechin exuded by *Centaurea maculosa* Lam. (Bais et al. 2002) and lactones released by *Echinochloa crusgalli* (L.) Beauv. (Xuan et al. 2006). Although these studies have examined only those compounds that are exuded through the plant root tissues, seeds are also able to rapidly release allelochemicals after the beginning of the imbibition process that contribute to the invasive behavior of some species (Ndakidemi and Dakora 2003 and references therein).

The tropical legume *Sesbania virgata* (Cav.) Pers (Syn. *S. marginata* Benthan) is a perennial, fast-growing shrub native to South America. It is 2–4 m in height and occurs in

Central, Southeast, and South Brazil, Argentina, Uruguay, and Paraguay. It produces a lot of seeds with long-term viability that are dispersed within indehiscent legume fruits that float in the water (Pott and Pott 1994). It has been described as invasive in flood and damp soil, especially in inundated rice plantations (Kissmann and Groth 1999). This species has been used for revegetation of riparian forests, soil erosion control, and rehabilitation of degraded areas (Pott and Pott 1994).

Previous studies have demonstrated that *S. virgata* seed leachates can affect the germination and growth of the radicle of some crop species (lettuce, tomato, and radish; Simões and Braga, unpublished data). It also has been reported that the seeds of various other *Sesbania* species contain toxic compounds that are able to restrict the growth of other plant species (Buta 1983; Powell et al. 1990; Van Staden and Grobbelaar 1995). *Sesbania* species generally present abundant seed production and rapid growth, occurring naturally near rivers, in flood places, and in modified soils. *S. punicea* (Cav) Benth. is described as a noxious weed in South Africa, invading abandoned fields and out-competing natural vegetation, and forming a dense population. This behavior seems to be associated with the presence of the potent alkaloid sesbanimide in the seeds that can inhibit the seedling growth of several species (Gorst-Allman et al. 1984; Van Staden and Grobbelaar 1995). As reported by Singh et al. (2007), under field conditions, intercropping of *Sesbania* species with cultivated plants reduces the density of grass weeds. Due to its invasive behavior and demonstrated ability to release phytotoxins from its seeds, *S. virgata* may fit the seed-related allelopathic invasiveness pattern of this genus; however, the chemicals responsible for the phytotoxicity have not yet been identified.

Despite the fact that seed leachates of *S. virgata* contain large amounts of sugars released during the mobilization of the storage cell wall carbohydrates by the developing embryo (Buckeridge and Dietrich 1996), no microbial contamination has been observed during the germination process. This lack of a microbial presence suggests the release of antimicrobial compounds during the early stages of germination. As the phytotoxic compounds produced are unknown, the aim of the present work was to isolate and identify phytotoxins present in the *S. virgata* seed leachates. We tested compounds on *Arabidopsis thaliana* and rice under laboratory conditions. We report that (+)-catechin is the major compound released by *S. virgata* seeds.

Methods and Materials

Seed Germination Seeds of *S. virgata* were collected from natural populations in the region of São Paulo, Brazil. Six thousand seeds were selected, scarified, and placed on two Whatman 1 filter papers in 15-cm-diameter Petri dishes along with 0.7 ml of distilled water per seed (50 seeds per dish). The plates were incubated for 3 days at 25°C with a photoperiod of 12 h.

Extraction and Isolation During the third day of germination, seeds were removed from the Petri dishes, the leachates were collected, and the filter papers were washed ×3 with 105 ml of distilled water (representing three times the initial water volume added to each dish). The leachates of each dish were combined, filtered through a *nylon* membrane, freeze-dried, and weighed (6 g). The resultant powder was dissolved in 300 ml distilled water and extracted sequentially with the same volume of hexane and ethyl acetate (×3 each). The aqueous extract was freeze-dried and re-dissolved in methanol, and evaporated under a vacuum. The ethyl acetate extracts were chromatographed in a regular Merck silica gel 60 (0.063–0.200 mm) column (80×3 cm) and eluted with a gradient of dichloromethane (DCM) and MeOH (0→100% MeOH). Fifty fractions were collected and assembled according to their thin-layer chromatography (TLC) profiles to yield ten fractions (A–J). One of the largest phytotoxic fractions collected (fraction H, 110 mg) was re-fractioned in a flash chromatography system with a silica gel column (1×20 cm) and DCM/MeOH (9:1) as an isocratic solvent system.

Seeds removed from the Petri dishes were also freeze-dried, triturated with a Walita mixer, extracted ×3 with pure ethanol (0.7 ml per seed), evaporated, and partitioned with hexane, ethyl acetate, and methanol as described above. The ethyl acetate fraction (600 mg) was also submitted to a regular Merck silica gel 60 (0.063–0.200 mm) column (2× 20 cm) with a gradient with DCM and MeOH (0→100% MeOH). The 60 fractions collected were compiled into 15 fractions according to their TLC profiles, and one fraction (six) that contained pure quercetin was detected.

Circular Dichroism Spectroscopy and ^1H NMR Analyses Quercetin and catechin isolated from *S. virgata* seeds and leachates were identified by ^1H NMR spectra analyses in CD_3OD and compared to standards purchased from Sigma by using a Varian INOVA 400-MHz Fourier spectrometer. Catechin was also analyzed by its circular dichroism (CD) spectrum in methanol on an Aviv model 202 spectrometer and compared with the standard. (+)-Catechin showed a cotton effect at 280 nm ($\Delta\epsilon$=−1.511; Korver and Wilkins 1971).

Quantification of Isolated Compounds Seeds of *S. virgata* were germinated as described above, and every day, between the beginning of the imbibition and the final day

of germination (1–6 days), a set of 40 seeds was removed from the Petri dishes. Tissues were separated (seed coat, endosperm, and embryo), freeze-dried, and extracted ×3 with ethanol (0.7 ml per seed). Ethanol was evaporated, and the extracts were suspended in 1 ml distilled water and partitioned with 1 ml ethyl acetate (×4). After drying, extracts were solubilized in MeOH (high-performance liquid chromatography, HPLC, grade) and injected into a C18 Dionex Acclaim column (150×4.6 mm) in an HPLC mass spectrometry (HPLC-MS) consisting of a system P680 pump, an ASI-100 autosampler, and a UVD170U UV detector coupled to a Thermo Finnigan Surveyor MSQ mass spectral detector. The samples were eluted with acetic acid (0.1%) in water (eluent A) and 0.1% acetic acid in methanol (eluent B) in a linear gradient from 10% of B (3 min) to 90% of B (90 min). Compounds were quantified by using Sigma standards for catechin and quercetin chromatographed under the same conditions. Leachates obtained from each germination day were also collected and extracted with ethyl acetate and analyzed by HPLC-MS as described above.

Phytotoxicity Bioassays Seeds of *A. thaliana* (L.) Heynh. ecotype Columbia (Col-0) purchased from Lehle Seed Co. (Texas, USA) were surface-sterilized with 20% commercial sodium hypochlorite (0.4% active chloride) for 2 min, washed ×4 in sterile distilled water, and further germinated on solid Murashige and Skoog (1962) basal medium in Petri dishes at 25±2°C and a 16:8 h L/D photoperiod. After 5 days, the *A. thaliana* seedlings were relocated to sterile 12-well plates (one plant per well) that contained 1 ml of liquid MS basal media. They were placed on an orbital shaker under the growth conditions described above to oxygenate the roots properly. Six replicates were used per treatment. After 1 day, catechin and other fractions obtained from *S. virgata* seed leachates and a commercial standard of (+)-catechin (Sigma) were applied to the liquid MS containing the *A. thaliana* seedlings. The substances were dissolved in methanol or ethanol, and 10 μl of the extracts containing different concentrations were applied to the wells. Controls were performed using only the solvents. After 7 days, the length and biomass of the plant root tissues were evaluated.

Seeds of inundated rice (*Oryza sativa* L.) cultivar IAC 106 (Instituto Agronômico de Campinas, SP, Brazil) were washed rapidly with 80% ethanol and then surface sterilized with 20% commercial sodium hypochlorite (0.4% active chloride) for 20 min. After that, seeds were washed three times with sterile distilled water then immersed in a 0.2% fungicide solution (Derosal Bayer) for 30 min and subsequently washed three times with sterile distilled water. Rice seeds were germinated in 250-ml flasks that contained solid MS basal medium at 25±2°C with 16:8 h L/D photoperiod. After 6 days, seedlings were transferred to tubes (2.3×15 cm) that contained 2 ml of liquid MS basal medium (one seedling per tube). The bioassay was performed as described for *A. thaliana.*

Statistical Analyses All statistical analyses were performed by using Winstat software (Microsoft, USA). Growth parameters were subjected to analysis of variance (ANOVA), and significant differences between means were determined by using *LSD* test at 1% probability.

Results

Fractions A–J obtained from the ethyl acetate extract of the seed leachates caused significant reduction in root length and root biomass in *A. thaliana* plants grown in MS media when compared to the controls (Fig. 1). The ^{1}H NMR spectrum from one of the largest fractions collected (H) and also from the G fraction was identical to that of the standard bioflavonoid catechin. When fraction H was rechromatographed, two of the 20 fractions were pure catechin (F2 and F3, containing 23 and 17 mg, respectively). ^{1}H NMR of these fractions confirmed that the isolate compound from *S. virgata* seed leachates was catechin, and the CD spectrum analyses revealed that the molecule was the enantiomer (+)-catechin (data not shown).

Different concentrations of (+)-catechin isolated from *S. virgata* seed leachates were tested against *A. thaliana* seedlings, and activity was compared with commercial (+)-catechin. Statistical analyses (*LSD*, ANOVA, P=0.01) showed that there were no differences between the treatments with catechin from Sigma and from *S. virgata* (Fig. 2). The effect of the concentrations of 50 and 100 μg ml^{-1} on root length was not different from the effect of methanol alone on control plants (Fig. 2a); however, differences were detected in root biomass of plants among these treatments (Fig. 2b). (+)-Catechin appeared to be phytotoxic to root tissues of *A. thaliana* at the concentration of 50 μg ml^{-1}. The concentrations of 200 and 500 μg ml^{-1} were toxic, causing a darkening of the plant roots, a total inhibition of lateral root formation (not shown), and drastic reduction in root biomass (Fig. 2).

Toxicity of (+)-catechin purified from *S. virgata* seed leachates was also observed on rice, and this level of toxicity was similar to that found with catechin purchased from Sigma. With both, toxicity was detected only in the response of root biomass (Fig. 3). This effect was statistically different from control plants when a concentration of 100 μg ml^{-1} of catechin or higher was used (Fig. 3b). Root damage, however, was observed even with the lowest concentration (50 μg ml^{-1}).

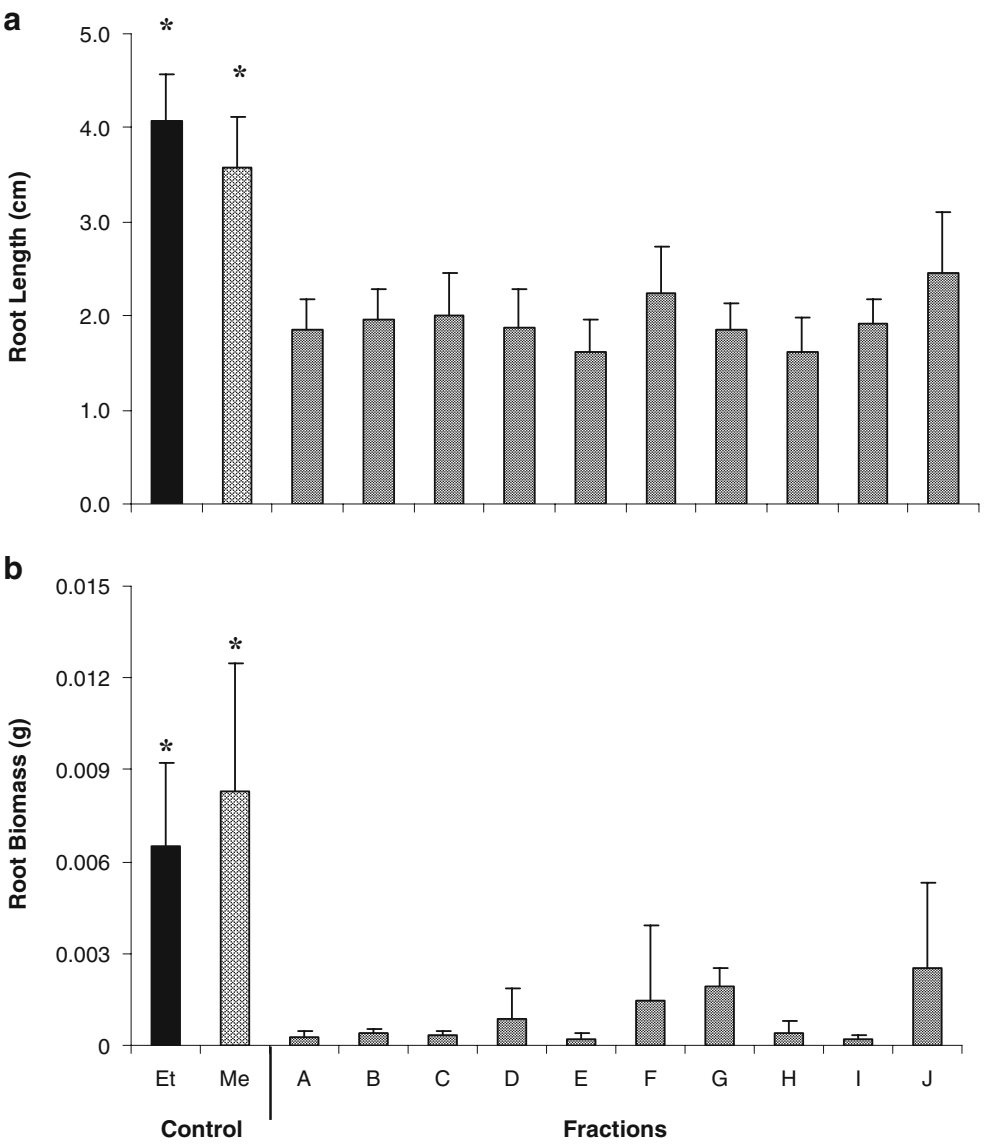

Fig. 1 Root length (**a**) and root biomass (**b**) of *A. thaliana* seedlings grown for 7 days in liquid MS media containing one of the fractions A–J collected from the *S. virgata* seed leachates' chromatography. Each fraction was administered at a concentration of 200 μg ml^{-1} of MS media. Control plants were treated with the same volume of ethanol (*Et*) used to solubilize fractions A–D or methanol (*Me*) used to solubilize fractions E–J. The *asterisk* indicates a significant difference between the means of the treatments and the controls determined by the test *LSD* (ANOVA, $P=0.01$). *Bars* represent the SD of the means ($N=6$)

The flavonoid quercetin was isolated from the seed extracts (25 mg), but it was not exuded by *S. virgata* seeds (Fig. 4). It was phytotoxic to *A. thaliana* (data not shown). Both (+)-catechin and quercetin are located in the seed coat tissues of *S. virgata*; these compounds were not detected by HPLC-MS analyses in the extracts of the embryo or endosperm tissues (data not shown).

During the germination of *S. virgata*, we found that (+)-catechin was rapidly released from the seed coat tissues on the first day of imbibition at levels up to 235 μg per seed (Fig. 4). After this first release, levels in the seed leachates decreased from the second day of imbibition. Based on the data of Fig. 4, the amount of (+)-catechin released from one seed was estimated as higher than 400 μg until the sixth day after the beginning of imbibition. The quantity of catechin and quercetin in the seed coat also decreased during germination, but the amount of (+)-catechin in the seed coat was considerably lower than that measured in the seed leachates (Fig. 4).

Discussion

High concentrations of constitutive phenolic compounds in the seed coat have been reported in *Sesbania* species. Some are rapidly released during imbibition and are found in the soil surrounding germinating seeds (Ceballos et al. 1998). We found that (+)-catechin was rapidly released from seed coat tissues of *S. virgata* on the first day of imbibition at much higher (approximately ten times) levels than those reported for other species of *Sesbania* (Ceballos et al. 1998) and *Lespedeza* (Buta and Lusby 1986).

Catechin has shown phytotoxic effects on a variety of plants, but the level of phytotoxicity varies depending

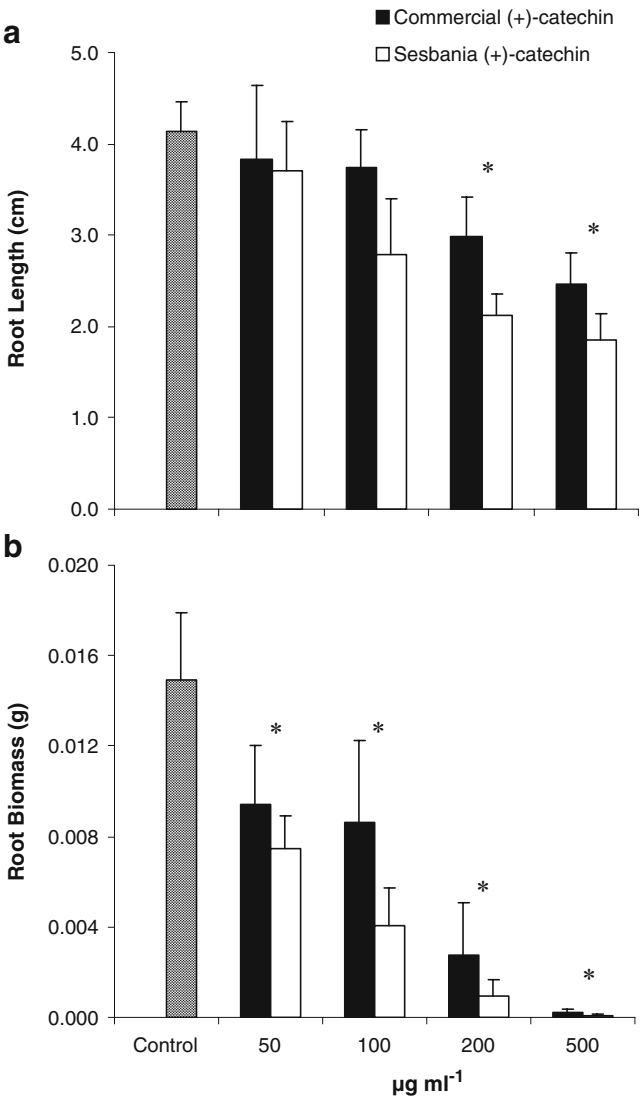

Fig. 2 Effect of different concentrations of (+)-catechin derived from *S. virgata* seed leachates and commercial (+)-catechin on the length (**a**) and root biomass (**b**) of *A. thaliana* seedlings after 7 days of treatment. Control plants were treated only with methanol. The *asterisk* indicates a significant difference between the means of the treatments and the control determined by the test *LSD* (ANOVA, P=0.01). *Bars* show the SD of the means (N=6)

on the species tested and the experimental conditions (Buta and Lusby 1986; Bais et al. 2002, 2003; Iqbal et al. 2003; Perry et al. 2005a; Thelen et al. 2005; D'Abrosca et al. 2006; Thorpe 2006; Weir et al. 2006; Furubayashi et al. 2007). In addition, (+)-catechin has antimicrobial activity and inhibits the growth of the pathogenic soil bacteria *Erwinia carotovora*, *E. amylovora*, *Xanthomonas campestris*, and *Pseudomonas fluorescens* (Veluri et al. 2004). (±)-Catechin also acts as an autoregulator of seed germination and seedling development in *Centaurea maculosa* and *Lespedeza* sp. (Buta and Lusby 1986; Perry et al. 2005b). Seed germination in *S. virgata* seems not to be affected by its coat leachates, but additional experiments are needed to definitively determine this.

The inhibitory effect of (+)-catechin isolated from seed leachates of *S. virgata* on *A. thaliana* growth was similar to that observed by using commercial catechin. Treated plants showed a reduction in root biomass (Fig. 2b) when compared to control plants. This was most likely due to the characteristic effect of the bioflavonoid catechin in inhibiting the development of lateral roots of seedlings of *A. thaliana*. The phytotoxicity of catechin on root cell tissues of *A. thaliana* has been previously described as caused by condensation of the cytoplasm generated by the rapid induction of reactive oxygen species, followed by a subsequent increase of $Ca^{2+}$and acidification of the cyto-

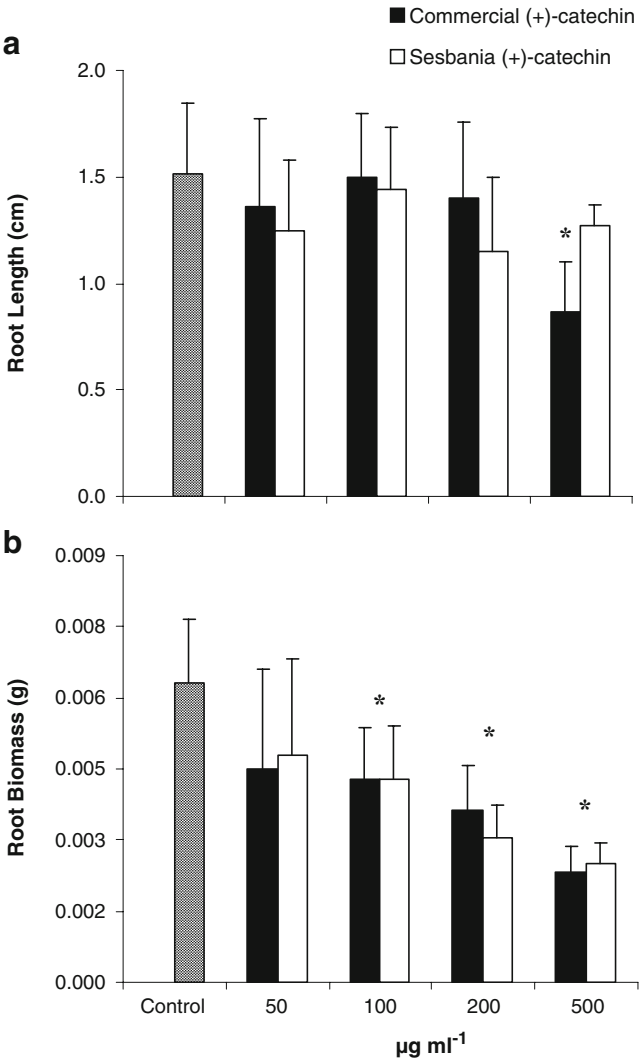

Fig. 3 Effect of different concentrations of (+)-catechin derived from *S. virgata* seed leachates and commercial (+)-catechin on root length (**a**) and root biomass (**b**) of rice seedlings after 7 days of treatment. Control plants were treated only with methanol. The *asterisk* indicates a significant difference between the means of the treatments and the control determined by the test *LSD* (ANOVA, P=0.01). *Bars* show the SD of the means (N=6)

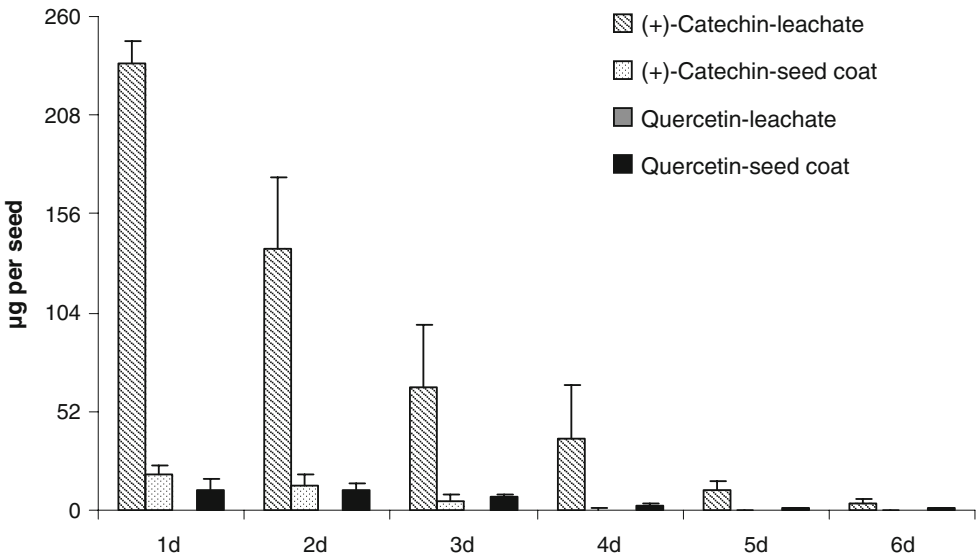

Fig. 4 Quantification of quercetin and catechin present in the seed coat and in the seed leachates of *S. virgata* during the beginning of imbibition (1 day—first day) to the ending of the germination process (6 days—sixth day). Analyses were performed by HPLC-MS, and compounds were quantified and detected by using a commercial standard. *Bars* represents the SD of the means (N=3)

plasm, which induces cell death (Bais et al. 2003). We also found phytotoxic effects of seed leachate catechin on rice seedlings (Fig. 3).

S. virgata is considered an invasive species in flood and damp soils, especially inundated rice plantations (Kissmann and Groth 1999). Therefore, the presence of this toxin in seed leachates may indicate an allelopathic strategy during germination and may contribute to the protection of *S. virgata* seeds against the attack of potential pathogens.

S. virgata is a pioneer species that produces many long-term viable seeds within indehiscent legume fruits. These fall on the ground and are further dispersed by water and/or wind, as reported for other *Sesbania* species (Pott and Pott 1994; Ceballos et al. 1998). The species thus forms a transitory seedbank, and its allelochemicals are released during seed imbibition. As described by Ceballos et al. (1998), the anatomical organization of *Sesbania* seeds is finely adapted to facilitate the rapid deployment of chemicals present in the seed coat, contributing to the rapid mobilization of these compounds toward the zone around the imbibed seed. Although these chemicals can be altered by reactions with other substances in the soil, microbial breakdown, and compound stability, the presence of active substances in the seed coat and their early release in high amounts at the beginning of imbibition suggest that they may play an ecological role. An early pulse of high catechin concentrations could contribute to enabling the species to rapidly and temporarily control the growth of nearby plants under specific environmental conditions. Early allelopathic effects may be advantageous to the competitive outcome; and resource depletion depends on the size of competing plants (Laterra and Bazzalo 1999). Moreover, antimicrobial activity of (+)-catechin could provide a hostile environment to potential microbial invaders.

Root-secreted (±)-catechin may contribute to the invasive behavior of the noxious weed *Centaurea maculosa*, which shows a devastating effect on species native to the Northeast of USA (Bais et al. 2002). Despite recent findings that indicate that the amounts of catechin found in soils around *C. maculosa* are lower than those previously reported to be phytotoxic (Blair et al. 2005, 2006), probably due to the low stability of the catechin in the soil, a short-term pulse of high amounts could be sufficient to confer some advantage at critical periods of development. This has been observed recently in the field for root exudates of *C. maculosa* (Perry et al. 2007). It is also important to note that the dynamics of root exudation is different from the release of substances from seed coats. As reported by Phillips et al. (2006), plants are able to recover part of their root-exuded compounds, sometimes with an influx rate that exceeds their efflux. *S. virgata* produces seeds with the ability to control water imbibition in early stages of germination, and as reported for other species of *Sesbania*, the anatomical structure of the seed coat plays a role in the establishment of an allelochemical-rich zone during imbibition (Ceballos et al. 1998). Additionally, seed coats remain in the environment, possibly increasing the amounts of soil catechin. The compound continues to be released up to 6 days after the beginning of imbibition as shown in Fig. 4.

The detection of other phytotoxic fractions derived from the *S. virgata* seed leachates that do not contain (+)-catechin in their molecular composition (Fig. 1) suggests that this seed can likely release other prospective phytotoxins that may relate to the invasive character and to adaptive mechanisms. Our data indicate that the seed coat of *S. virgata* contains the bioflavonoids (+)-catechin and quercetin, but that only (+)-catechin seems to operate as an allelochemical. Additional experiments are needed to

determine the presence of other active compounds and also the possibility of co-interactions that could potentiate phytotoxic effects.

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References

BAIS, H. P., WALKER, T. S., STERMITZ, F. R., HUFBAUER, R. A., and VIVANCO, J. M. 2002. Enantiomeric-dependent phytotoxic and antimicrobial activity of (+)-catechin. A rhizosecreted racemic mixture from spotted knapweed. *Plant Physiol.* 128:1173–1179.

BAIS, H. P., VEPACHEDU, R., GILROY, S., CALLAWAY, R. M., and VIVANCO, J. M. 2003. Allelopathy and exotic plant invasion: from molecules and genes to species interactions. *Science* 301:1377–1380.

BLAIR, A. C., HANSON, B. D., BRUNK, G. R., MARRS, R. A., WESTRA, P., NISSEN, S. J., and HUFBAUER, R. A. 2005. New techniques and finds in the study of a candidate allelochemical implicated in invasion success. *Ecol. Lett.* 8:1039–1047.

BLAIR, A. C., NISSEN, S. J., BRUNK, G. R., and HUFBAUER, R. A. 2006. A lack of evidence for an ecological role of the putative allelochemical (±)-catechin in spotted knapweed invasion success. *J. Chem. Ecol.* 32:2327–2331.

BUCKERIDGE, M. S., and DIETRICH, S. M. C. 1996. Mobilisation of the raffinose family oligosaccharides and galactomannan in germinating seeds of *Sesbania marginata* Benth. (Leguminosae-Faboideae). *Plant Sci.* 117:33–43.

BUTA, G. J. 1983. Linoleic acid as a plant growth inhibitor from seeds of *Sesbania punicea*. *J. Nat. Prod.* 46:775.

BUTA, J. G., and LUSBY, W. R. 1986. Catechins as germination and growth inhibitors in *Lespedeza* seeds. *Phytochemistry* 25: 93–95.

CEBALLOS, L., HOSSAERT-MCKEY, M., MCKEY, D., and ANDARY, C. 1998. Rapid deployment of allelochemicals in exudates of germinating seeds of *Sesbania* (Fabaceae): roles of seed anatomy and histolocalization of polyphenolic compounds in antipathogen defense of seedlings. *Chemoecology* 8:141–151.

D'ABROSCA, B., DELLAGRECA, M., FIORENTION, A., ISIDORI, M., MONACO, P., and PACIFICO, S. 2006. Chemical constituents of the aquatic plant *Schoenoplectus lacustris*: evaluation of phytotoxic effects on the green alga *Selenatrum capricornutum*. *J. Chem. Ecol.* 32:81–96.

FURUBAYASHI, A., HIRADATE, S., and FUJII, Y. 2007. Role of catechol structure in the adsorption and transformation reactions of L-Dopa in soils. *J. Chem. Ecol.* 33:239–250.

GORST-ALLMAN, C. P., STEYN, P. S., VLEGGAAR, R., and GROBBELAAR, N. 1984. Structure elucidation of sesbanimide using high-field NMR spectroscopy. *J Chem. Soc.* 1:1311–1314.

IQBAL, Z., HIRADATE, S., NODA, A., ISOJIMA, S., and FUJII, Y. 2003. Allelopathic activity of buckwheat: isolation and characterization of phenolics. *Weed Sci.* 51:657–662.

KISSMANN, K. G., and GROTH, D. 1999. Plantas Infestantes e nocivas. BASF, São Bernardo do Campo.

KORVER, O., and WILKINS, C. K. 1971. Circular dichroism spectra of flavonols. *Tetrahedron* 27:5459–5465.

LATERRA, P., and BAZZALO, M. E. 1999. Seed-to-seed allelopathic effects between two invaders of burned Pampa grasslands. *Weed Res.* 39:297–308.

MURASHIGE, T., and SKOOG, F. 1962. A revised medium for rapid growth and bioassay with tissue culture. *Physiol. Plant.* 15:473–497.

NDAKIDEMI, P. A., and DAKORA, F. D. 2003. Legume seed flavonoids and nitrogenous metabolites as signals and protectants in early seedling development. *Funct. Plant Biol.* 30:729–745.

NELSON, E. B. 2004. Microbial dynamics and interactions in the spermosphere. *Annu. Rev. Phytopathol.* 42:271–309.

PERRY, L. G., JOHNSON, C., ALFORD, E. R., VIVANCO, J. M., and PASCHKE, M. W. 2005a. Screening of grassland plants for restoration after spotted knapweed invasion. *Restor. Ecol.* 13:725–735.

PERRY, L. G., THELEN, G. C., RIDENOUR, W. M., WEIR, T. L., CALLAWAY, R. M., PASCHKE, M. W., and VIVANCO, J. M. 2005b. Dual role for an allelochemical: (±)-catechin from *Centaurea maculosa* root exudates regulates conspecific seedling establishment. *J. Ecol.* 93:1126–1135.

PERRY, L. G., THELEN, G. C., RIDENOUR, W. M., CALLAWAY, R. M., ASCHKE, M. W., and VIVANCO, J. M. 2007. Concentrations of the allelochemical (±)-catechin in *Centaurea maculosa* soils. *J. Chem. Ecol.* 33:2337–2344.

PHILLIPS, D. A., FOX, T. C., and SIX, J. 2006. Root exudation (net efflux of amino acids) may increase rhizodeposition under elevated CO_2. *Global Change Biol.* 12:561–567.

POTT, A., and POTT, V. J. 1994. Plantas do Pantanal. EMPRAPA/CPAP/SPI, Corumbá.

POWELL, R. G., PLATTNER, R. D., and SUFFNESS, M. 1990. Ocurrence of sesbanimide in seeds of toxic *Sesbania* species. *Weed Sci.* 38:148–152.

SINGH, S., LADHA, J. K., GUPTA, R. K., BHUSHAN, L., RAO, A. N., SIVAPRASAD, S., and SINGH, P. P. 2007. Evaluation of mulching, intercropping with *Sesbania* and herbicide use for weed management in dry-seeded rice (*Oryza sativa* L.). *Crop Protect* 26:518–524.

THELEN, G. C., VIVANCO, J. M., NEWINGHAM, B., GOOD, W., BAIS, H. P., LANDRES, P., CAESAR, A., and CALLAWAY, R. M. 2005. Insect herbivory stimulates allelopathic exudation by an invasive plant and the suppression of natives. *Ecol. Lett.* 8:209–217.

THORPE, A. 2006. Biochemical effects of *Centaurea maculosa* on soil nutrient cycles and plant communities. PhD dissertation, University of Montana, Missoula.

VAN STADEN, J., and GROBBELAAR, N. 1995. The effect of sesbanimide and *Sesbania* seed extracts on germination and seedling growth of a number of plant species. *Environ. Exp. Bot.* 35:321–329.

VELURI, R., WEIR, T. L., BAIS, H. P., STERMITZ, F. R., and VIVANCO, J. M. 2004. Phytotoxic and antimicrobial activities of catechin derivatives. *J. Agric. Food Chem.* 52:1077–1082.

WEIR, T. L., BAIS, H. P., STULL, V. J., CALLAWAY, R. M., THELEN, G. C., RIDENOUR, W. M., BHAMIDI, S., STERMITZ, F. R., and VIVANCO, J. M. 2006. Oxalate contributes to the resistance of *Gaillardia grand flora* and *Lupinus sericeus* to a phytotoxin produced by *Centaurea maculosa*. *Planta* 223:785–795.

XUAN, T. D., CHUNG, M. III, KHANH, T. D., and TAWATA, S. 2006. Identification of phytotoxic substances from early growth of barnyard grass (*Echinochloa crusgalli*) root exudates. *J. Chem. Ecol.* 32:895–906.

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Allelopathic Enhancement and Differential Gene Expression in Rice under Low Nitrogen Treatment

Biqing Song · Jun Xiong · Changxun Fang · Long Qiu · Riyu Lin · Yiyuan Liang · Wenxiong Lin

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Abstract The allelopathy-competition separation (ACS) based approach was used to explore the biointerference relationship between rice accessions and barnyardgrass exposed to different nitrogen (N) supplies in hydroponics. Rice accession PI312777 exhibited high allelopathic potential to suppress the growth of accompanying weeds, especially when the culture solution had low N content. The non-allelopathic rice Lemont showed an opposite result. Additionally, subtractive hybridization suppression (SSH) was used to construct a forward SSH-cDNA library of PI312777 to investigate gene expression profiles under low N treatment. A total of 35 positive clones from the SSH-cDNA library were sequenced and annotated. According to the function category, 24 genes were classified into five groups related to primary metabolism, phenolic allelochemical synthesis, plant growth/cell cycle regulation, stress response/signal transduction, and protein synthesis/degradation. Among them, two up-regulated genes that encode PAL and cytochrome P450 were selected. Their transcript abundance at low N level was compared further between the allelopathic rice and its counterpart by utilizing real-time quantitative polymerase chain reaction (qRT-PCR). The transcription levels of the two genes increased in both rice accessions when exposed to low N supply, but PI312777 at a higher magnitude than Lemont. At 1, 3, and 7 days of the treatments, the corresponding relative expression levels of *PAL* were 11.38, 4.83, and 3.57 fold higher in PI312777 root, but there were 1.15, 2.74, and 2.94 fold increases for Lemont, compared with the control plants fed with regular nutrient. The same trend was found for *cytochrome P450*. These findings suggest that the stronger ability of PI312777 to suppress target weeds, especially in low N nutrient conditions, might be attributed to the stronger activation of the genes that function in *de novo* synthesis of allelochemicals.

Keywords Rice allelopathy · Low nitrogen · Suppression subtractive hybridization (SSH) · Gene expression · Real time PCR

B. Song · J. Xiong · C. Fang · L. Qiu · R. Lin · Y. Liang · W. Lin
Key Laboratory of Biopesticide and Chemical Biology,
Fujian Agriculture and Forestry University,
C/O Ministry of Education,
Fuzhou, China

B. Song · J. Xiong · C. Fang · L. Qiu · R. Lin · Y. Liang · W. Lin (✉)
School of Life Sciences, FAFU,
Fuzhou 350002, China
e-mail: wenxiong181@163.com

Introduction

Rice monocultural practices along with the heavy use of chemicals that include herbicides are characteristic of modern agriculture and are inducing herbicide tolerant and/or resistant weed biotypes, such as barnyardgrass (*Echinochloa crus-galli* L.), a major pest plant in rice production. Allelopathy has attracted increasing attention as a feasible option for controlling field weeds with an increasing awareness of environmental protection and human health. The relevant studies on rice allelopathy began about 20 years ago in the United States. Since then, progress has occurred in understanding the genetics, chemistry, and physiology of allelopathy (Olofsdotter et al. 2002; Bi et al. 2007).

Previous studies showed that allelopathic potential in the suppression of target weeds could be enhanced in response

to external biotic and abiotic factors such as pest attack, weak solar radiation, higher temperature shock, and nutrient starvation (Einhellig 1999; Wu et al. 1999; Belz 2007). Kim et al. (2000) first reported that the allelopathic ability in rice (*Oryza sativa* L.) was induced by higher densities of accompanying weeds. Besides biotic factors, several abiotic factors have been proposed to regulate the expression of crop allelopathy. These include agricultural production input factors and environmental conditions (He et al. 2004; Xiong et al. 2005; Belz 2007). Liu et al. (2006) found that production of allelochemicals was stimulated in *Alexandrium tamarense* after exposure to N- and P-limited conditions. The target plant *Prorocentrum donghaiense* was killed by the donor plant *A. tamarense* after co-culture for 4 days under nutrient-limited conditions but survived under nutrient-sufficient conditions. Shen and Lin (2007) also observed the inducible phenomenon in rice allelopathy, i.e., stronger inhibition of the target weed *E. crus-galli*, as P nutrient level dropped to below the optimum in hydroponic mixtures of rice/weed. Although the associated mechanism is not clear, these studies suggest that stress factors may elicit allelochemical biosynthesis in competing crops, which is similar to plant defensive responses when attacked by insects or pathogens. Thus, the triggering of differential induction of biosynthetic pathways by using specific stress factors can be applied to elicit the production of allelopathic compounds. Furthermore, such an approach may assist in the design of strategies for practical application of crop allelopathy (An et al. 2003; Xu et al. 2004; Friebe et al. 2006; Belz 2007).

This study evaluated the effect of allelopathic function of different rice accessions by using the allelopathy-competition separation approach (ACS) (Xiong et al. 2005). Additionally, a combined analysis of subtractive hybridization suppression (SSH) and real-time quantitative polymerase chain reaction (qRT-PCR) was performed to investigate differential gene expression patterns in allelopathic and non-allelopathic rice accessions treated with low N nutrient conditions in rice/weed mixtures.

Methods and Materials

Plant Materials Rice (*O. sativa*) accession PI312777 (high allelopathic potential) and Lemont (low allelopathic potential) introduced from the USA were selected as donor plants. Barnyardgrass (*E. crus-galli* L.) was used as the receiver plant.

Experiment 1: Allelopathic Performance in Response to N Treatments Treatments followed Xiong et al. (2005) with minor modification. Seeds of the two rice accessions and barnyardgrass were germinated on seedling trays. When the seedlings reached the 3-leaf (rice) and 2-leaf (barnyardgrass) stages, respectively, forty uniform seedlings of each were selected, transplanted into a Styrofoam plate (holes spaced at 5×6 cm^2), and the seedlings stabilized with a cotton plug inserted into each. The Styrofoam plate was floated on a pot ($45\times35\times15$ cm^3) filled with 10l Hoagland nutrient solution (normal nutrient condition) (Fajer et al. 1992; Belz and Hurle 2004; Kim et al. 2005; Xiong et al. 2005). PI312777 and Lemont seedlings were grown under the same condition. After 7 d of recovery in the Hoagland nutrient solution, 30 rice seedlings and 10 weed seedlings were transplanted into a Styrofoam plate to form a hydroponic rice-weed mixture with the barnyardgrass seedlings in the center surrounded by the rice seedlings. This setting can induce rice allelopathy (Belz and Hurle 2004; Kim et al. 2005; Xiong et al. 2005). Three N treatments in Hoagland's solution were prepared and consisted of normal N (20 mg•l^{-1}, denoted as 1N), half normal N (10 mg•l^{-1}, 1/2N), and quarter normal N (5 mg•l^{-1}, 1/4N). pH values of the treatment solutions were all maintained at 5.5 throughout the experiment by using 1M NaOH or 0.5M H_2SO_4 (Kim et al. 2005; Shen and Lin 2007). A monoculture of barnyardgrass under the same condition was used as control (ck) for each treatment.

Barnyardgrass, the target plant, including control plants, were sampled 7 d after the start of co-culture. Harvested tissues were snap-killed in an oven at 105°C for 20 min, and then dried at 70°C until constant weight. Dry weight (DW) was used to determine inhibition rates (IR) above controls and to evaluate the biointerference (BI) that consisted of the allelopathic effect (AE) and resource competition (RC) (Olofsdotter 1998; Xiong et al. 2005). IR was calculated as: IR = (ck - treatment)/ck × 100%, which indicates inhibition when IR> 0 and promotion when IR< 0.

After barnyardgrass seedlings were harvested, the used nutrient solutions from the three treatments were collected and their nitrogen (N), phosphorus (P), and potassium (K) concentrations were all reconstituted to full Hoagland levels. These treated solutions, denoted as A, B, and C corresponding to the treatments 1/4 N, 1/2 N, and 1N, respectively, were then used for hydroponic barnyardgrass monoculture to determine whether its growth response was mediated by the root exudate residuals from the two rice accessions. Nine barnyardgrass seedlings at the 3-leaf stage were transplanted into each pot. Seedlings grown in fresh Hoagland solution were used as control (ck). The pH of the culture solution and residual levels of N, P, and K were measured and balanced weekly. Barnyardgrass in all treatments was sampled on d 14. The DW of barnyardgrass from each treatment was recorded. IR differences were used to evaluate allelopathic effects of the two rice accessions on barnyardgrass. BI was separated into AE and RC using the equation: BI = AE + RC.

Table 1 Comparison of biointerference (BI) of barnyardgrass by two rice accessions under different nitrogen treatments

Hydroponic N level (mg•l^{-1})	BI of barnyardgrass by PI312777 (%)	BI of barnyardgrass by Lemont (%)
5	72.65±6.45Aa	67.12±4.76Aa
10	73.70±4.02Aa	19.15±0.51Bb
20	64.86±3.91Aa	12.66±0.14Bb

BI = (1-(DW of target weed in the mixture)/ DW of the target weed in monoculture) × 100%. Values in the table are means ± SD of three replicates. The small letters represent significant differences at the 5% level; capital letters represent significant differences at 1% level. The same convention was used for all the tables unless stated otherwise.

Experiments were conducted in triplicate in a greenhouse of the Agroecological Institute, Fujian Agriculture and Forestry University, Fuzhou, China between 2004 and 2006. All data were subjected to statistical analysis using Least Significant Difference Test.

Experiment 2 - Differential Expression of Allelopathy-Associated Genes To detect the differential expression of genes associated with biointerference in the rice/weed interaction, a combination of SSH and qRT-PCR was used. Seedling preparation and subsequent treatment conditions were the same as the above mixtures, except that culture solutions had two N levels – 5 mg•l^{-1}N (the lower N treatment; 1/4 strength Hoagland, denoted as 1/4 N) and 20 mg•l^{-1} N (normal N; 1N as full strength Hoagland). Roots of the two rice accessions were sampled on d 1, 3, and 7 after N treatment, respectively. Roots were frozen in liquid N_2, and stored at –80°C until analysis.

Construction of Subtractive Library SSH was used to construct a subtractive library (Diatchenko et al. 1996). Total RNA was extracted with Trizol Reagent (Invitrogen), and genomic DNA was removed with *DNaseI* (TaKaRa). Tester and driver RNA samples were prepared by pooling equal amounts of RNA from rice roots grown in 1/4N and 1N solutions. cDNA was synthesized according to the method of SMART PCR cDNA Synthesis. LD-PCR products were purified and digested with 15 unit *RsaI* at 37°C for 4 hr. SSH was carried out by using the PCR-Select cDNA Subtraction Kit (Clontech) based on the manufacturer's instructions. After two hybridizations, a nested PCR was performed to selectively amplify the differentially expressed fragments. Products of PCR were purified by E.Z.N.A. Cycle-Pure Kit (OMEGA), ligated into pMD18-T Vector (TaKaRa), and then transformed into *E. Coli* DH5α cells by $CaCl_2$. The bacteria were plated onto ampicillin-containing Luria-Bertani (LB) agar plates, which were overlaid with X-Gal and isopropyl-beta-D-thiogalactopyranoside (IPTG). After incubation (overnight at 37°C), white colonies (putative positive clones) were picked and cultured in 1.5 ml microfuge tubes with 500 μl LB liquid medium (AMP^+).

Screening and Identification of Positive Clones Rice clones were amplified by PCR by using primer 1 and primer 2R in the Clontech PCR-select cDNA subtraction Kit. PCR products were blotted onto Hybond-N + nylon membranes (Amersham), which were dried for 2 hr at 80°C under vacuum. Tester cDNA and driver cDNA were used as forward and reverse probes, respectively; both were hybridized with the selected clones. Colonies that showed strong hybridization signal with the forward SSH probe, but not with the reverse probe were positive clones. Reverse northern blots were performed according to the manufacturer's instructions of DIG-High Prime DNA Labeling and Detection Starter Kit® (Roche). The hybridization membranes were scanned by ArtixScan 1010 (Microtek).

Sequence Analysis Thirty-five clones were selected and sequenced at Shanghai Sangon Biological Engineering Technology and Service Co., Ltd. The identity of these genes was searched by blastn and blastx in the National Center for Biotechnology Information, USA (NCBI) database.

Confirmation of Up-Regulated Gene Expression qRT-PCR was used to validate the results from SSH analysis. First strand cDNA was synthesized from the same DNA-free-total RNA extracts used for SSH with ExScript RT reagent Kit (TaKaRa). After dilution (1:10, v/v), cDNA samples were amplified with gene-specific primers (5′-CTCGCCGTTCCA CTCCTTG-3′ and 5′-GCTCGGCTGCGTATTCCT-3′ for *PAL*; 5′-TGCTGTATCATGGGAAACTAAA-3′ and 5′-AGT CATAGATAGCCAAGAGGGT-3′ for *cytochrome P450*). RT-PCR was performed according to the method of SYBR Green Iwith the *SYBR Premix Ex* Taq (TaKaRa). *Actin* was

Table 2 Effects of the used solutions for culturing two rice accessions (PI312777 and Lemont) on the inhibition rate (IR) of barnyardgrass in monoculture

Treatment solution	IR of barnyardgrass over control mediated by PI312777 (%)	IR of barnyardgrass over control mediated by Lemont (%)
A	54.16±2.97Aa	3.65±0.66Aa
B	44.58±3.16Aab	13.81±7.58Aa
C	38.54±0.00Ab	9.45±0.06Ba

Treatment solution A, B, C in this monoculture refer to the used solutions collected from the mixture trial and were all reconstituted to the same N, P, and K levels as full Hoagland solution, but putatively contained different levels of allelochemicals released from allelopathic rice accession and its counterpart in the mixture under different nitrogen treatments (5, 10, and 20 mg•l^{-1} denoted as 1/4 N, 1/2 N, and 1N) as described in experiment 1

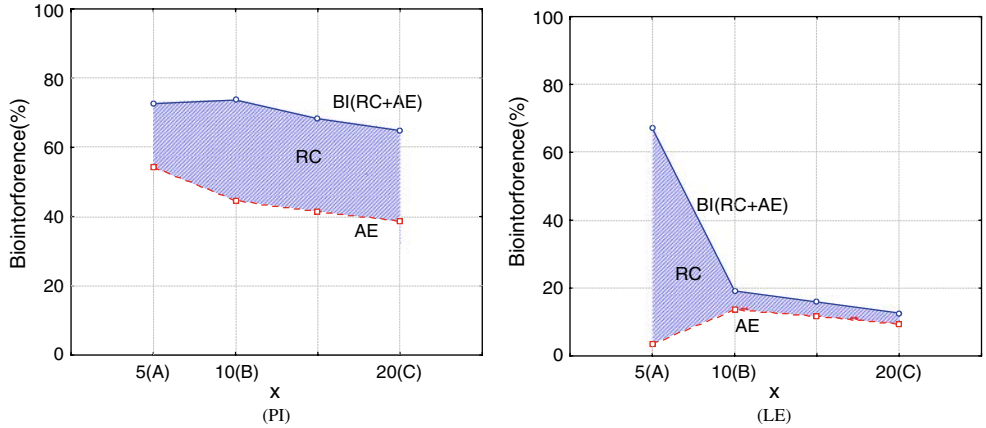

Fig. 1 The biointerference of allelopathic rice PI312777 (*PI*) and its counterpart Lemont (*LE*) with the accompanying barnyardgrass exposed to different nitrogen levels. Note: biointerference (*BI*) = allelopathic effect (*AE*) + resource competition (*RC*). *A*, *B*, *C* in this monoculture refer to the used solutions collected from the mixture trial and were all reconstituted to the same N, P, and K levels as full Hoagland solution, but putatively contained different levels of allelochemicals released from allelopathic rice accession and its counterpart in the mixture under different nitrogen treatments (5, 10, and 20 mg•l^{-1} denoted as 1/4 N, 1/2 N, and 1N) as described in experiment 1

used as the reference gene detected with primers 5′-TGTAAGCAACTGGGATGA-3′ and 5′-CCTTCGTAGATTGGGACT-3′. The amplification procedure was: an initial denaturation at 95°C for 10 sec followed by 41 cycles of 95°C for 5 sec and 60°C for 20 sec. The specificity of the amplification was verified by the melting curve at the end of the PCR cycle. Fluorescence was read at every temperature increment of 0.2°C with a hold time of 2 sec. The relative quantification (ratio) of a target gene was calculated with the following formula: ratio = $2^{-\Delta\Delta Ct}$ (Pfaffl 2001) and was analyzed using software SPSS 11.5.

Results

Allelopathy and Resource Competition Biointerference (BI) of allelopathic rice PI312777 was much greater with barnyardgrass under the normal N hydroponic solution compared with the non-allelopathic rice Lemont (Table 1). As N decreased, biointerference of barnyardgrass by both rice accessions appeared to increase, but the increase by PI312777 was not statistically significant. In contrast, the biointerference of barnyardgrass by Lemont was dramatic (Table 1). When N levels were reduced to 5 mg•l^{-1} (i.e., the low N level), the BI of neither rice accession was significant). Barnyardgrass in the different treated solutions in subsequent monoculture experiment were all inhibited to some extent compared with control plants growing in fresh nutrient solutions (Table 2). Treated solutions derived from PI312777 strongly suppressed target plants and accounted for ca. 60-71% of the total biointerference; the allelopathic effect increased from C to A treatment (Table 2). This suggests that the allelopathic effect of PI312777 could be enhanced under limited N conditions. Treated solutions derived from Lemont only slightly inhibited target plants (Table 2). Instead, Lemont showed high resource competition under N-limited conditions,

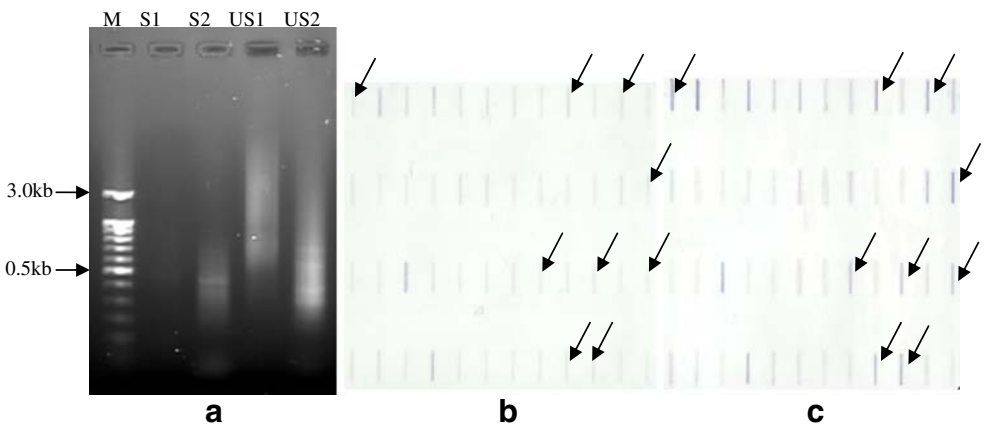

Fig. 2 The results of SSH (**a**) and the screening of positive clones by reverse northern blot (**b** and **c**). **a** *M*, marker; *S1*, first PCR product of subtracted tester cDNA; US1, first PCR product of unsubtracted tester cDNA; S2, second PCR product of subtracted tester cDNA; *US2*, second PCR product of unsubtracted tester cDNA. **b** Reverse hybridization. **c** Forward hybridization. *Arrows* pointed out positive clones

explaining approx. 95% of total biointerference (Table 1, Table 2, and Fig. 1).

Induced Gene Expression in Allelopathic Rice The forward subtracted cDNA library was constructed by SSH to detect the molecular response of PI312777 to low N (1/4 N) treatment (see Fig. 2a). A total of 994 clones were obtained from the forward subtractive library; 188 of them were selected for reverse northern blots after PCR (see Fig. 2b and c). Gene expression in 35 PI312777 clones were positive for up-regulation. These clones were sequenced, and the cDNA sequences were searched against standard databases (www.ncbi.nlm.nih.gov). According to nucleic acid homologies and encoded protein sequences, 32 out of the 35 clones were successfully assigned. The putative functions of the assigned expressed sequence tags (ESTs) are given in Table 3. Clones 312, 403, and 891 encode for the same putative triosephosphate isomerase, and clone 323 showed the highest homology to putative glycine hydroxymethyltransferase from rice (*O. sativa*), all of which are involved in plant primary metabolism. Clones 278, 547, and 663 had homology to phenylalanine ammonia-lyase

Table 3 Proposed identities of genes up-regulated in allelopathic rice accession PI312777

Clone	Length (bp)	Accession Number	Best Homologue in Database	Score	ID%/E Value	Source
Primary metabolism						
312	619	ABR25842	Putative triosephosphate isomerase	576	99/0	*O.sativa*
323	386	NM_001057746	Putative glycine hydroxymethyltransferase	341	99/0	*O. sativa*
403	705	ABR25842	Putative triosephosphate isomerase	580	99/0	*O. sativa*
891	593	NM_001048551	Putative triosephosphate isomerase	535	100/0	*O. sativa*
Phenolic synthesis						
278	498	ABR25322	Phenylalanine ammonia-lyase	460	99/0	*O. sativa*
547	441	NM_001071389	Putative o-methyltransferase	404	99/0	*O. sativa*
663	542	BAE45261	Cytochrome P450	80	72/1.2	*P. xylostella*
Plant growth/cell cycle regulation						
34	504	AAM08829	Putative SCARECROW gene regulator-like	113	99/2e-55	*O. sativa*
84	391	NM_001051791	No apical meristem (NAM) protein	231	100/7e-126	*O. sativa*
257	544	ABA98006	Subitilisin-chymotrypsin inhibitor	192	97/2e-102	*O. sativa*
642	572	NM_001059257	DNA binding protein S1FA family protein	359	98/0	*O. sativa*
935	551	NM_001060636	S1/P1 Nuclease	120	86/1e-59	*O. sativa*
489	552	BAD81918	BolA-like protein	285	100/6e-158	*O.sativa*
272	290	NM_001065974	putative CLB1 protein (calcium-dependent lipid binding)	220	98/2e-119	*O.sativa*
Stress resistance/Signal transduction						
803	384	NM_001070187	Putative protein kinase interactor 1	240	99/3e-131	*O.sativa*
943	623	A55092	Catalase 2 (CAT-2)	182	97/3e-12	*Zea mays*
243	621	NM_001052425	Myosin-like protein	328	100/0	*O.sativa*
201	232	AAB70546	Metallothionein-like protein type 1	150	98/8e-78	*O. sativa*
120	230	AAB70546	Metallothionein-like protein type 1	157	99/6e-82	*O. sativa*
715	618	NM_001075076	Metallothionein-like protein type 1	287	95/7e-147	*O. sativa*
917	522	NM_001057541	Expressed protein	465	98/0	*O. sativa*
Protein synthesis/degradation						
177	466	ABR25449	Putative 40S ribosomal protein S13	458	99/0	*O.sativa*
188	415	NM-001056613	60S ribosomal protein L22–2, putative	327	99/0	*O.sativa*
341	459	NM-001056613	60S ribosomal protein L22–2, putative	404	100/0	*O.sativa*
486	736	ABA94602	40S ribosomal protein S9, putative	404	98/0	*O. sativa*
399	383	A2WXX3	putative 60S ribosomal protein L5	265	94/3e-146	*O. sativa*
349	507	BAD36074	Putative chaperonin 10	254	99/2e-139	*O. sativa*
640	372	NM-001056613	60S ribosomal protein L22–2, putative	276	98/9e-153	*O. sativa*
657	410	ABR25449	Putative 40S ribosomal protein S13	154	100/4e-81	*O.sativa*
622	634	NM_001062753	Putative apoptosis-related protein	532	98/0	*O.sativa*
743	406	NM_001056776	HSP20-like chaperone	341	99/0	*O. sativa*
Function unknown						
308	486	NM_001070166	Function unknown	252	100/2e-138	*O.sativa*
645	391		No significant similarity found			
700	395		No significant similarity found			
298	329		No significant similarity found			

(PAL), putative o-methyltransferase from *O. sativa* and cytochrome P450 from *Plutella xylostella,* respectively. These enzymes were associated with *de novo* synthesis of phenolic-based allelochemicals. Clones 34, 84, 257, 642, 935, 489, and 272 showed the highest homologies to putative SCARECROW gene regulator-like, no apical meristem (NAM) protein, subitilisin-chymotrypsin inhibitor, DNA binding protein S1FA family protein, S1/P1 nuclease, BolA-like protein, and putative CLB1 protein (calcium-dependent lipid binding), respectively from *O. sativa,* which participate in plant growth and cell cycle regulation. Clones 803, 943, and 243 had the greatest homologies to putative protein kinase interactor 1, catalase 2 (CAT-2), and myosin-like protein. In contrast, clones 201, 120, 715, and 917 had homologies to the same metallothionein-like protein type 1, and an expressed protein from *O. sativa* that plays a critical role in stress resistance and signal transduction. Clones 188, 341, 640, 177, and 657 encode for the same putative 60S ribosomal protein L22–2 and 40S ribosomal protein S13, respectively, but clones 486, 399, 349, 622, and 743 showed the highest homologies to putative 40S ribosomal protein S9, putative 60S ribosomal protein L5, putative chaperonin 10, putative apoptosis-related protein, and HSP20-like chaperone, respectively, from *O. sativa*. Those enzymes function in protein synthesis and degradation. The four assigned sequences encoded for by clones 298, 308, 645 and 700 have no known function (Table 3).

Differential Expression of Two Genes by qRT-PCR Gene expression of both *PAL* and *P450*, which are involved in *de novo* allelochemical synthesis, increased in PI312777 and Lemont when exposed to nutrient conditions with limited N availability. The relative expression levels of the two genes in PI312777, however, were higher than those in Lemont, showing that the relative transcript abundance of *PAL* in root of PI312777 increased by 11.38, 4.83, and 3.57 times, while in Lemont they increased by 1.15, 2.74 and 2.94 times on day 1, 3, and 7 after treatment with low N, respectively. The same was true in the expression of *P450* gene for the two rice accessions as shown in Fig. 3. This finding further confirms the findings of SSH analysis (Table 3).

Discussion

The present study shows that rice PI312777 has higher allelopathic potential in the suppression of target weed growth under all N treatments, accounting for 59.57-78.83% of the total biointerference. The reverse was true for rice Lemont that had a lower allelopathic effect (4–14% in terms of barnyardgrass inhibition) in all N treatments, but dramatically increased resource competition (by up to 64% when N was reduced to 5 $mg \bullet l^{-1}$) and explained nearly 95% of the total biointerference (Fig. 1). It is suggested that the allelopathic potential of PI312777 is enhanced under N-limited conditions. The change in allelopathic potential seems to result from differential gene expression mediated by different N conditions, since N deprivation and other stress factors can induce gene expression associated with secondary metabolism in many crops (Sheveleva et al. 1997; Scheible et al. 2004). Up-regulation of the putative genes that encode for PAL, O-methyltransferase, triosephosphate isomerase, and cytochrome P450, which are involved in *de novo* synthesis of phenolic allelochemicals and detoxification of toxic substances, was detected in PI312777 by SSH at low N. PAL is the first key enzyme in phenylpropanoid metabolism that can be regulated by various biotic and abiotic factors to different extents depending on different plant species (Dixon and Paiva 1995; Dixon et al. 2002). P450 is directly involved in the formation of *p*-coumaric acid (Anterola et al. 2002), an intermediate for methylated polyphenolic synthesis, and detoxification of plant autotoxic substances (Li et al. 1997). Triosephosphate isomerase is vital in the glycolytic pathway, and provides substrate for phenylpropanoid metabolism (Xu and Hall 1993). It is suggested that allelopathic potential can be enhanced under N-limited conditions,

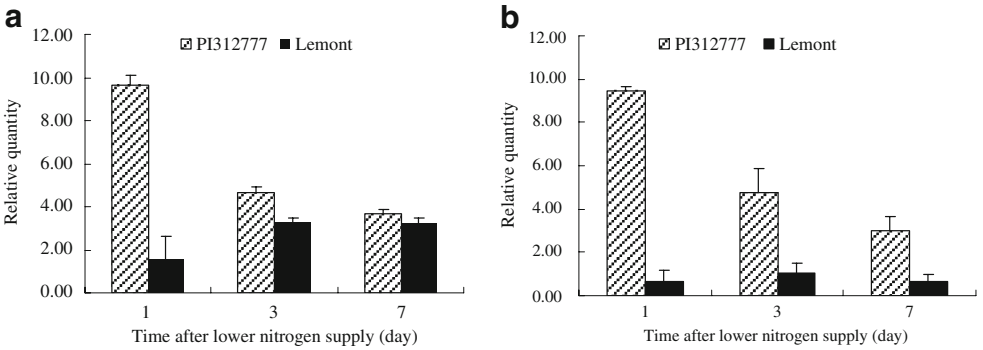

Fig. 3 Relative expression levels of the genes encoding PAL (**a**) and Cytochrome P450 (**b**) in allelopathic rice accession PI312777and its counterpart Lemont under low nitrogen treatment by using qRT-PCR analysis

which may result in the activation of genes that encode for PAL and P450. In turn, this may lead to increased phenolic allelochemicals that can suppress the growth of accompanying weeds under limited N conditions in hydroponic solution.

Phenolic allelochemicals are derived mainly from L-phenylalanine via *t*-cinnamate (Razal et al. 1996; Scheible et al. 2004). Stimulation of phenylpropanoid metabolism can be triggered by changes in N levels and mediated by the induction of enzymes in the early steps of phenylpropanoid biosynthesis (Chishake and Horiguchi 1997; Fritz et al. 2006; Kovacik et al. 2007). Under decreased N availability, phenolic content and PAL activity in plants may increase because of decreased demand for proteins required for growth. In this process, ammonium ions released by PAL can be assimilated to increase N cycling, for example, via the GS/GOGAT system (Razal et al. 1996). The resulting N-free carbon skeletons of *t*-cinnamate can be shunted into phenylpropanoid metabolism (Bryant et al. 1987; Reichardt et al. 1991). This hypothesis seems to explain why the allelopathic potential increases in response to low N stress by phenolic compounds that have low turnover rates, but not by terpenoids that have higher turnover rates (Reichardt et al. 1991; Mihaliak et al. 1991).

In addition, the present work shows that molecular changes in plant growth and the cell cycle are also involved in the enhancement of rice allelopathic potential under limited N conditions. For example, the putative SCARE CROW (SCR) gene regulator-like is involved in cell division (Wysocka-Diller et al. 2000). Subitilisin-chymotrypsin inhibitor in rice is one of bifunctional α-amylase/subtilisin inhibitors, which functions in the regulation of seed development and root growth of rice seedlings (Yamagata et al. 1998). The genes that encode for putative CLB1 protein and BolA-like protein appear to be involved in the modulation of the cell cycle (Luo et al. 1994; Kasai et al. 2004). The up-regulation of these genes detected by SSH in the present study suggests that root growth of allelopathic rice was facilitated in low N conditions, which, in turn, led to the increased root/shoot ratio as auto adaptation to the limiting nutrient conditions (data not shown here).

The present study also indicates that other genes were upregulated in PI312777 when exposed to low N. For instance, the genes that encode for putative protein kinase interactor 1, metallothionein-like protein type 1, catalase 2 (CAT-2), ribosomal proteins, and S1/P1 nuclease were detected simultaneously in PI312777 (Table 3). These genes are involved in signal transduction, stress resistance and detoxification, protein synthesis, and programmed cell death-associated endonuclease (Elias et al. 2002; Bauwe and Kolukisaoglu 2003). Therefore, the transcriptional activation of various biosynthetic pathway genes can be an important step for regulating the accumulation of phenylpropanoids during a plant's response to nutrient deficiency. Extensive molecular work is still needed to understand the network of signal transduction and the associated control for increasing chemical defense and growth adaptation to the limiting nutrient conditions in rice/weed mixtures (Dixon and Paiva 1995; Dixon et al. 2002).

In summary, rice allelopathy has been confirmed as an inducible genetic trait (Bi et al. 2007) that is associated with molecular regulation of secondary metabolic pathways. It may be feasible to trigger allelopathic potential through alteration of agricultural practice or manipulation of environmental conditions. Further study needs to be directed to this end to elucidate the molecular genetics and enzymology of associated biosynthetic pathways, and to establish a series of specific management strategies that can be applied to crop production to avoid down-regulation of the relevant genes associated with allelopathic activity (Belz 2007; Macias et al. 2007).

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References

AN, M., LIU, D. L., JOHNSON, I. R., and LOVETT, J. V. 2003. Mathematical modelling of allelopathy: II. The dynamics of allelochemicals from living plants in the environment. *Ecol. Model* 161:53–66.

ANTEROLA, A. M., JEON, J. H., DAVIN, L. B., and LEWIS, N. G. 2002. Transcriptional control of monolignol biosynthesis in Pinus taeda: factors affecting monolignol ratios and carbon allocation in phenylpropanoid metabolism. *J. Biol. Chem* 277:18272–18280.

BAUWE, H., and KOLUKISAOGLU, U. 2003. Genetic manipulation of glycine decarboxylation. *J. Exp. Bot* 54:1523–1535.

BELZ, R. G. 2007. Allelopathy in crop/weed interactions – an update. *Pest Manag. Sci* 63:308–326.

BELZ, R. G., and HURLE, K. 2004. A novel laboratory screening bioassay for crop seedling allelopathy. *J. Chem. Ecol* 30:175–198.

BI, H. H., ZENG, R. S., SU, L. M., AN, M., and LUO, S. M. 2007. Rice allelopathy induced by methyl jasmonate and methyl salicylate. *J. Chem. Ecol* 35:1089–1103.

BRYANT, J. P., CHAPIN, F. S. III, REICHARDT, P. B., and CLAUSEN, T. P. 1987. Response of winter chemical defense in Alaska paper birch and green alder to manipulation of plant carbon/nutrient balance. *Oecologia* 72:510–514.

CHISHAKE, N., and HORIGUCHI, T. 1997. Responses of secondary metabolism in plants to nutrient defiency. *Soil Plant Nutr* 43:987–991.

DIATCHENKO, L., LAU, Y. F. C., CAMPBELL, A. P., CHENCHIK, A., MOQADAM, F., HUANG, B., LUKYANOV, S., LUKYANOV, K., GURSKAYA, N., SVERSLOV, E. D., and SIEBERT, P. D. 1996. Suppression subtractive hybridization: A method for generating

differentially regulated or tissue-specific cDNA probes and libraries. *Proc. Natl. Acad. Sci. USA* 93:6025–6030.

DIXON, R. A., and PAIVA, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7:1085–1097.

DIXON, R. A., ACHNINE, L., KOTA, P., LIU, C. J., REDDY, M. S. S., and WANG, L. J. 2002. The phenylpropanoid pathway and plant defense-a genomics perspective. *Mol. Plant Pathol* 3:371–390.

EINHELLIG, F. A. 1999. An integrated view of allelochemicals amid multiple stress, pp. 479–494, in Ingerjit, K. M. M. Dakshini, and C. L. Foy (eds.). Principles and practices of plant ecology: Allelochemical InteractionsCRC Press LLC, Boca Raton, FL.

ELIAS, M., POTOCK, M., CVROKOVA, F., and ZARSKY, V. 2002. Molecular diversity of phospholipase D in angiosperms. *BMC Genomics* 3:1471–2164.

FAJER, E. D., BOWERS, M. D., and BAZZAZ, F. A. 1992. The effect of nutrients and enriched CO_2 environments on production of carbon-based allelochemicals in plantago: A test of the carbon/nutrient balance. *Am. Nat* 140:702–723.

FRIEBE, A. 2006. Brassinosteroids in induced resistance and induction of tolerance to abiotic stress in plants, pp. 233–242, in A. M. Rimando, and S. O. Duke (eds.). Natural Products for Pest ManagementAmerican Chemical Society, Washington, DCACS Symposium Series No. 927.

FRITZ, C., PALACIOS-ROJAS, N., FEIL, R., and STITT, M. 2006. Regulation of secondary metabolism by the carbon–nitrogen status in tobacco: nitrate inhibits large sectors of phenylpropanoid metabolism. *Plant J* 46:533–548.

HE, H. Q., SHEN, L. H., XIONG, J., JIA, X. L., LIN, W. X., and WU, H. 2004. Conditional genetic effect of Allelopathy in rice (Oryza sativa L.) under different environmental conditions. *Plant Growth Regul* 44:211–218.

KASAI, T., INOUE, M., KOSHIBA, S., YABUKI, T., AOKI, M., NUNOKAWA, E., SEKI, E., MATSUDA, T., MATSUDA, N., TOMO, Y., SHIROUZU, M., TERADA, T., OBAYASHI, N., HAMANA, H., SHINYA, N., TATSUGUCHI, A., YASUDA, S., YOSHIDA, M., HIROTA, H., MATSUO, Y., TANI, K., SUZUKI, H., ARAKAWA, T., CARNINCI, P., KAWAI, J., HAYASHIZAKI, Y., KIGAWA, T., and YOKOYAMA, S. 2004. Solution structure of a BolA-like protein from Mus musculus. *Protein Sci* 13:545–548.

KIM, K. U., SHIN, D. H., LEE, I. J., and KIM, H. Y. 2000. Rice Allelopathy in Korea, pp. 57–82, in K.U. Kim, and D.H. Shin (eds.). Rice AllelopathyChan-Suk Park Publish, Korea.

KIM, S. Y., MADRID, A. V., PARK, S. T., YANG, S. J., and OLOFSDOTTER, M. 2005. Evaluation of rice allelopathy in hydroponics. *Weed Res* 45:74–79.

KOVACIK, J., KLEJDUS, B., BACKOR, M., and REPCA, M. 2007. Phenylalanine ammonia-lyase activity and phenolic compounds accumulation in nitrogen-deficient Matricaria chamomilla leaf rosettes. *Plant Sci* 172:393–399.

LI, L., POPKO, J. L., ZHANG, X. H., OSAKABE, K., TSAI, C. J., JOSHI, C. P., and CHIANG, V. 1997. A novel mutifuctional *O-methyltransferase* implicated in a dual methylation pathway associated with lignin biosynthesis in loblolly pine. *Proc. Natl. Acad. Sci. USA* 94:5461–5466.

LIU, J. S., XIE, J., YANG, W. D., and LI, L. X. 2006. Allelopathic effect of alexandrium tamarense on prorocentrum donghaiense under limited nutrient conditions. *J. Trop. Subt. Bota* 14:207–212 (in Chinese).

LUO, Q., MICHAELIS, C., and WEEKS, G. 1994. Overexpression of a truncated cyclin B gene arrests Dictyostelium cell division during mitosis. *J. Cell Sci* 107:3105–3114.

MACIAS, F. A., MOLINILLO, J. M. G., VARELA, R. M., and GALINDO, J. C. G. 2007. Allelopathy - a natural alternative for weed control. *Pest Manag. Sci* 63:327–348.

MIHALIAK, C. A., GERSHENZON, J., and CROTEAU, R. 1991. Lack of rapid monoterpene turnover in rooted plants: implication of theories of plant defense. *Oecologia* 87:373–376.

OLOFSDOTTER, M. 1998. Allelopathy in rice, pp. 1–5, in M. Olofsdotter (ed.). Allelopathy in RiceInternational Rice Research Institute, Manila, Philippines.

OLOFSDOTTER, M., JENSEN, L. B., PAMPLONA, R., NAVAREZ, D., LEE, S. B., and PHENG, S. 2002. Towards utilization of allelopathy-the rice example, pp. 197–207, in M. J. Reigosa, and N. Pedrol (eds.). Allelopathy-from molecules to ecosystemsScience Publishers Inc. Enfield (NH), USA.

PFAFFL, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nuc. Acids Res* 29:2002–2007.

RAZAL, R. A., ELLIS, S., SINGH, S., LEWIST, N. G., and TOWERS, G. H. N. 1996. Nitrogen recycling in phenylpropanoid metabolism. *Phtochemistry* 41:31–35.

REICHARDT, P. B., CHAPIN, P. S., BRYANT, J. P., MATTER, B. R., and CLAUSEN, T. P. 1991. Carbon/nutrient balance as a predictor of plant defense in Alaska balsam poplar: potential importance of metabolite turnover. *Oecologia* 88:401–406.

SCHEIBLE, W. R., MORCUENDE, R., CZECHOWSKI, T., FRITZ, C., OSUNA, D., PALACIOS-ROJAS, N., SCHINDELASCH, D., THIMM, O., UDVARDI, M. K., and STITT, M. 2004. Genome-wide reprogramming of primary and secondary metabolism, protein synthesis, cellular growth processes, and the regulatory infra-structure of Arabidopsis in response to nitrogen. *Plant Physiol* 136:2483–2499.

SHEN, L. H., and LIN, W. X. 2007. Effects of phosphorus levels on allelopathic potential of rice co-cultured with barnyardgrass. *Allelopathy J* 19:393–402.

SHEVELEVA, E., CHMARA, W., BOHNERT, H. J., and JENSEN, R. G. 1997. Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiol* 115:1211–1219.

WU, H. W., PRATLEY, J., LEMERLE, D., and AN, M. 1999. Crop cultivars with allelopathy capability. *Weed Res* 39:171–180.

WYSOCKA-DILLER, J. W., HELARIUTTA, Y., FUKAKI, H., MALAMY, J. E., and BENFEY, P. N. 2000. Molecular analysis of SCARECROW function reveals a radial patterning mechanism common to root and shoot. *Development* 127:595–603.

XIONG, J., LIN, W. X., ZHOU, J. J., WU, M. H., CHEN, X. X., and HE, H. Q. 2005. Studies on biointerference between barnyardgrass and rice accessions at different nitrogen regimes, pp. 501–504, in J. D. I. Harper, M. An, H. Wu, and J. H. Kent (eds.). Proceedings Fourth World Congress on AllelopathyCharles Sturt University, Wagga Wagga, NSW, Australia.

XU, M., HILLWIG, M. L., PRISIC, S., COATES, R. M., and PETERS, R. J. 2004. Functional identification of rice syn-copalyl diphosphate synthase and its role in initiating biosynthesis of diterpenoid phytoalexin/allelopathic natural products. *Plant J* 39:309–318.

XU, Y., and HALL, T. C. 1993. Cytosolic triosephosphate isomerase is a single gene in rice. *Plant Physiol* 101:683–687.

YAMAGATA, H., KUNIMASTU, K., KAMASUKA, H., KURAMOTA, T., and IWASKI, T. 1998. Rice bifunctional a-amylase/subitilisin inhibitor: characterization, localization and changes in develop-mental and germinating seeds. *Biosci. Biotech. Biochem* 62:978–985.

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ERRATUM

Spectrum of Cyanide Toxicity and Allocation in *Heliconius erato* and *Passiflora* Host Plants

Mirian Medina Hay-Roe · James Nation

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We regret inadvertently overlooking and failing to cite a study of cyanogenesis in the body parts of *Heliconius* species completed much earlier than our published paper. That earlier study, entitled "Chemical Ecology of Passion Vine Butterflies: Sequestration of Cyanogens and Patterns of Host-plant Specialization by *Heliconius* Butterflies" by Dr. Helene S. Engler, was accepted as her Ph.D. Dissertation in 1998 by the University of Texas at Austin.

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M. M. Hay-Roe · J. Nation
Department of Entomology and Nematology,
University of Florida,
P.O. Box 110650, Gainesville, FL 32611-0650, USA

Present address:
M. M. Hay-Roe (✉)
McGuire Center for Lepidoptera and Biodiversity,
Florida Museum of Natural History,
P.O. Box 112710, Gainesville, FL 32611-2710, USA
e-mail: mmhr@ufl.edu

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Relationships Between Sex and Stress Hormone Levels in Feces and Marking Behavior in a Wild Population of Iberian Wolves (*Canis lupus signatus*)

Isabel Barja · Gema Silván · Juan Carlos Illera

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Abstract Feces deposited by the breeding alpha pair on exposed substrates and/or zones may act as visual and olfactory marks associated with social dominance in wolves. The aim of this study was to determine if there was a correlation between marking behavior, sex hormone levels, and physiological stress in a wild population of Iberian wolves in Northwest Spain. The glucocorticoid and sex hormone levels were measured in feces collected as a function of exposure (conspicuous/inconspicuous), height (above ground level/at ground level), and strategic location in the habitat (at crossroads/off crossroads), as well as the frequency of re-marking. The feces, believed to serve as marking cues, had higher glucocorticoid levels (cortisol) and sex hormones (testosterone, progesterone, and estradiol). The results suggest that in Iberian wolves, the alpha pair is subject to higher social stress than subordinate individuals, and that the reproductive suppression of subordinates is not mediated by chronic glucocorticoid elevation.

Keywords Fecal marking behavior · Iberian wolf · Sex hormones · Social rank · Stress responses

I. Barja (✉)
Unidad Zoología, Departamento de Biología,
Facultad de Ciencias, Universidad Autónoma de Madrid,
Madrid, Spain
e-mail: isabel.barja@uam.es

G. Silván · J. C. Illera
Departamento de Fisiología (Fisiología Animal),
Facultad de Veterinaria, Universidad Complutense de Madrid,
Madrid, Spain

Introduction

Vertebrates may use chemical signs in different contexts and under different environmental conditions, and these can be of particular importance in situations (at night, underground, or in dense vegetation) when visual and auditory signs are difficult to detect (Gorman and Trowbridge 1989). A number of the features of these chemical signals, such as spatial range, localizability, temporal parameters, intensity, detectability, and information content, may be adjusted for particular social functions (Alberts 1992).

In carnivores, marking behavior may serve many functions, which include the identification of individuals, their reproductive state, and/or social status, as well as information with respect to territoriality (Gorman and Trowbridge 1989). An association between marking and dominance has been reported for a large number of species (Ralls 1971; Gorman and Trowbridge 1989), and in some cases only high-ranking individuals (alpha pair) exhibit marking behavior (Peters and Mech 1975; Rothman and Mech 1979) through which they continually announce their status to subordinates (Gorman and Trowbridge 1989).

Although many species use urine and feces as visual and scent marks (Macdonald 1980; Gorman 1990), it is often difficult to distinguish between normal excretion and their use in communication. However, one feature is that those used as signals are usually produced in small quantities and are often left in prominent sites (Kleiman 1966; Macdonald 1985; Gorman and Trowbridge 1989; Barja and De Miguel 2004; Barja et al. 2004, 2005), such as junctions on frequently used tracks, where they may be detected by animals traveling in different directions (Peters and Mech 1975; Vilà et al. 1994; Zub et al. 2003; Barja et al. 2004).

In social species, agonistic and aggressive interactions between individuals may be a source of stress (Creel 2001; Sands and Creel 2004; Creel 2005), initiating a cascade of physiological responses that results in glucocorticoid secretion (cortisol/corticosterone) by the adrenal cortex (Axelrod and Reisine 1984; Stewart 2003). Short-term glucocorticoid secretions have been related to adaptive responses of animals to stressors, and are considered beneficial for the organism. However, long-term glucocorticoid secretion may lead to pathologies (e.g. reproductive and/or endocrine disruption, suppression of the immune system, and gastrointestinal ulcerations) (Stewart 2003) that could significantly reduce overall fitness, by affecting survival and reproductive success (Sapolsky 1992; Möstl and Palme 2002). Consequently, glucocorticoids have been used as physiological indicators of stress in several species (Möstl and Palme 2002; Stewart 2003), and it has been suggested that such assays may provide a noninvasive method for evaluating adrenal and gonadal functions (Wasser et al. 2000; Young et al. 2004).

Wolves are social animals, and packs can be characterized as dominance hierarchies that consist of a dominant male and female (the alpha pair), with the remainder of the pack usually made up of their offspring from several litters (Mech and Boitani 2003). Only a small proportion of all wolf scats found in the habitat are in "exposed" sites, and if they serve as cues left by the alpha pair (the only individuals that reproduce in the group), then one would predict that levels of sex hormones in the putative marking scats would be significantly higher than in those found in unexposed sites. Furthermore, one would predict that glucocorticoid levels would also differ, reflecting different stress levels between dominant and subordinate members of the pack. The present study was designed to test these hypotheses.

Methods and Materials

Study Site The research was conducted between June 2004 and August 2005 in the Macizo Central Ourensano in Galicia (NW Spain), a 120-km^2 mountainous area, 850–1,707 m above sea level with a continental (hot summers and cold winters) climate. The area was occupied by a pack of Iberian wolves, with an alpha pair that reared pups in both years.

Fecal Sample Analysis and Collection Fecal samples were collected once each month along roads and trails frequently used by the wolves, but away from human habitation to minimize the chances of collecting domestic dog feces. Wolf feces were identified by their size and shape, and in cases that there was any doubt as to the origin of specific scats (<5% of the total found), they were not collected for the subsequent analyses. In each monthly sampling period, transects were visited twice, at dusk and the following morning, to ensure that only fresh scats (< 12 hr), characterized by a layer of mucus, a strong smell, and no signs of dehydration, were sampled. Approximately 10 g were collected from each scat and held at −20°C until assayed. The fact that wolves are nocturnal ensured that the scats collected each morning had only been exposed to low night time temperatures, thereby reducing potential losses of glucocorticoids, as the concentrations detected may be influenced by prevailing temperature conditions (Möstl et al. 2002; Möstl and Palme 2002).

The spatial characteristics of the deposition site for each scat found was recorded, using substrate type, height, and positions of fecal markings, as well as the incidence of re-marking. Substrates were classified as inconspicuous or conspicuous, the latter being those that, for a human observer, stood out from the surroundings including rocks, plants, and mounds of soil. All others were regarded as inconspicuous (Barja et al. 2005; Barja and List 2006). Feces were considered as true marking cues if they occurred on an exposed substrate >4 cm above ground level (Barja et al. 2005; Barja and List 2006), at an intersection where two or more trails crossed (Barja et al. 2004) and/or they occurred on previous fecal marks, usually those that were on conspicuous substrates.

The fecal samples were analyzed to quantify the metabolite levels of cortisol, testosterone, progesterone, and estradiol. Hormones were extracted by using previously established methods (Brown et al. 1994; Young et al. 2004; Barja et al. 2008), and the efficiency of extraction was tested by the addition of radiolabeled hormones (3H-cortisol, 3H-testosterone, 3H-progesterone, and 3H-estradiol, 4,000–8,000 dpm, ICN, CA, USA) to a parallel set of fecal samples. Hormone levels were determined by using an enzyme immunoassay developed by us and validated for

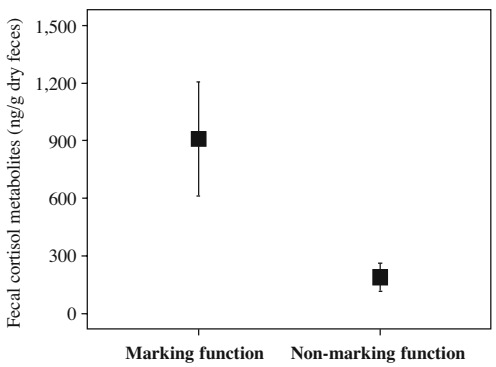

Fig. 1 Comparison of fecal glucocorticoid levels in Iberian wolf scats with (N=21), and without (N=39), a presumed marking function. The fecal samples were collected from June 2004 to August 2005 in Montes do Invernadeiro Natural Park (NW Spain). Mean ± SE is shown for both groups

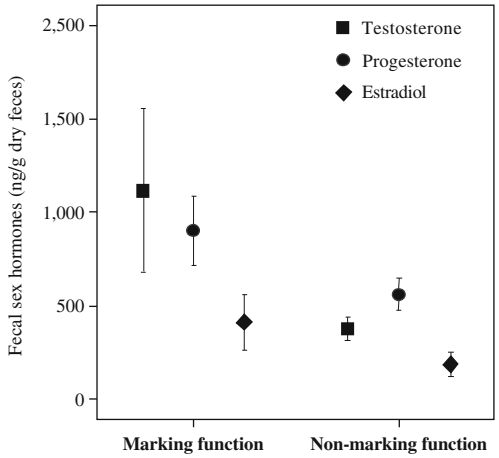

Fig. 2 Comparison of fecal sex hormone concentrations (androgens, progestins, and estrogens) for fecal samples with and without a presumed marking function. Data are presented as mean ± SE

this study system (Barja et al. 2008). Standard dose–response curves were constructed by plotting the binding percentage (B/Bo×100) against the standard hormone concentrations added. Fecal hormone metabolite concentrations are presented as nanogram per gram of dry fecal matter.

Statistical Analyses Kolmogorov–Smirnov–Lilliefors and Levene tests were used to determine the normality of data and homogeneity of variances, respectively. All hormone metabolite concentrations were log-transformed to normalize the data and to decrease heterogeneity of variances. Samples were divided into two groups, depending on whether they were considered to have a marking function or not. *t* tests were used to test for significant differences between two groups. Pearson correlation coefficients were computed to assess the relationship between cortisol metabolite concentrations and sex steroid hormone levels. The data are given as means ± SE and the level of significance for statistical tests was $P<0.05$. The statistical analyses were conducted with the software SPSS 15.0.

Results

Scats presumed to have a marking role had significantly higher levels of glucocorticoids (Fig. 1) and sex hormones (Fig. 2) than those that did not. Furthermore, these significant differences were evident regardless of the criteria used to identify those scats assumed to play a marking role, except for testosterone and progesterone in feces from re-marked and non-re-marked sites (Table 1). Both male and female hormone levels rise at the beginning of the reproductive period, and these changes are associated with the expression of reproductive behavior (Seal et al. 1979; Asa and Valdespino 1998; Mech and Boitani 2003). Consequently, it is of interest to note that in the scats assumed to play a marking role, the mean concentrations of testosterone (1,636.6±819.5 vs. 1,028.4±462.9 ng/g of dry feces; $N=21$, $t=9.7$, $P<0.001$) and estradiol: 441.5±387.6 vs. 409.9±158.5 ng/g of dry feces; $N=21$, $t=7.2$, $P<0.001$) were higher during the breeding than in the non-breeding season. However, the mean levels of progesterone were higher during the non-breeding period (772.1±641.1 vs. 919.0±225.1 ng/g of dry feces, $N=21$, $t=15.4$, $P<0.001$).

Significant positive correlations were found between cortisol metabolite levels, an indicator of stress, and the three sex hormones examined: testosterone ($r=0.249$, $P=0.055$, $N=60$), progesterone ($r=0.594$, $P<0.001$, $N=60$) and estradiol ($r=0.623$, $P<0.001$, $N=60$).

Discussion

The higher levels of sex hormones in the scats in exposed sites, presumed to play a marking role, would support the hypothesis that these were deposited by the breeding alpha pair, as it has been well documented that, in wolves, they are usually the only individuals that reproduce within group (Mech 1999; Mech and Boitani 2003).

Table 1 Mean levels (± SE) of steroid hormones found in wolf scats at Montes do Invernadeiro Natural Park (NW Spain) In relation to physical characterization deposition site

Substrate/Position	Cortisol	Testosterone	Progesterone	Estradiol	No. Samples ($N=60$)
Conspicuous	786.1±340.9*	1,737.6±795.9*	1,380.4±367.0*	467.4±173.7*	10
Inconspicuous	372.1±115.8	412.8±78.4	540.0±73.0	225.9±64.6	50
> 4 cm above ground	936.7±412.0*	2,113.2±956.9*	1,277.6±327.3*	550.8±207.5*	8
< 4 cm above ground	364.9±111.5	406.0±75.7	588.1±90.5	222.3±62.2	52
At crossroads	744.5±294.2*	1,599.6±630.8*	852.4±238.7*	398.2±142.6*	13
Off crossroads	357.2±117.3	366.4±65.0	632.4±100.1	229.6±67.9	47
Re-marking	1,776.0±912.2*	637.2±225.2	575.0±64.8	914.7±518.3*	5
No re-marking	618.6±173.9	858.0±404.8	738.4±145.5	257.1±59.4	21

Fecal hormone metabolite concentrations are presented as ng/g of dry fecal matter
* Indicates a significant difference ($P<0.001$) between the two samples.

Early research with mice (Louch and Higginbotham 1967) and squirrel monkeys (Manogue 1975) in captivity suggested that subordinate individuals were subjected to higher stress. Also, in captive timber wolves, urinary cortisol levels were similar in dominant and subordinate individuals (McLeod et al. 1996). In wolves, it is known that losing fights can increase glucocorticoid secretion (Creel 2005), and it has been argued that chronic stress might underlie reproductive suppression of social subordinates in cooperative breeders (Reyer et al. 1986).

However, the elevated glucocorticoid concentrations that we found, together with the higher levels of sex hormones, in marking scats of the Iberian wolf do not support the psychological castration hypothesis. Instead, our findings, including the positive correlations between the concentrations of cortisol metabolites and the sex hormones, support the notion that maintaining dominance, which yields significant benefits from access to food and mates that result in increased reproductive success, comes at a cost (Creel 2001; Sands and Creel 2004). This is similar to the results of other studies on wild populations of dwarf mongoose (Creel et al. 1992, 1996), African wild dogs (Creel et al. 1996, 1997), and the gray wolf (Sands and Creel 2004; Creel 2005) that showed that dominant individuals suffer higher levels of stress than subordinates. The apparent disparity between the results that use captive and wild populations may be explained by the fact that in captivity subordinate individuals are forced to remain close to dominants, whereas in the wild, moving away is a common means of terminating an attack when behavioral appeasement does not work (Creel 2001).

We believe that a noninvasive method of quantifying glucocorticoid and reproductive steroid levels in fecal samples will be a useful tool for future field studies. This will allow us to obtain both ecological and physiological data, and the combined information will provide the basis for elaboration of more effective and successful conservation programs (Wikelski and Cooke 2006).

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References

ALBERTS, A. C. 1992. Constraints on the design of chemical communication systems in terrestrial vertebrates. *Am. Nat.* 139: S62–S69.

ASA, C. S., and VALDESPINO, C. 1998. Canid reproductive biology: An integration of proximate mechanisms and ultimate causes. *Am. Zool.* 38:251–259.

AXELROD, J., and REISINE, T. D. 1984. Stress hormones: their interaction and regulation. *Science* 224:452–459.

BARJA, I., and DE MIGUEL, F. J. 2004. Variation in stimulus, seasonal context and response to urine marks by captive Iberian wolves (*Canis lupus signatus*). *Acta Ethol.* 7:51–57.

BARJA, I., and LIST, R. 2006. Faecal marking behaviour in ringtails (*Bassariscus astutus*) during the non-breeding period: spatial characteristics of latrines and single faeces. *Chemoecology* 16:219–222.

BARJA, I., DE MIGUEL, F. J., and BÁRCENA, F. 2004. Importance of the crossroads in faecal marking behaviour of the wolves (*Canis lupus*). *Naturwissenschaften* 91:489–492.

BARJA, I., DE MIGUEL, F. J., and BÁRCENA, F. 2005. Faecal marking behaviour of Iberian wolf in different zones of their territory. *Folia Zool.* 54:21–29.

BARJA, I., SILVÁN, G., ROSELLINI, S., PIÑEIRO, A., GONZÁLEZ-GIL, A., CAMACHO, L., and ILLERA, J. C. 2007. Stress physiological responses to tourist pressure in a wild population of European pine marten. *J. Steroid Biochem. Mol. Biol.* 104:136–142.

BARJA, I., SILVÁN, G., ROSELLINI, S., PIÑEIRO, A., ILLERA, M. J., and ILLERA, J. C. 2008. Quantification of sexual steroid hormones in faeces of Iberian wolf (*Canis lupus signatus*): a non-invasive sex typing method. *Reprod. Domest. Anim.* (in press), April.

BROWN, J. L., WASSER, S. K., WILDT, D. E., and GRAHAM, L. H. 1994. Comparative aspects of steroid hormone metabolism and ovarian activity in felids, measured non invasively in feces. *Biol. Reprod.* 51:776–786.

CREEL, S. 2001. Social dominance and stress hormones. *Trends Ecol. Evol.* 16:491–497.

CREEL, S. 2005. Dominance, aggression, and glucocorticoid levels in social carnivores. J. *Mammal.* 86:255–264.

CREEL, S., CREEL, N. M., and MONFORT, S. L. 1996. Social stress and dominance. *Nature* 379:12.

CREEL, S., CREEL, N. M., WILDT, D. E., and MONFORT, S. L. 1992. Behavioral and endocrine mechanisms of reproductive suppression in Serengeti dwarf mongooses. Ani. Behav. 43:231–245.

CREEL, S., CREEL, N. M., MILLS, G. M. N., and MONFORT, S. L. 1997. Rank and reproduction in cooperatively breeding African wild dogs: behavioral and endocrine correlates. *Behav. Ecol.* 8:298–306.

GORMAN, M. L. 1990. Scent marking strategies in mammals. *Rev. Suisse Zool.* 97:3–30.

GORMAN, M. L., and TROWBRIDGE, B. J. 1989. The role of odor in the social lives of carnivores, pp. 57–139, in J. L. Gittleman (ed.). Carnivore Behavior, Ecology and Evolution. Cornell University Press, New York.

KLEIMAN, D. G. 1966. Scent marking in the canidae. *Symp. Zool. Soc. Lond.* 18:167–177.

LOUCH, C. D., and HIGGINBOTHAM, M. 1967. The relation between social rank and plasma corticosterone levels in mice. *Gen. Comp. Endocrinol.* 8:441–444.

MACDONALD, D. W. 1980. Patterns of scent marking with urine and faeces amongst carnivore communities. *Symp. Zool. Soc. Lond.* 45:107–139.

MACDONALD, D. W. 1985. The carnivore: Order carnivora, pp. 619–722, in R. E. Brown, and D. W. Macdonald (eds.). Social Odours in Mammals Clarendon Press, Oxford.

MANOGUE, K. R. 1975. Dominance status and adrenocortical reactivity to stress in squirrel monkeys (*Saimiri sciureus*). *Primates* 14:457–463.

MCLEOD, P. J., MOGER, W. H., RYON, J., GADBOIS, S., and FENTRESS, J. C. 1996. The relation between urinary cortisol levels and social behaviour in captive timber wolves. *Can. J. Zool.* 74:209–216.

MECH, L. D. 1999. Alpha status, dominance and division of labor in wolf packs. *Can. J. Zool.* 77:1196–1203.

MECH, L. D., and BOITANI, L. 2003. Wolves: Behavior, Ecology and Conservation. University of Chicago press, Chicago.

MÖSTL, E., and PALME, R. 2002. Hormones as indicators of stress. *Domest. Anim. Endocrinol.* 23:67–74.

MÖSTL, E., MAGGS, J. L., SCHRÖTTER, G., BESENFELDER, U., and PALME, R. 2002. Measurement of cortisol metabolites in faeces of ruminants. *Vet. Res. Commun.* 26:127–39.

PETERS, R. P., and MECH, L. D. 1975. Scent-marking in wolves. *Am. Sci.* 63:628–637.

RALLS, K. 1971. Mammalian scent marking. *Science* 171:443–449.

REYER, H. U., DITTAMI, J. P., and HALL, M. R. 1986. Avian helpers at the nest: are they psychologically castrated? *Ethology* 71:216–228.

ROTHMAN, R. J., and MECH, L. D. 1979. Scent-marking in lone wolves and newly formed pairs. *Anim. Behav.* 27:750–760.

SANDS, J., and CREEL, S. 2004. Social dominance, aggression and faecal glucocorticoid levels in a wild population of wolves, *Canis lupus*. *Anim. Behav.* 67:387–396.

SAPOLSKY, R. 1992. Neuroendocrinology of the stress response, pp. 287–324, in J. B. Becker, S. M. Breedlove, and D. Crews (eds.). Behavioral Endocrinology MIT Press, Massachusetts.

SEAL, U. S., PLOTKA, E. D., PACKARD, J. M., and MECH, L. D. 1979. Endocrine correlates of reproduction in the wolf. I. Serum progesterone, estradiol and LH during the estrous cycle. *Biol. Reprod.* 21:1057–1066.

STEWART, P. M. 2003. The adrenal cortex, pp. 491–551, in P. R. Larsen, H. M. Kronenberg, S. Melmed, and K. S. Polonsky (eds.). Williams Textbook of Endocrinology Saunders, Philadelphia.

VILÀ, C., URIOS, V., and CASTROVIEJO, J. 1994. Use of faeces for marking in Iberian wolves (*Canis lupus*). *Can. J. Zool.* 72:374–377.

WASSER, S. K., Hunt, K. E., BROWN, J. L., COOPER, K., CROCKETT, C. M., BECHERT, U., MILLSPAUGH, J. J., LARSON, S., and MONFORT, S. 2000. A generalized fecal glucocorticoid assay for use in a diverse array of non domestic mammalian and avian species. *Gen. Comp. Endocrinol.* 120:260–275.

WIKELSKI, M., and COOKE, S. J. 2006. Conservation physiology. *Trends Ecol. Evol.* 21:38–46.

YOUNG, K. M., WALKER, S. L., LANTHIER, C., WADDELL, W. T., MONFORT, S. L., and BROWN, J. L. 2004. Noninvasive monitoring of adrenocortical activity in carnivores by fecal glucocorticoid analyses. *Gen. Comp. Endocrinol.* 137:148–165.

ZUB, K., THEUERKAUF, J., JEDRZEJEWSKI, W., JEDRZEJEWSKA, B., SCHMIDT, K., and KOWALCZYK, R. 2003. Wolf pack territory marking in the Bialowieza primeval forest (Poland). *Behaviour* 140:635–648.

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Distance Communication of Sexual Status in the Crayfish *Orconectes quinebaugensis*: Female Sexual History Mediates Male and Female Behavior

William S. Durgin · Kelly E. Martin · Heather R. Watkins · Lauren M. Mathews

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Abstract Chemical communication plays an important role in mediating social interactions of many taxa, particularly arthropods. Many individuals communicate information about their reproductive status to potential mates through distance and/or contact pheromones, an ability that may be advantageous to both signalers and receivers. In this paper, we describe tests of two hypotheses on the role of distance communication in the reproductive behaviors of crayfish (*Orconectes quinebaugensis*). First, we hypothesized that male crayfish would show stronger attraction towards virgin females (females with no viable sperm) than towards non-virgin females because of the fitness costs (to males) associated with sperm competition. Second, we hypothesized that female crayfish should show differential responses to mature male signals depending on their own sexual history: virgin females should be more strongly attracted to male signals than should non-virgin females because they must mate at least once to be able to fertilize eggs in the spring. Data from two Y-maze experiments yielded support for both hypotheses: males were attracted to signals from virgin females, but not to signals from non-virgins. Likewise, virgin females were attracted to signals from males, but non-virgin females were not. We discuss our data in the context of the potential costs and benefits of mate searching and suggest that distance chemical communication of sexual status may be particularly advantageous when the costs of mate searching are high.

W. S. Durgin · K. E. Martin · H. R. Watkins · L. M. Mathews (✉)
Department of Biology, Worcester Polytechnic Institute,
100 Institute Road,
Worcester, MA 01609, USA
e-mail: Lmathews@wpi.edu

Keywords Chemical communication · Distance pheromone · Sexual status · Crayfish · *Orconectes*

Introduction

In many species, interactions among individuals are governed by signals that are normally undetectable by humans, including visual (Papke et al. 2007), acoustic (Conner 1999; Fenton 2003), and chemical (Wyatt 2003) signals. In particular, chemical signals are a primary mode of communication in both aquatic and terrestrial arthropods (Greenfield 2002). Although chemical cues may function in many behavioral interactions, their roles in mediating reproductive interactions are of particular interest. Sex pheromones may function in gender recognition during physical contact between conspecifics and/or at a distance and, in some taxa, affect sexual receptivity of one or both sexes (Schiestl and Ayasse 2000; Stebbing et al. 2003; Houck et al. 2007) and to play a role in mate choice by one or both sexes (Widemo and Johansson 2006; Costanzo and Monteiro 2007).

If individuals can accurately send or receive information about sexual status at a distance, this could reduce the risk of costly mate-searching decisions. Males of some taxa can distinguish low- from high-value (e.g., virgin from mated) females by physical contact (Kelly et al. 1998; Uhía and Cordero Rivera 2005; Friberg 2006). However, distance communication of mating history has not been widely reported. In aquatic habitats, distance chemical signals may be subject to complex dispersion patterns (Atema 1995). Nevertheless, much research indicates that waterborne signals play important roles in mediating social interactions of aquatic animals, particularly arthropods (Greenfield 2002). In crayfish, chemical signals carried in the urine

function in dominance interactions among conspecifics (Bergman et al. 2005; Bergman and Moore 2005). However, their roles in reproductive interactions are unclear, although Acquistapace et al. (2002) found that female chemical cues alone were insufficient to elicit responses in males of *Austropotamobius pallipes*, while Stebbing et al. (2003) reported evidence for a sex pheromone released by adult females of the crayfish *Pacifastacus leniusculus*. A number of other crayfish species use pheromones to communicate gender or sexual status (Bechler 1995).

In this study, we explore the role of distance chemical signaling in the reproductive behavior of the crayfish *Orconectes quinebaugensis* (Mathews and Warren 2008), a taxon that may be subject to high mate-search costs (e.g., an increased risk of predation: Garvey et al. 1994; Hill and Lodge 1999). We were interested in determining how chemical signaling and behavioral responses to chemical signals change in response to the sexual histories of females. In this species, mating is temporally decoupled from fertilization, and females store ejaculates in their spermathecae between the autumn mating and spring egg-laying seasons (Hamr 2002). We first hypothesized that sexually mature males would prefer waterborne signals of virgin females (females carrying no viable sperm) over the signals of non-virgin females because of the potential costs of sperm competition. Second, we hypothesized that virgin females would be more attracted to signals from sexually mature males than would non-virgin females because females must mate with at least one male to produce any fertile eggs and should be under selection to associate with sexually mature males. We tested these hypotheses by using a Y-maze apparatus.

Methods and Materials

Collection and Care of Crayfish Crayfish were collected by hand or by seine from the Quinebaug River in Sturbridge, MA, USA. We considered females to be "virgins" at the time they were collected (August) because we have not observed mating behavior before late September either in the field or in the laboratory and have never obtained fertile eggs from isolated females collected in late summer (personal observation). Females were held in two tanks, one with no males and one with a ~1:1 sex ratio. All were ≥25 mm carapace length and sexually mature. Our assumptions that all "virgin" females were unmated (at the time of collection) and that all "non-virgin" females were mated (while in the holding tanks) may not be correct. However, false assignment of the sexual status of females would bias our data in support of our null hypothesis. Experimental males were collected between August and November. All males were in form I (sexually mature; Hamr 2002) and were housed in two tanks with no access to females. All holding tanks were ~190-l and were maintained at light/dark cycles that mimicked external conditions and at ambient temperatures. Clay pots were provided as shelter for the crayfish, and the animals were fed a diet of Wardley shrimp pellets daily.

Design and Testing of Y-Maze Apparatus We designed and constructed a Y-maze apparatus to allow experimental subjects to choose between two water sources (Fig. 1a). The apparatus comprised a pair of Y-mazes constructed of black ABS piping cut in half longitudinally and connected with ABS cement. A wire gate was placed ~15 cm from the end of each stem to contain the crayfish during an acclimation period before the start of each trial. The two Y-mazes were each connected to two water sources (Fig. 1a,b). The sources comprised two components (Fig. 1b): the lower reservoir container (57 l) and the upper secondary container (7.6 l). The secondary containers each had a hole in the bottom, plugged with a 10-cm diameter polyvinyl chloride standpipe elevated 4 cm above the bottom of the secondary container. A 150-W submerged electric pump moved water from the reservoir to the secondary container where the standpipe regulated water depth and, therefore, the rate of flow of water through a valve and into rubber tubing connecting to the Y-mazes.

Experimental Procedure In experiment 1, there were three treatments. In two, experimental males were presented with a choice between control (unconditioned) water and water conditioned by either virgin females or non-virgin females. In the third, males were given a choice between water conditioned by virgin females and water conditioned by non-virgin females. In experiment 2, there were two treatments. In one, virgin females were presented with a choice between control water and water conditioned by mature males, and in the other, non-virgin females were presented with a choice between control water and water conditioned by mature males. In both experiments, crayfish were distributed among treatments haphazardly with respect to carapace length.

Treated water was conditioned for 24 h before use. In the field, crayfish live in environments with some water exchange (particularly in streams), and therefore, a small volume of water conditioned for 24 h may result in an unnaturally high concentration of chemical signals, though such signals may also be volatile and undergo degradation over a 24-h period. We chose a 24-h conditioning period in part for logistic reasons (it simplified coordination among multiple testers), to increase the chance that chemical cues were present in the conditioned water and to reduce the risk of contamination by alarm signals, which have been shown

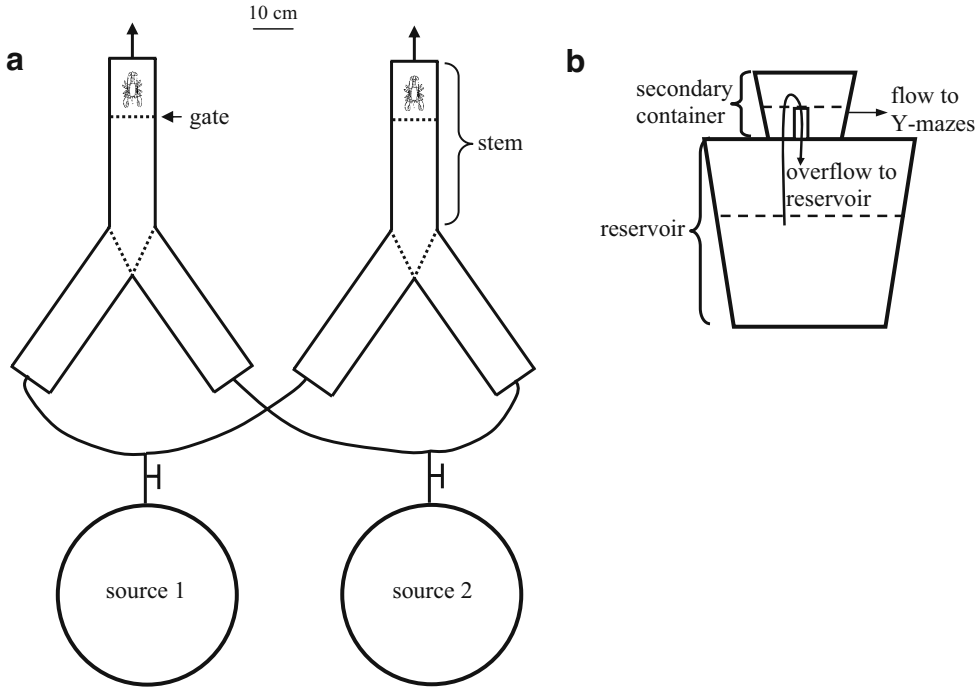

Fig. 1 Diagram of Y-maze apparatus. The apparatus comprised a pair of Y-mazes connected to two water sources. **a** Water flow from each source container was controlled by a stopcock and was split and delivered to one arm of each of two Y-mazes where it flowed down the arm, mixed with water from the other arm in the stem, and finally exited through the rear of the stem. **b** Detailed diagram of a single source container. Each source container comprised a larger reservoir and a smaller secondary container. Water was pumped from the reservoir to the secondary container where it flowed out through tubing to the two Y-mazes. Water depth in the secondary container was controlled by a 4-cm standpipe, which allowed water to flow back into the reservoir when it reached a depth of 4 cm

to affect behavior in the sister species *O. virilis* for >15 min (Hazlett 1990). Other investigators using Y-maze designs to study responses to chemical cues in crayfish have used variable exposure times to treat water sources, ranging at least from 3 h (Zulandt Schneider et al. 1999) to 48 h (Tierney and Dunham 1982).

A 38-l aquarium was filled with 4 l of tap water. Four crayfish of the appropriate treatment group were selected haphazardly from their holding tank and were placed in the conditioning tank with four clay pots. Control water was treated identically, except that no crayfish were added. After 24 h, 2 l of water was removed by siphoning, and water was used in trials within 20 min. Before adding the conditioned waters, the entire apparatus was flushed with tap water. Next, in each reservoir, 2 l of treated or control water was mixed with 57 l of tap water to yield a large enough volume of water for 7 min of data collection. One experimental crayfish was placed behind the wire gates in the neutral zone of each Y-maze (Fig. 1); after a 5-min acclimation period, the outflow valves were opened and the gates removed. All movements were recorded by a video camera above the Y-mazes.

Data Analysis For each treatment, we quantified the total time the experimental crayfish spent in each arm of the Y-maze for the first 7 min of each trial. A crayfish was considered to have entered or exited an arm when the tip of its rostrum passed over the seam that joined the arms in the Y-maze (Fig. 1a). For each trial, we subtracted the time spent in one arm from the time spent in the other. For most treatments, this was the time in the experimental arm minus the time spent in the control arm, but for one treatment in experiment 1, this was the time in the virgin female arm minus the time in the non-virgin female arm. We compared these values with the expected values of zero (no difference in time spent in the two arms of the maze) with Wilcoxon signed-ranks tests in SPSS 14.0, with $\alpha = 0.05$.

The Y-maze apparatus was designed (because of logistical constraints) such that two Y-mazes, each with a focal crayfish, received water from the same control and experimental sources. To investigate the impact of this pseudo-replication on our data set, statistical analyses were run in two ways: first, with each focal crayfish considered to be independent and second, with half the data (one from each set of paired replicates) randomly eliminated for each treatment. In all treatments, random elimination of one sample from each pair of replicates resulted in qualitatively similar inferences (i.e., the same statistical inference was reached with the partial data set as with the entire data set). Therefore, we report means and Z and P values based on the entire data set for each treatment.

Results

In both experiments, all focal crayfish were responsive, spending at least some of the time in one or each of the arms of the Y-maze. In experiment 1, males spent significantly more time in the arm containing water treated by virgin females than in the arm containing control water, but appeared to behave randomly with respect to water conditioned by non-virgin females and control water (Fig. 2). In addition, when presented simultaneously with signals from both virgin and non-virgin females, males spent significantly more time in water conditioned by virgin females than in water conditioned by non-virgin females (Fig. 2).

In experiment 2, virgin and non-virgin females behaved differently towards signals from sexually mature males (Fig. 3). In one treatment, virgin females spent significantly more time in water conditioned by males than in control water, while in the other, females spent more time in control than in water conditioned by males, though this difference was not statistically significant.

Discussion

Our data indicate that both male and female behavior in *O. quinebaugensis* is mediated by the previous mating histories of females. Males showed a significant preference for water treated by virgin females, while they showed no preference for water treated by non-virgins. This suggests that unmated females may represent a more valuable resource to males; mated females may either have low reproductive value to males, or they may not produce chemical signals that advertise their locations to males. There are a number of possible factors that could decrease a female's perceived

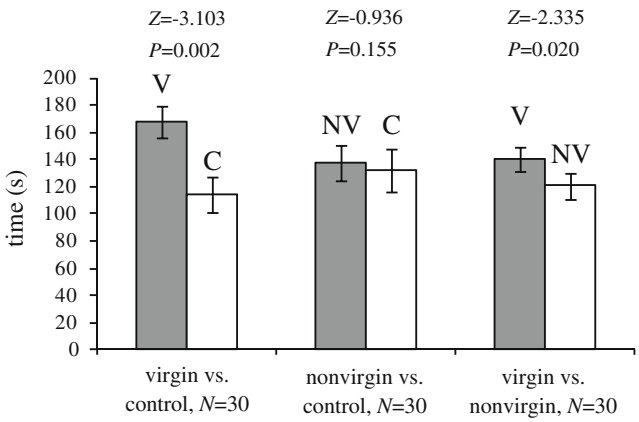

Fig. 2 Mean times spent in each of two arms of Y-maze by males in three treatments. *V* virgin female water, *NV* non-virgin female water, *C* control water. *Z* and *P* values from Wilcoxon signed-ranks tests are shown *above the bars*; sample sizes are listed *below bars*. *Error bars* show standard error of the mean

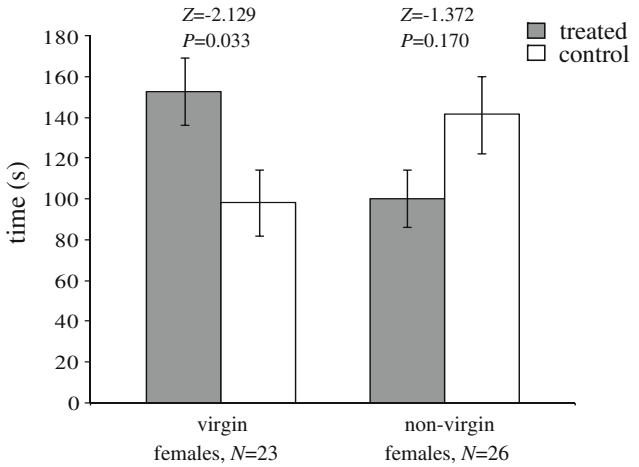

Fig. 3 Mean times spent in each of two arms of Y-maze by virgin or non-virgin females. In each treatment, females were presented with a choice between water treated by exposure to a mature male and control water. *Z* and *P* values from Wilcoxon signed-ranks tests are shown *above the bars*; sample sizes are listed *below bars*. *Error bars* show standard error of the mean

value to males once she has mated. If the first male to mate with a female has an advantage in sperm competition, then males that selectively mate with virgin females would have higher fitnesses. First male sperm precedence has been demonstrated in a number of arthropods, including both spiders (Elgar 1998) and insects (Simmons and Siva-Jothy 1998), but in decapod crustaceans, most investigations have indicated that last-male precedence is most common (Koga et al. 1993; Urbani et al. 1998; Murai et al. 2002). Alternatively, mating males may deposit sperm plugs, making it energetically costly (because of the need to remove the sperm plug) or impossible for other males to mate with the same female. Sperm plugs have been described in other crustaceans (Jivoff 1997; Aiken et al. 2004; Oh and Hankin 2004) and may occur in other orconectid crayfish (Berrill and Arsenault 1984). Finally, already-mated females may be less likely to mate than virgin females, particularly if the costs of polyandry outweigh the potential benefits. If mated females cannot often or easily be coerced into mating again, then males may be less inclined to invest in searching for and courting them.

In addition, our data show that virgin and non-virgin females responded differently when exposed to the signals of mature males. Virgin females spent significantly more time in water conditioned by males than in control water, while non-virgins showed no significant difference in the time they spent in control or treated water, indicating that virgin and non-virgin females may differ in the extent to which they actively seek potential mates. Once mated, females are already assured of having a supply of sperm with which to fertilize their eggs in the spring and may be less willing to engage in costly mate searching, although they may accept matings from some courting males, e.g.,

for potential genetic benefits or as bet-hedging against male infertility.

The importance of chemical signals in communicating detailed information with conspecifics has probably long been underestimated by behavioral ecologists because such signals are so difficult to observe directly. Here, we have provided indirect evidence for distance communication of recent mating history through chemical signals. Although males of many arthropods are known to assess female reproductive status through physical contact, the importance of distance pheromones for assessment of conspecifics is less well known. In addition, distance chemical signaling has been considered to be problematic in aquatic taxa because the direction of water flow is often unpredictable, and therefore, the sources of signals may not be located with great accuracy (Breithaupt 1999; Kozlowski et al. 2003). However, in some other decapod crustaceans in which mating is tied to the female's molt, males show an ability to detect distance signals from pre-molt females (Gleeson 1991; Mathews 2003), and therefore, males may exploit part of a normal endocrine process as a signal of a female's value as a mate. In addition, female lobsters may use signals in the urine of males to make mate-choice decisions based on male dominance status (Bushman and Atema 2000). Similarly, female rock shrimp (*Rhynchocinetes typus*) may use chemical cues to locate dominant males (Diaz and Thiel 2004).

In general, individuals of many taxa may exchange a great deal of information about one another without any contact that is perceptible to humans. Distance chemical communication may be of particular importance whenever movement through space is especially energetically costly or risky, as is likely to be the case for individuals of *O. quinebaugensis*. Under such circumstances, there should be a strong selective advantage in being able to identify at a distance those individuals with which physical contact would be most beneficial. Future research, for example, into the complexity of personal information that can be transmitted in broadcast signals, will reveal insight into the importance of distance chemical signaling in many taxa.

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References

ACQUISTAPACE, P., AQUILONI, L., HAZLETT, B. A., and GHERARDI, F. 2002. Multimodal communication in crayfish: Sex recognition during mate search by male *Austropotamobius pallipes*. *Can. J. Zool* 80:2041–2045.

AIKEN, D. E., WADDY, S. L., and MERCER, S. M. 2004. Confirmation of external fertilization in the American lobster, *Homarus americanus*. *J. Crustac. Biol* 24:474–480.

ATEMA, J. 1995. Chemical signals in the marine environment: Dispersal, detection, and temporal signal analysis. *Proc. Natl. Acad. Sci. U. S. A.* 92:62–66.

BECHLER, D. L. 1995. A review and prospectus of sexual and interspecific pheromonal communication in crayfish. *Freshwater Crayfish* 8:657–667.

BERGMAN, D. A., MARTIN, A. L., and MOORE, P. A. 2005. Control of information flow through the influence of mechanical and chemical signals during agonistic encounters by the crayfish, *Orconectes rusticus*. *Anim. Behav.* 70:485496.

BERGMAN, D., and MOORE, P. 2005. Prolonged exposure to social odours alters subsequent social interactions in crayfish (*Orconectes rusticus*). *Anim. Behav.* 70:311–318.

BERRILL, M., and ARSENAULT, M. 1984. The breeding behavior of a northern temperate orconectid crayfish, *Orconectes rusticus*. *Anim. Behav.* 32:333–339.

BREITHAUPT, T. 1999. Urine release in freely moving catheterized lobsters (*Homarus americanus*) with reference to feeding and social activities. *J. Exp. Biol.* 202:837–844.

BUSHMANN, P. J., and ATEMA, J. 2000. Chemically mediated mate location and evaluation in the lobster, *Homarus americanus*. *J. Chem. Ecol.* 26:883–899.

CONNER, W. E. 1999. 'Un chant d'appel amoureux': Acoustic communication in moths. *J. Exp. Biol.* 202:1711–1723.

COSTANZO, K., and MONTEIRO, A. 2007. The use of chemical and visual cues in female choice in the butterfly *Bicyclus anynana*. *Proc. R. Soc. Lond. B* 274:845–851.

DIAZ, E. R., and THIEL, M. 2004. Chemical and visual communication during mate searching in rock shrimp. *Biol. Bull.* 206:134–143.

ELGAR, M. A. 1998. Sperm competition and sexual selection in spiders and other arachnids, pp. 307–339, in T. R. Birkhead, and A. P. Møller (eds.). Sperm Competition and Sexual SelectionAcademic, San Diego, California.

FENTON, M. B. 2003. Eavesdropping on the echolocation and social calls of bats. *Mamm. Rev.* 33:193–204.

FRIBERG, U. 2006. Male perception of female mating status: Its effect on copulation duration, sperm defence and female fitness. *Anim. Behav* 72:1259–1268.

GARVEY, J. E., STEIN, R. A., and THOMAS, H. M. 1994. Assessing how fish predation and interspecific prey competition influence a crayfish assemblage. *Ecology* 75:532–547.

GLEESON, R. A. 1991. Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*, pp. 17–32, in R. T. Bauer, and J. W. Martin (eds.). Crustacean Sexual BiologyColumbia University Press, New York, New York.

GREENFIELD, M. D. 2002. Signalers and receivers: Mechanisms and evolution of arthropod communication. Oxford University Press, Oxford.

HAMR, P. 2002. *Orconectes*, pp. 585–608, in D. M. Holdich (ed.). Biology of Freshwater CrayfishBlackwell Science, Oxford.

HAZLETT, B. A. 1990. Source and nature of disturbance—Chemical system in crayfish. *J. Chem. Ecol.* 16:2263–2275.

HILL, A. M., and LODGE, D. M. 1999. Replacement of resident crayfishes by an exotic crayfish: the roles of competition and predation. *Ecol. Appl.* 9:678–690.

HOUCK, L. D., PALMER, C. A., WATTS, R. A., ARNOLD, S. J., FELDHOFFF, P. W., and FELDHOFFT, R. C. 2007. A new vertebrate courtship pheromone, PMF, affects female receptivity in a terrestrial salamander. *Anim. Behav.* 73:315–320.

JIVOFF, P. 1997. The relative roles of predation and sperm competition on the duration of the post-copulatory association between the

sexes in the blue crab, *Callinectes sapidus*. *Behav. Ecol. Sociobiol.* 40:175–185.

KELLY, L. S., SNELL, T. W., and LONSDALE, D. J. 1998. Chemical communication during mating of the harpacticoid *Tigriopus japonicus*. *Philos. Trans. R. Soc. Lond. B* 353:737–744.

KOGA, T., HENMI, Y., and MURAI, M. 1993. Sperm competition and the assurance of underground copulation in the sand-bubbler crab *Scopimera globosa* (Brachyura, Ocypodidae). *J. Crustac. Biol.* 13:134–137.

KOZLOWSKI, C., VOIGT, R., and MOORE, P. A. 2003. Changes in odour intermittency influence the success and search behavior during orientation in the crayfish *Orconectes rusticus*. *Mar. Freshw. Behav. Physiol.* 36:97–110.

MATHEWS, L. M. 2003. Tests of the mate-guarding hypothesis for social monogamy: Male snapping shrimp prefer to associate with high-value females. *Behav. Ecol.* 14:63–67.

MATHEWS, L. M. and WARREN, A. H. 2008. A new crayfish of the genus *Orconectes* Cope, 1872 from southern New England (Crustacea: Ddecapoda: Cambaridae). *P. Biol. Soc. Wash.* (in press)

MURAI, M., KOGA, T., and YONG, H. S. 2002. The assessment of female reproductive state during courtship and scramble competition in the fiddler crab, *Uca paradussumieri*. *Behav. Ecol. Sociobiol.* 52:137–142.

OH, S. J., and HANKIN, D. G. 2004. The sperm plug is a reliable indicator of mating success in female Dungeness crabs, *Cancer magister*. *J. Crustac. Biol.* 24:314–326.

PAPKE, R. S., KEMP, D. J., and RUTOWSKI, R. L. 2007. Multimodal signalling: Structural ultraviolet reflectance predicts male mating success better than pheromones in the butterfly *Colias eurytheme* L. (Pieridae). *Anim. Behav.* 73:47–54.

SCHIESTL, F. P., and AYASSE, M. 2000. Post-mating odor in females of the solitary bee, *Andrena nigroaenea* (Apoidea, Andrenidae), inhibits male mating behavior. *Behav. Ecol. Sociobiol.* 48:303–307.

SIMMONS, L. W., and SIVA-JOTHY, M. T. 1998. Sperm competition in insects: Mechanisms and the potential for selection, pp. 341–434, in T. R. Birkhead, and A. P. Møller (eds.). Sperm Competition and Sexual SelectionAcademic, San Diego.

STEBBING, P. D., BENTLEY, M. G., and WATSON, G. J. 2003. Mating behavior and evidence for a female released courtship pheromone in the signal crayfish *Pacifastacus leniusculus*. *J. Chem. Ecol.* 29:465–475.

TIERNEY, A. J., and DUNHAM, D. W. 1982. Chemical communication in the reproductive isolation of the crayfishes *Orconectes propinquus* and *Orconectes virilis* (Decapoda, Cambaridae). *J. Crustac. Biol.* 2:544–548.

UHÍA, E., CORDERO, and RIVERA, A. 2005. Male damselflies detect female mating status: Importance for postcopulatory sexual selection. *Anim. Behav.* 69:797–804.

URBANI, N., SAINTE-MARIE, B., SEVIGNY, J. M., ZADWORNY, D., and KUHNLEIN, U. 1998. Sperm competition and paternity assurance during the first breeding period of female snow crab (*Chionoecetes opilio*) (Brachyura: Majidae). *Can. J. Fish. Aquat. Sci.* 55:1104–1113.

WIDEMO, F., and JOHANSSON, B. G. 2006. Male-male pheromone signaling in a lekking *Drosophila*. *Proc. R. Soc. Lond. B* 273:713–717.

WYATT, T. D. 2003. Pheromones and animal behaviour: Communication by smell and taste. Cambridge University Press, Cambridge.

ZULANDT, SCHNEIDER, R. A., SCHNEIDER, R. W. A., and MOORE, P. A. 1999. Recognition of dominance status by chemoreception in the red swamp crayfish, *Procambarus clarkia*. *J. Chem. Ecol.* 25:781–794.

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Identification of the Airborne Aggregation Pheromone of the Common Bed Bug, *Cimex lectularius*

Eric Siljander · Regine Gries · Grigori Khaskin · Gerhard Gries

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Abstract Adults and juveniles of the common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), return to and aggregate in harborages after foraging for hosts. We tested the hypothesis that the aggregation is mediated, in part, by an airborne aggregation pheromone. Volatiles from experimental *C. lectularius* harborages were captured on Porapak Q, fractionated by liquid chromatography, and bioassayed in dual-choice, still-air olfactometer experiments. Of 14 compounds with >100 pg abundance in gas chromatography–mass spectrometry analyses of two bioactive fractions, 10 compounds [nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, benzaldehyde, (+)- and (−)-limonene, sulcatone, benzyl alcohol] proved to be essential components of the *C. lectularius* airborne aggregation pheromone.

Keywords *Cimex lectularius* · Cimicidae · Bed bug · Aggregation pheromone · Octanal · Nonanal · Decanal · (*E*)-2-Hexenal · (*E*)-2-Octenal · (2*E*,4*E*)-Octadienal · (2*E*,4*Z*)-Octadienal · Benzaldehyde · Benzyl acetate · Benzyl alcohol · Limonene · Sulcatone · Geranylacetone

Introduction

The common bed bug, *Cimex lectularius* L. (Hemiptera: Cimicidae), has been an ectoparasitic pest of humans throughout recorded history (Usinger 1966; Panagiotakopulu and Buckland 1999; Reinhardt and Siva-Jothy 2007). Recently, it has experienced a global resurgence (Krueger 2000; Boase 2001) with large economic impact due to costly control efforts, lost business, and litigation (Doggett et al. 2004). Considering that all stages of *C. lectularius* bloodfeed, an investigation of their pheromonal communication was warranted (Reinhardt and Siva-Jothy 2007) to determine whether synthetic pheromones could be exploited for detection or management of infestations.

Previous studies demonstrated that *C. lectularius* produce an alarm pheromone [(*E*)-2-hexenal and (*E*)-2-octenal] and an adult 'assembling scent' (Marx 1955; Levinson and Bar Ilan 1971; Levinson et al. 1974a,b). More recent research has shown that the 'assembling scent' is perceived only by contact chemoreception (Siljander et al. 2007) and thus is an arrestant rather than an attractant.

Cimex lectularius aggregates within refugia and returns to these harborages after each blood meal (Kemper 1936). This behavior has been attributed to a specific 'nest odor' (Marx 1955). However, there is no experimental evidence in support of an airborne aggregation pheromone. In the kissing bug, *Triatoma infestans*, both airborne attractant and chemotactile arrestant pheromones mediate aggregation behavior (Schofield and Patterson 1977; Lorenzo Figueiras et al. 1994; Lorenzo and Lazzari 1996; Lorenzo Figueiras and Lazzari 1998). We postulated that *C. lectularius* also might utilize both contact and airborne aggregation pheromones.

In this study, we show that *C. lectularius* uses an airborne aggregation pheromone and report the identification of components of the pheromone.

Methods and Materials

Experimental Insects Cimex lectularius were supplied by, or collected from, three sources: (1) Harold Harlan,

E. Siljander · R. Gries · G. Khaskin · G. Gries (✉)
Department of Biological Sciences, Simon Fraser University,
8888 University Drive,
Burnaby, British Columbia V5A 1S6, Canada
e-mail: gries@sfu.ca

Crownsville, MD, USA; (2) George Keeney, Ohio State University, Columbus, OH, USA; and (3) natural populations in human dwellings, Vancouver, BC, Canada. Insects from all three sources were combined and kept in wide-mouth glass jars (=colony jars; 9×9.5 cm) containing strips of cardboard folded accordion-style to provide a surface on which they could walk and oviposit. Insects were reared at 21–28°C, 25–50% RH, and a 16L/8D photoperiod. They were blood-fed through an artificial membrane (Montes et al. 2002) or on human volunteers. The membrane system used heparinated chicken blood as a food source. For feeding on humans, groups of insects were placed into jars (3.5×5.5 cm) topped with nylon mesh and allowed to feed to repletion through the mesh. Third- to fifth-instar juveniles were randomly selected and fed at least 2 wk before use in experiments, thus allowing them time to digest partially their blood meal and molt to the subsequent instar or adult stage. Mating status of adults used in experiments was not known, unless specified.

Collection of Volatiles To collect airborne semiochemicals for bioassays and chemical analyses, colony jars containing 500–700 *C. lectularius* of mixed stage, age, and gender, exuviae, eggs, and cardboard strips were placed in a cylindrical Pyrex glass chamber (15.5×26 cm). A pump drew charcoal-filtered air at ~2 l/min through each chamber and through a glass column (14×1.3 cm OD) containing 0.5 g of Porapak Q (50–80 mesh; Waters Associates, Inc., Milford, MA, USA). After 72 hr, volatiles were eluted from the Porapak Q trap with 2 ml of pentane. Jar aeration extracts (JAEs) contained ~43,200 bug hour equivalents (1 BHE=volatiles released by one *C. lectularius* during 1 hr) and were stored under darkness at −15°C.

To determine the stage and gender of *C. lectularius* that emitted volatiles, groups of 100 adult males, 100 adult females, or 100 juveniles 1- to 3-wk post-molt were placed on folded filter paper (Whatman no. 1; 9 cm diameter) in separate cylindrical Pyrex glass chambers (10×18 cm) and aerated for 6 d. Each Porapak Q trap was eluted with 2 ml pentane, which was concentrated under a nitrogen stream to a volume of 100 µl for subsequent chemical analysis.

To determine the enantiomer(s) of limonene produced by *C. lectularius*, 50 adult males aged 1–6 wk were placed on folded filter paper (Whatman no. 1; 9 cm diameter) in a cylindrical Pyrex glass chamber (10×5 cm) and aerated for 6 d. Males were chosen because they appeared to release the largest quantity of limonene in previous volatile collections. The Porapak Q trap was eluted with 2 ml pentane, and extract was stored in the dark at −15°C. Porapak Q extracts from four aerations were obtained and fractionated on silica gel. Fractions containing limonene were pooled and concentrated to a volume of 10 µl for subsequent analysis.

Collection of Contact Pheromone Twenty-five juvenile (fourth- or fifth-instar) *C. lectularius* were placed into a glass jar (5×8 cm) containing a folded Whatman no. 1 filter paper disc (5.5 cm diameter). Five such jars were kept for 14 d at room temperature under a 16L/8D photoperiod, allowing insects to walk, rest, defecate, and/or deposit juvenile contact pheromone (Siljander et al. 2007). Each disc received 8,400 contact pheromone hour equivalents (1 CPHE=contact pheromone deposited by one *C. lectularius* during 1 hr). To extract the pheromone, each exposed paper disc was submerged in methanol (HPLC-grade) for 1 hr at room temperature. The supernatant was withdrawn with a pipette, and the five samples were pooled and stored at room temperature until needed.

Analytical Methods Aliquots of JAE or adult male aeration extracts were concentrated under a nitrogen stream and fractionated on silica gel (0.5 g) in a glass column (14×0.5 cm ID). After pre-rinsing the silica gel with pentane, aeration extract aliquots were applied and then eluted with 2 ml each of pentane/ether (100:0, 90:10, 75:25, 50:50, and 0:100) generating five fractions that contained analytes of increasing polarity.

Aliquots of JAEs, bioactive fractions (see olfactometer experiments), and insect aerations were analyzed by coupled gas chromatography–mass spectrometry (GC-MS) in full-scan electron impact mode by using a Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m×0.25 mm ID, J&W Scientific, Folsom, CA, USA). The temperature program started at 50°C for 2 min, then increased at 10°C per min to 280°C. All volatile constituents of >100 pg abundance in two bioactive fractions were considered candidate pheromone components. They were identified by comparing their retention indices (Van den Dool and Kratz 1963) and mass spectra with those reported in the literature [(*E*)-2-hexenal, benzaldehyde, 6-methyl-5-hepten-2-one (sulcatone), octanal, limonene, benzyl alcohol, nonanal, benzyl acetate, decanal, and geranylacetone: Adams 1989; (*E*)-2-octenal: Marques et al. 2000; Staples et al. 2002; (2*E*,4*E*)- and (2*E*,4*Z*)-octadienal: Aldrich et al. 1999; Adolph et al. 2003] and with those of authentic standards.

To determine the enantiomeric composition of limonene emitted by adult male *C. lectularius*, the GC-MS was fitted with a Cyclodex-B™ column (30 m×0.25 mm ID, J&W Scientific), and aliquots of extracts were chromatographed isothermally at 80°C for 15 min. The presence of each enantiomer was determined by comparing retention times and intensity ratios of selected ions (*m*/*z* 93, 121, 136) in the analyte with those of authentic (+)- and (−)-limonene.

Authentic Standards Octanal, nonanal, decanal, 6-methyl-5-hepten-2-one (sulcatone), geranylacetone, benzyl alcohol, benzyl acetate, (+)- and (−)-limonene were purchased from

Sigma-Aldrich (Oakville, ON, Canada). (*E*)-2-Hexenal, (*E*)-2-octenal, and (2*E*,4*E*)-octadienal were purchased from Bedoukian Research (Danbury, CT, USA), and benzaldehyde was purchased from Fisher Scientific (Ottawa, ON, Canada). All commercial compounds were >95% pure.

(2*E*,4*Z*)-Octadienal was synthesized (Fig. 1). 2,3-Dihydropyran (11.45 g) and trifluoroacetic acid (50 µl) were added to *cis*-2-butene-1,4-diol (Aldrich; Fig. 1, compound **1**, 10.00 g, 113.6 mmol). After stirring for 3 hr, ether **2** (7.7 g, 79%) was separated on silica gel (100 g SiO_2, 20–70% ether in hexane as eluent) from unreacted diol **1** and the di-THP derivative. Alcohol **2** (3.0 g, 17.4 mmol) was oxidized to aldehyde **3** by stirring 1 hr in 40 ml of dichloromethane with 5.8 g (26.4 mmol) of pyridinium chlorochromate (PCC) and 3.6 g (26 mmol) of potassium carbonate. Aldehyde **3** was separated from the reaction mixture on silica gel with ether/hexane (1:9) as eluent. Immediately after separation, the *E*- and *Z*-isomers were present in a ratio of 91:9 based on GC. After standing 24 hr in 50 ml dry tetrahydrofuran (THF), only the *E*-isomer of aldehyde **3** (1.7 g, 10.0 mmol, 57.5% yield, >98% pure) was detectable by GC. A solution of aldehyde **3** (0.75 g, 4.40 mmol) in 22 ml THF was then added at −70°C to a stirred suspension (in 100 ml THF) of the ylid generated from butyltriphenylphosphonium bromide (4.0 g, 10.0 mmol) and 10.0 mmol sodium bis-(trimethylsilyl) amide (Aldrich) in 10 ml THF at −30°C to −10°C. The reaction mixture was allowed to warm to 0°C, quenched with water, and the products were extracted with ether/hexane (1:1). The organic layer was washed [water/methanol (3:1), brine], dried, and evaporated. Purification by flash chromatography (10 g silica gel, 30% ether in hexane as eluent) afforded 0.40 g of 1-*O*-tetrahydropyranyl-(2*E*,4*Z*)-octadienol (**4**) (90% pure, GC) to which methanol (100 ml) and trifluoroacetic acid (20 µl) were added. The resulting (2*E*,4*Z*)-octadienol was partially purified by flash chromatography (10 g silica gel, 25–50% ether in hexane as eluent) (0.23 g, 1.82 mmol, >95% by GC) and oxidized in dichloromethane with PCC (2.74 mmol) in the presence of K_2CO_3 (1.37 mmol) to yield 0.15 g of 85% pure (2*E*,4*Z*)-octadienal (**5**). Purification by flash chromatography (10 g silica gel, 5% ether/hexane) afforded 0.12 g of known aldehyde **5** (22% yield based on aldehyde **3**; overall yield, 10%; Adolph et al. 2003; Khrimian 2005). Aldehyde **5** was purified by high performance liquid chromatography (HPLC) using a Waters LC 626 HPLC equipped with a Waters 486 variable-wavelength UV/visible detector set to 210 nm, HP Chemstation software (Rev. A.07.01), and a reversed-phase Nova-Pak® C18 column (60 Å, 4 µm, 3.9× 300 mm, Waters Corporation), eluting with 0.75 ml/min of acetonitrile/water (1:1). Purified aldehyde **5** (>95%) contained only the *E*,*E*-isomer as an impurity.

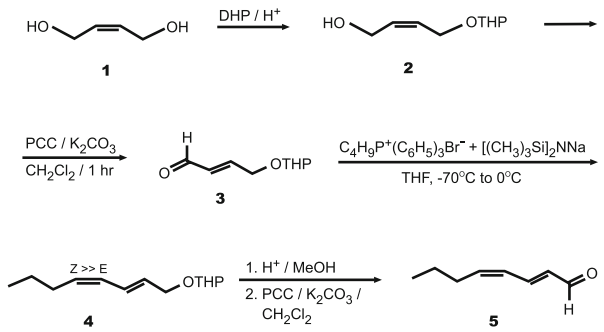

Fig. 1 Scheme for the synthesis of (2*E*,4*Z*)-octadienal

Olfactometer Experiments Responses of *C. lectularius* to test stimuli were bioassayed in still-air olfactometers because *C. lectularius* inhabits enclosed microhabitats with little or no air movement and avoid moving air (Kemper 1936; Johnson 1941; Usinger 1966; E.S., personal observation). The olfactometers consisted of three glass chambers (each 3.5×10 cm ID) linearly interconnected by glass tubes (each 2.5×1 cm ID; Takács and Gries 2001). Test stimuli were randomly assigned to one of the two lateral chambers, each containing a paper towel disc (9 cm diameter; folded eight times for corrugation) with a tab (2.5×0.7 cm) extending through to the central chamber to facilitate inter-chamber movement of bioassay insects. Stimuli were pipetted onto the discs, with treatment and control discs receiving the same amount of solvent. For each of the 23–50 replicates in each of experiments 1–44 (Table 1), one randomly selected 1- to 3-wk post-molt *C. lectularius* was released in the central chamber of the olfactometer 3 hr before the end of scotophase, when *C. lectularius* are most active (Mellanby 1939b). An insect was classed as a responder when it was found 16–18 hr later during photophase on a paper disc or within a lateral glass tube in a state of akinesis. To rigorously compare the effectiveness of test stimuli, experiments were often run in parallel to minimize the effect of any confounding variables, such as weather or time since last feeding. All experiments were conducted at 22–24°C, 25–40% RH, under an 18-hr photoperiod of 3D/15L. After each replicate, olfactometers were washed thoroughly with hot water and Sparklene™ detergent and oven-dried at 125°C for 3 hr.

To determine whether the JAE elicited behavioral responses, experiments 1–3 tested the responses of adult males, adult females, and juveniles (fourth- or fifth-instar) to 10 JAE (~2,000 BHE). Experiment 4 tested the effect of a lower dose (100 BHE) on the response of juveniles. In experiment 5, olfactometers were stacked (3 rows×3 columns) inside yellow plastic bins (38×31×11 cm; Columbia Plastics Ltd.®, Vancouver, BC, CA) covered with dark plastic trays (45×35×2 cm) to determine whether the absence of moving air and directional light improved the response of juveniles to the test stimulus (~200 BHE).

Table 1 Details on experimental insects and stimuli tested in three-chambered olfactometer and cage bioassays (experiments grouped by brackets were run in parallel)

Experiment No.	Treatment Stimulus	Control Stimulus[c]	Responder	Number of Replicates
Three-chambered Olfactometer Bioassays				
1	Porapak Q extract at 2,000 BHE [a,b]	Solvent	Adult ♂	43
2	2,000 BHE[a]	Solvent	Adult ♀	50
3	2,000 BHE[a]	Solvent	Juvenile	40
4	100 BHE[a]	Solvent	Juvenile	40
5	200 BHE	Solvent	Juvenile	36
6	All 5 silica gel fractions recombined	Solvent[d]	Juvenile	30
7	All *minus* fraction 1	Solvent[d]	Juvenile	30
8	All *minus* fraction 2	Solvent[d]	Juvenile	30
9	All *minus* fraction 3	Solvent[d]	Juvenile	30
10	All *minus* fraction 4	Solvent[d]	Juvenile	30
11	All *minus* fraction 5	Solvent[d]	Juvenile	30
12	Synthetic Blend[e] (SB)	Solvent	Juvenile	36
13	SB	Solvent	Adult ♂	45
14	SB	Solvent	Mated adult ♀	45
15	SB	Solvent	Virgin adult ♀	36
16	SB	Solvent	Juvenile	23
17	SB *minus* monoterpenes	Solvent	Juvenile	24
18	SB *minus* benzyl derivatives	Solvent	Juvenile	23
19	SB *minus* ketones	Solvent	Juvenile	23
20	SB *minus* saturated aldehydes	Solvent	Juvenile	23
21	SB *minus* monoene-aldehydes	Solvent	Juvenile	23
22	SB *minus* diene-aldehydes	Solvent	Juvenile	23
23	SB	Solvent	Juvenile	33
24	SB *minus* (*E*)-2-hexenal	Solvent	Juvenile	32
25	SB *minus* henzaldehyde	Solvent	Juvenile	31
26	SB *minus* sulcatone	Solvent	Juvenile	33
27	SB *minus* octanal	Solvent	Juvenile	32
28	SB *minus* (+)-limonene	Solvent	Juvenile	35
29	SB *minus* (–)-limonene	Solvent	Juvenile	32
30	SB *minus* benzyl alcohol	Solvent	Juvenile	32
31	SB *minus* (*E*)-2-octenal	Solvent	Juvenile	30
32	SB *minus* (2*E*,4Z)-octadienal	Solvent	Juvenile	32
33	SB *minus* nonanal	Solvent	Juvenile	32
34	SB *minus* benzyl acetate	Solvent	Juvenile	33
35	SB *minus* decanal	Solvent	Juvenile	30
36	SB *minus* geranylacetone	Solvent	Juvenile	33
37	New Synthetic Blend[f] (NSB)	Solvent	Juvenile	34
38	NSB *minus* (2*E*,4Z)-octadienal	Solvent	Juvenile	34
39	NSB *minus* benzyl acetate	Solvent	Juvenile	34
40	NSB *minus* geranylacetone	Solvent	Juvenile	34
41	NSB *minus* (2*E*,4Z)-octadienal *minus* benzyl acetate	Solvent	Juvenile	34
42	NSB *minus* (2*E*,4Z)-octadienal *minus* geranylacetone	Solvent	Juvenile	34
43	NSB *minus* benzyl acetate *minus* geranylacetone	Solvent	Juvenile	33
44	NSB *minus* (2*E*,4Z)-octadienal *minus* benzylacetate *minus* geranylacetone	Solvent	Juvenile	33
Cage Bioassays				
45	SB	Solvent	10 Juveniles	10
46	SB *plus* Juvenile contact pheromone (720 CPHE[g])	Solvent *plus* Juvenile contact pheromone (720 CPHE)	10 Juveniles	10

[a] Olfactometers not placed in plastic bins for duration of bioassay.
[b] BHE=bug hour equivalents; 1 BHE=volatiles released by one *C. lectularius* during 1 hr.
[c] In all experiments, treatment and control stimuli received the same amount and composition of solvent(s).
[d] Solvent consisted of a 6:4 (*v*/*v*) mixture of pentane and ether.
[e] SB=synthetic blend of 14 components (200 BHE): see Table 2.
[f] NSB=new synthetic blend=SB *minus* octanal.
[g] CPHE=contact pheromone hour equivalents; 1 CPHE=contact pheromone deposited by one *C. lectularius* during 1 hr.

All subsequent still-air olfactometer experiments were conducted according to this protocol. Moreover, juveniles were chosen as bioassay insects because they showed the strongest response to JAE or synthetic blends, and their response was not affected by mating status, as was the case with mated adult females.

To determine candidate pheromone components (see Byers 1992), parallel-run experiments 6–11 tested the five recombined silica gel fractions of JAE vs. blends lacking single fractions. Taking the results of experiments 6–11 and those of GC-MS analyses into account, experiments 12–15 tested a synthetic blend (SB) of 14 candidate pheromone components [octanal, nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, (2*E*,4*Z*)-octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, (+)-limonene, (−)-limonene, sulcatone, and geranylacetone], combined in ratios resembling JAE at 200 BHE (Table 2), for ability to elicit behavioral responses from juveniles, males, mated females, and virgin females.

To identify essential components in the SB, parallel-run experiments 16–22 tested SB vs. blends that lacked groups of organic chemicals, such as monoterpenes (experiment 17), benzyl derivatives (experiment 18), ketones (experiment 19), saturated aldehydes (experiment 20), monoene aldehydes (experiment 21), or diene aldehydes (experiment 22). Similarly, parallel-run experiments 23–36 tested SB vs. blends lacking a single component. (2*E*,4*E*)-Octadienal could not be removed as a single component because it kept forming as a rearrangement product of (2*E*,4*Z*)-octadienal and could not be separated by HPLC. Considering the results of experiments 23–36, parallel-run experiments 37–44 tested a new synthetic blend (NSB; SB *minus* octanal) vs. NSB blends that lacked one, two, or all of the three components (2*E*,4*Z*)-octadienal, benzyl acetate, and geranylacetone.

Table 2 Percentage (mean±SE, n=3) of components in *Cimex lectularius* colony jar aeration extracts relative to the most abundant compound, (*E*)-2-octenal, and quantities present in synthetic blend (SB)

Compound	Relative Ratio[a] (±SE) JAE[c]	Quantity Present in SB[b] (ng) 200 BHE[d]
(*E*)-2-Octenal	100	32.1
(*E*)-2-Hexenal	64.7±5.8	18.4
Benzaldehyde	3.1±1.3	0.7
Sulcatone	2.1±0.6	0.4
Octanal	2.1±0.4	0.6
Limonene	6.5±2.2	1.8[e]
Benzyl alcohol	0.7±0.1	0.6
(2*E*,4*Z*)-Octadienal	0.8±0.2	0.3
Nonanal	6.6±0.2	3.6
(2*E*,4*E*)-Octadienal	1.1±0.4	0.4
Benzyl acetate	0.9±0.1	0.4
Decanal	3.8±0.2	2.0
Geranylacetone	6.6±0.3	2.4

[a] Peak areas were calculated based on the ion current in GC-MS analyses.
[b] Ion current was corrected for instrument response to each standard.
[c] JAE=*C. lectularius* colony jar aeration extract.
[d] BHE=bug hour equivalents; 1 BHE=volatiles released by one *C. lectularius* during 1 hr.
[e] Total quantity of racemic mixture: 0.9 ng of (+)-limonene *plus* 0.9 ng of (−)-limonene.

In experiment 45, the ability of the 14-component SB to attract or arrest juvenile *C. lectularius* was tested in Plexiglas cage (30×30×42 cm) olfactometers, with four mesh-covered holes (18 cm diameter) for ventilation and a sliding door (21×25 cm) for access. Cages were topped with a dark plastic tray (45×35×2 cm) and covered with sheer white silk fabric to diffuse the overhead lighting. The rough (sandpaper-treated) cage floor allowed the insects to walk easily to either of two paper disc shelters (6 cm diameter; folded six times for corrugation) in opposing corners of the cage. The treatment (2,000 BHE) and solvent control stimuli were randomly assigned and pipetted onto these discs, with treatment and control discs receiving the same amount of solvent (pentane). For each of 10 replicates in experiments 45–46 (Table 1), 10 randomly selected 1- to 3-wk post-molt juvenile *C. lectularius* were released in the center of the cage 3 hr before the end of scotophase, and the number of juveniles on each disc was recorded 16–18 hr later during photophase. Insects were classed as responders when they were found on a paper disc in a state of akinesis. All experiments were conducted at 22–24°C, 25–40% RH, under an 18-hr photoperiod of 3D/15L. After each replicate, the cages were sequentially washed with 70% ethanol and hexane and then dried for 5 hr. Follow-up experiment 46 tested whether the response of insects to airborne pheromone in experiment 45 could be enhanced by adding juvenile contact pheromone at 720 CPHE to both treatment and control discs.

Statistical Analyses Numbers of *C. lectularius* responding to treatment and control stimuli in three-chamber olfactometer experiments 1–44 were analyzed with the χ^2 goodness-of-fit test, using Yates correction for continuity (α=0.05; Zar 1999). The mean proportion of juvenile *C. lectularius* that responded to treatment and control stimuli in cage olfactometer experiments 45–46 was analyzed with the Wilcoxon paired-sample test (α=0.05; Zar 1999). Nonresponders were not included in statistical analyses.

Results

Porapak Q extracts of colony jar aerations (JAEs; Fig. 2) tested at 2,000 BHE attracted/arrested females and juveniles (Fig. 3, experiments 2–3) but failed to elicit a significant response from males (Fig. 3, experiment 1). The lower dose of 100 BHE was still significantly effective for juveniles

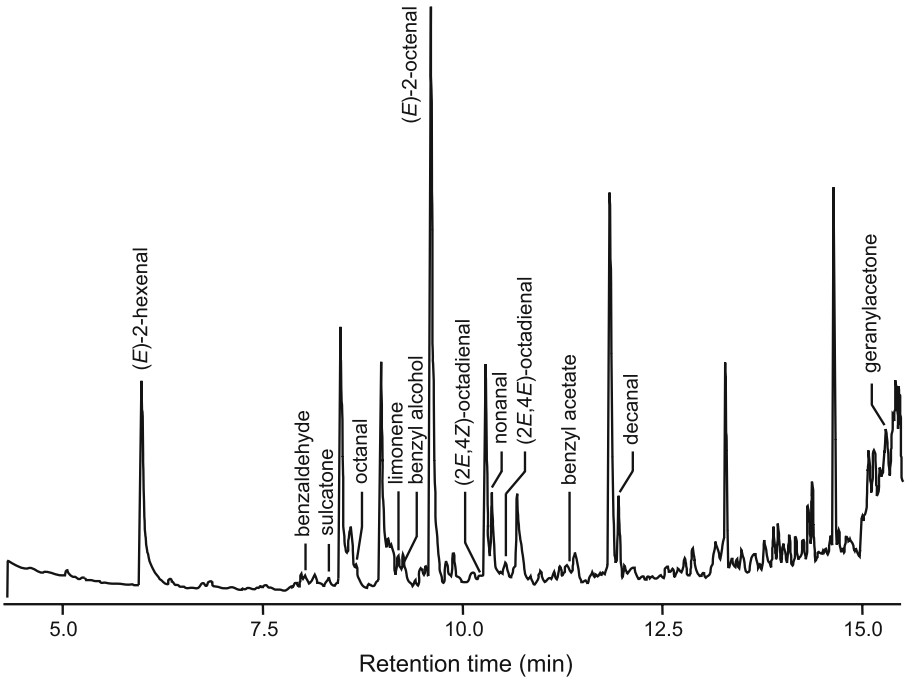

Fig. 2 Representative gas chromatogram of Porapak Q *Cimex lectularius* jar aeration extract. Chromatography: Varian Saturn 2000 Ion Trap GC-MS fitted with a DB-5 column (30 m×0.25 mm ID). Temperature program: 50°C (2 min), 10°C per min to 280°C

(Fig. 3, experiment 4). When bioassay conditions were modified so that olfactometers were housed within plastic bins, juveniles strongly responded to 200 BHE (Fig. 4, experiment 5).

The recombination of all five liquid chromatography fractions of JAE elicited significant responses from juveniles, as did blends lacking fractions 1, 3, or 5 (Fig. 4, experiments 6, 7, 9, and 11). JAE blends lacking either fraction 2 or 4 were not attractive (Fig. 4, experiments 8 and 10), indicating that fractions 2 and 4 contained the bioactive components. The components in fractions 2 and 4 were identified by comparing their GC-retention times and mass spectra with those of authentic standards. The 14 components identified included octanal, nonanal, decanal, (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, (2*E*,4*Z*)-octadienal, benzaldehyde, benzyl alcohol, benzyl acetate, (+)-limonene, (−)-limonene, sulcatone, and geranylacetone (Table 2). A SB of these 14 candidate pheromone components tested at 200 BHE (Table 2) elicited significant responses from juveniles, males, and virgin females (Fig. 5, experiments 12, 13, and 15) but not from mated females (Figure 5, experiment 14). Synthetic blends that lacked monoterpenes, benzyl derivatives, ketones, saturated aldehydes, monoene aldehydes, or diene aldehydes were not attractive (Fig. 6, experiments 16–22). Synthetic blends that lacked either octanal, (2*E*,4*Z*)-octadienal, benzyl acetate, or geranylacetone were still attractive (Fig. 7, experiments 27, 32, 34, and 36), whereas SBs lacking either (*E*)-2-hexenal, benzaldehyde, sulcatone, (+)-limonene, (−)-limonene, benzyl alcohol, (*E*)-2-octenal, nonanal, or decanal were not attractive (Fig. 7, experiments 24–26, 28–31, 33, and 35). A NSB (SB *minus* octanal) unexpectedly failed to attract juveniles (Fig. 8, experiment 37), and NSB blends that lacked (2*E*,4*Z*)-octadienal, benzyl acetate, or both were not active (Fig. 8, experiments 38, 39, and 41). However, all blends that lacked geranylacetone, including the NSB without the four

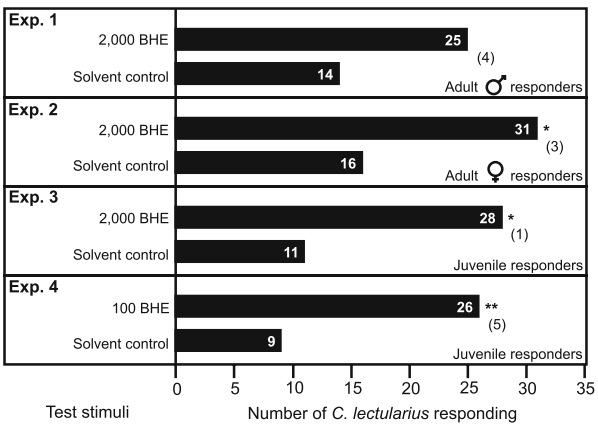

Fig. 3 Response of male (experiment 1), female (experiment 2), and juvenile (experiments 3 and 4) *Cimex lectularius* in three-chambered olfactometers to aliquots [100 or 2,000 bug hour equivalents (BHE)] of Porapak Q colony jar aeration extracts. 1 BHE=volatiles released by one *C. lectularius* during 1 hr. Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; *P<0.05; **P<0.01. The same amount of pentane (7.5 µl) was applied to treatment and control stimuli

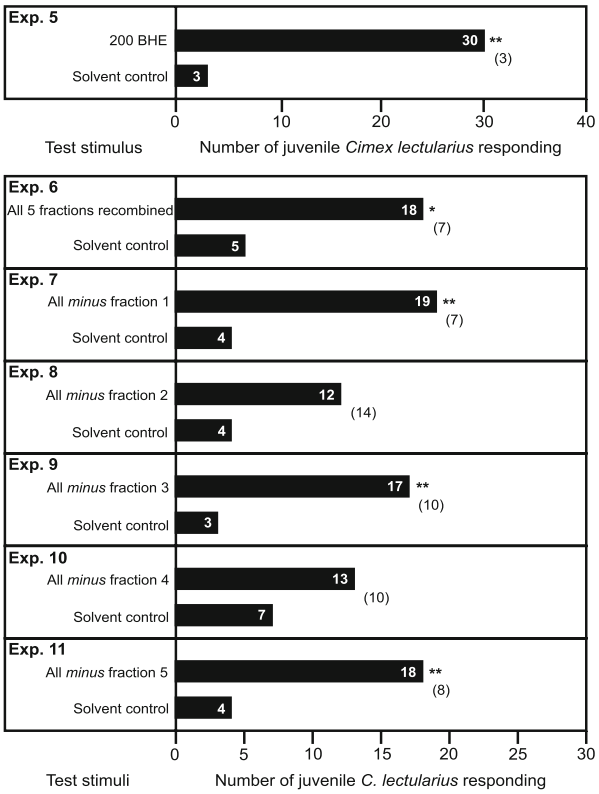

Fig. 4 Response of juvenile *Cimex lectularius* in three-chambered olfactometers to aliquots (200 BHE; see Fig. 2) of Porapak Q colony jar aeration extracts (experiment 5) or to silica gel fractions (200 BHE) of the same extract (experiments 6–11). Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; *$P<0.05$; **$P<0.01$. Experiments 6–11 were run in parallel; the same amount of pentane (10 µl, experiment 5; 50 µl, experiments 6–11) was applied to treatment and control stimuli

components octanal, (2*E*,4*Z*)-octadienal, benzyl acetate, and geranylacetone, elicited significant responses from juveniles (Fig. 8, experiments 40, 42–44).

In cage experiment 45, the proportions of juveniles that responded to SB at 2,000 BHE or to solvent control were not significantly different (Fig. 9). However, when juvenile contact pheromone at 720 CPHE was added to both treatment and control discs (experiment 46), SB at 2,000 BHE attracted/arrested a greater proportion of bioassay insects than did the solvent control (Fig. 9).

Of the 14 components tested in SB, all except benzyl alcohol and benzyl acetate were detected in Porapak Q extracts of adult males and females (Table 3). Adult males appear to release the largest quantity of volatiles (Table 3).

In GC-MS analyses of the limonene enantiomer(s) emitted by adult males, the combined ion (*m/z* 93, 121, and 136) current for (+)- and (−)-limonene revealed a 1:1.3 ratio of the two enantiomers.

Discussion

Our data provide evidence that adult male, virgin adult female, and juvenile *C. lectularius* respond to an airborne aggregation pheromone. With 10 essential components, the pheromone is unusually complex. Such a complex aggregation pheromone has been reported only for cocoon-spinning *Cydia pomonella* larvae (Jumean et al. 2005). Similarly complex sex pheromones occur in 14 species of the Lepidoptera, each deploying pheromone blends of six or more components (Byers 2006). The essential components were identified from juveniles, but we predict that adult males and virgin adult females will respond to the same components because all insects responded similarly to the SB (Fig. 5).

The origin of each of the ten essential pheromone components remains unknown, but (*E*)-2-hexenal, (*E*)-2-octenal, (2*E*,4*E*)-octadienal, benzaldehyde, nonanal, decanal, and sulcatone were all prevalent in headspace volatiles from adult male and adult female *C. lectularius* (Table 3). The trace component benzyl alcohol co-eluted with limonene in GC-MS analyses, rendering its detection and quantification difficult. (+)- and (−)-Limonene were present in above-background quantities in aerations only of adult males (Table 3), suggesting that males are the primary or sole emitters of limonene enantiomers.

The bioassay protocol took into account that *C. lectularius* are active during the scotophase and most active during the last few hours before photophase (Mellanby 1939b). Therefore, bioassays commenced 3 hr before

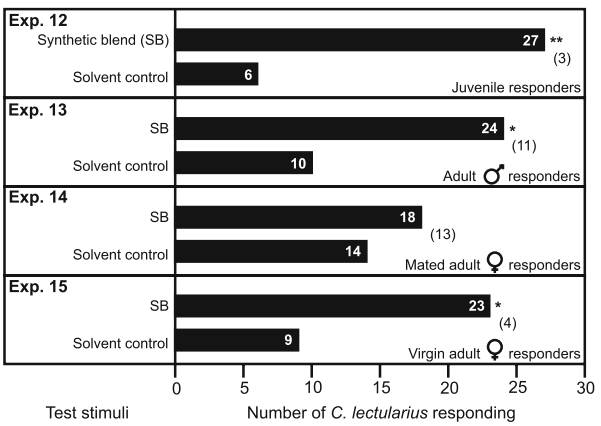

Fig. 5 Response of juvenile (experiment 12), male (experiment 13), mated female (experiment 14), and virgin female (experiment 15) *Cimex lectularius* in three-chambered olfactometers to a synthetic blend (*SB*) of 14 candidate pheromone components at 200 BHE. Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; *$P<0.05$; **$P<0.01$. Composition of SB is listed in Table 2. The same amount of pentane (10–15 µl) was applied to treatment and control stimuli

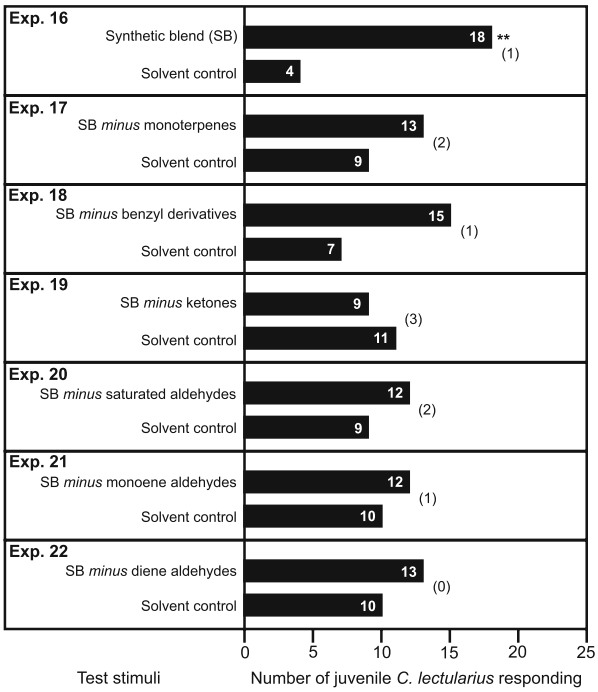

Fig. 6 Response of juvenile *Cimex lectularius* in three-chambered olfactometer experiments 16–22 to synthetic blends (*SB*) at 200 BHE (see Table 2), lacking groups of candidate pheromone components. Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; $*P<0.05$; $**P<0.01$. All experiments were run in parallel; the same amount of pentane (15 µl) was applied to treatment and control stimuli

photophase, when *C. lectularius* typically seek hosts and then return to their shelters. Levinson and Bar Ilan (1971) observed that *C. lectularius* sought shelter during the first 4 hr after release and then remained relatively still for the next 16 hr. In preliminary overnight experiments with three-chambered still-air olfactometers, we observed that bioassay insects did not change chambers after they became inactive, usually within the first hour of the photophase. They would remain for the duration of the bioassay in the chamber that they selected as a resting or aggregation site.

The concern that the ratio of synthetic pheromone components released from paper towel discs may differ from that of natural pheromone components released by insects was addressed by comparative analyses of headspace volatiles from both sources. These analyses revealed no significant difference in the blend composition of natural and synthetic pheromone.

The sensitivity of *C. lectularius* to their aggregation pheromone cannot be determined from this study. However, response of juveniles to JAEs with a 20-fold difference in applied dose (Fig. 3, experiments 3 and 4) and response of adults to JAE (Fig. 3, experiments 1–2) or SB (Fig. 5, experiments 13 and 15) that differed 10-fold in dose suggest that the airborne aggregation pheromone is effective over a concentration range of at least one order of magnitude.

The two most abundant aggregation pheromone components in *C. lectularius*, (*E*)-2-hexenal and (*E*)-2-octenal, have previously been reported as alarm pheromone (Levinson and Bar Ilan 1971; Levinson et al. 1974a,b). Their threshold for eliciting alarm behavior [1.3–2.3 µg/ml air for (*E*)-2-hexenal; 0.25–0.29 µg/ml air for (*E*)-2-octenal; measured at time of alarm reaction] greatly exceeds the physiological detection threshold, which for (*E*)-2-hexenal was estimated by receptor potential response to dose range to be 2×10^{10} molecules/ml air [3.25×10^{-6} µg/ml air] (Levinson et al. 1974b). The detection threshold for (*E*)-2-octenal would be similar because it elicited receptor potentials at the same dose as (*E*)-2-hexenal (Levinson et al. 1974b). In our study, assuming that all applied pheromone components evaporated and were contained within a single olfactometer chamber, the levels tested could not have exceeded 9.50×10^{-4} µg/ml air of (*E*)-2-hexenal or 1.56×10^{-3} µg/ml air of (*E*)-2-octenal. These levels are significantly lower than the alarm response threshold but higher than the proposed physiological detection threshold. These data and calculations suggest that (*E*)-2-hexenal and (*E*)-2-octenal serve as multifunctional pheromone components that are attractive/arrestant at

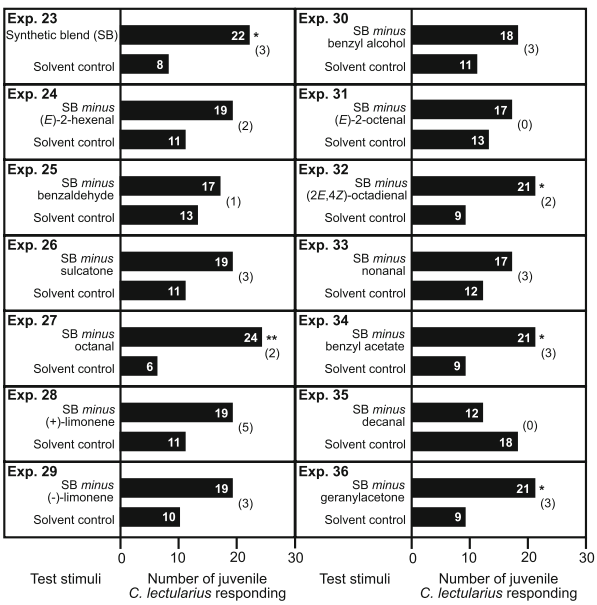

Fig. 7 Response of juvenile *Cimex lectularius* in three-chambered olfactometer experiments 23–36 to synthetic blends (*SB*) at 200 BHE (see Table 2), lacking single candidate pheromone components. Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; $*P<0.05$; $**P<0.01$. All experiments were run in parallel; the same amount of pentane (15 µl) was applied to treatment and control stimuli

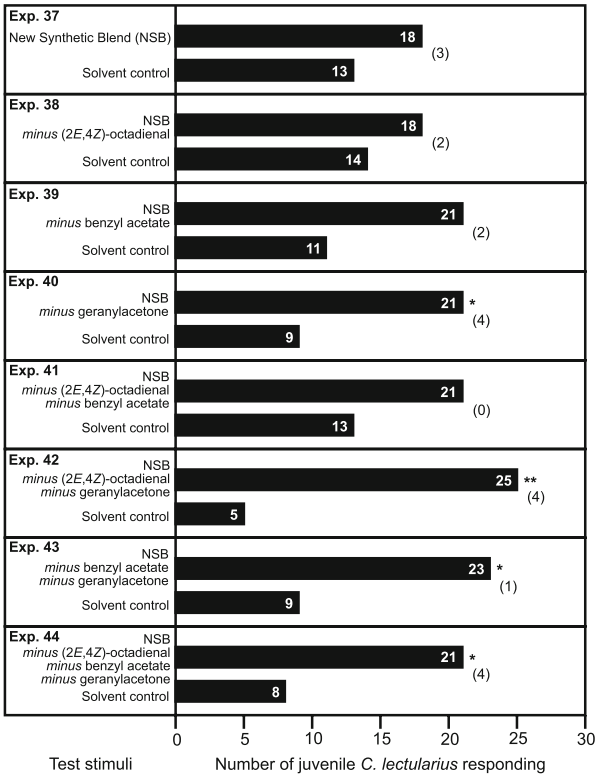

Fig. 8 Response of juvenile *Cimex lectularius* in three-chambered olfactometer experiments 37–44 to a new synthetic blend (*NSB*; blend as listed in Table 2 but without octanal) at 200 BHE, lacking one or more candidate pheromone components. Number of insects responding to each stimulus is given *within bars*; number of insects not responding in each experiment is given *in parentheses*. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; χ^2 test with Yates correction for continuity; $^*P<0.05$; $^{**}P<0.01$. All experiments were run in parallel; the same amount of pentane (10 µl) was applied to treatment and control stimuli

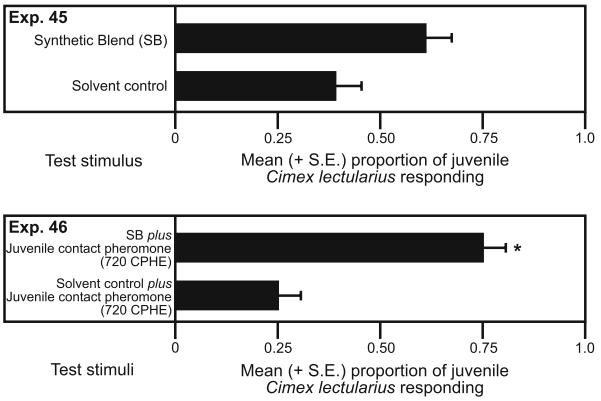

Fig. 9 Mean (+SE) proportion of 10 juvenile *Cimex lectularius* per replicate (10 replicates) responding in cage olfactometer experiments 45 and 46 to a synthetic blend (SB; see Table 2) at 2,000 BHE (experiment 45) or SB in combination with *C. lectularius* juvenile contact pheromone at 720 contact pheromone hour equivalents (CPHE; experiment 46). 1 CPHE=contact pheromone deposited by one *C. lectularius* during 1 hr. For each experiment, an *asterisk* indicates a significant response to the treatment stimulus; Wilcoxon paired-sample test; $^*P<0.05$. The same amount of pentane (50 µl) for SB application or methanol (100 µl) for CPHE application was applied to treatment and control stimuli

low concentrations but repellent at high concentrations (Borden 1985; Blum 1996). Multifunctional pheromones have also been reported in other Hemiptera, such as *Eurydema rugosa* (Ishiwatari 1974, 1976) and *Nezara viridula* (Lockwood and Story 1985).

Pheromones that mediate aggregation behavior may attract and/or arrest conspecifics. Our three-chambered and cage olfactometer experiments did not allow us to discern between attraction and arrestment responses. Anemotactic response to pheromone would demonstrate unambiguously long-range attractiveness, but such responses would be difficult to obtain because *C. lectularius* avoid moving air (Kemper 1936; Johnson 1941; Usinger 1966). However, results of cage experiments (Fig. 9) imply that the airborne aggregation pheromone may function mainly as an attractant. In the relatively large bioassay cage where treatment and control paper discs were not confined and sheltered, as they were in the small three-chambered olfactometers (experiments 1–44), SB alone appeared insufficient to attract and arrest a significant proportion of juveniles (experiment 45). However, when the juvenile contact pheromone (Siljander et al. 2007) was added to both treatment and control discs, SB was preferred significantly over the solvent control (experiment 46). If the juvenile contact pheromone were to arrest juveniles at the site of their first choice, then the airborne aggregation pheromone must have functioned as an attractant. In natural settings, the airborne aggregation pheromone may direct refuge-seeking *C. lectularius* toward established harborages,

Table 3 Percentage of components in aerations of adult male, adult female, and juvenile *Cimex lectularius*, relative to the most abundant compound, (*E*)-2-octenal, emitted by males

Compound	Relative Ratios[a]		
	Male	Female	Juvenile
(*E*)-2-Octenal	100	21.4	1.6
(*E*)-2-Hexenal	9.9	2.3	–[b]
Benzaldehyde	0.1	Trace	–
Sulcatone	0.5	0.2	Trace
Octanal	0.9	0.2	0.1
Limonene	0.2	Trace	Trace
Benzyl alcohol	–	–	–
(2*E*,4*Z*)-Octadienal	0.2	0.1	Trace
Nonanal	9.3	1.1	0.7
(2*E*,4*E*)-Octadienal	0.4	0.1	0.0
Benzyl acetate	–	–	–
Decanal	1.7	1.1	0.4
Geranylacetone	1.2	0.7	Trace

[a] Peak areas were calculated based on ion current in GC-MS analyses.
[b] A dash (–) indicates that the component was not detected in GC-MS analyses.

whereas the stage-specific contact pheromones arrest them at the source.

Aggregation pheromones likely have evolved due to the Allee effect, which is a positive relationship between any component of individual fitness and either numbers or density of conspecifics (Stephens et al. 1999; Wertheim et al. 2005). The use of pheromones by *C. lectularius* to locate aggregations of conspecifics within refugia may benefit individuals by marking harborages with suitable microclimates, potential mates, or protection from predators. Aggregated *C. lectularius* are less sensitive to desiccation (Benoit et al. 2007), and aggregated juveniles may also benefit from a higher rate of development at lower temperatures, a phenomenon observed in nymphs of *N. viridula* (Lockwood and Story 1986). Moreover, aggregation may facilitate mate-finding for adults, which do not appear to possess a sex pheromone that mediates mate attraction, and adults in aggregations may benefit from communal calling that increases the active space of pheromone (Wertheim et al. 2005). Finally, individual *C. lectularius* in aggregations may benefit from increased vigilance for natural enemies, which would help explain the use of both aggregation and alarm pheromones (Wertheim et al. 2005).

The pheromone elicited behavioral responses from juveniles, males, and virgin females (Fig. 5; experiments 12, 13, and 15), but not mated females. The fact that mated females did not respond to pheromone (Fig. 5; experiment 14) might be attributed to the injurious nature of mating in *C. lectularius*. During traumatic insemination, the male pierces the abdomen of the female with a needle-like intromittent organ, the paramere, and releases sperm into her body cavity (Carayon 1966). Females have a specialized structure (spermalege) that receives sperm and contributes to wound-healing and defense against infection, but frequent rematings reduce longevity and reproductive success (Mellanby 1939a; Stutt and Siva-Jothy 2001; Morrow and Arnqvist 2003; Reinhardt et al. 2003). Considering that females may have little or no control over remating frequency (Stutt and Siva-Jothy 2001), gravid females may avoid repeated traumatic insemination by seeking new refugia devoid of conspecifics. Dispersal of gravid females would explain observations that most nonaggregated individuals in natural populations are females (E.S., unpublished; R. Cooper, personal communication). It would also explain the spread of populations. Wingless *C. lectularius* are reliant on host transportation from place to place on clothing, luggage, and furniture (Usinger 1966; Doggett et al. 2004; Reinhardt and Siva-Jothy 2007). The likelihood of transportation on any of these objects depends upon *C. lectularius* actively seeking new refugia, as opposed to returning to an old one. Therefore, mated females not returning to established harborages and exploiting new refugia may be passively dispersed more often than males or juveniles. This hypothesis is supported by findings that most dispersing bat and bird bugs detected on the bodies of host bats or birds are females (Loye 1985; Heise 1988; Brown and Brown 2005; Reinhardt and Siva-Jothy 2007). Whether this concept applies to *C. lectularius* is unknown and would require interception data (Doggett et al. 2004) including stage, gender, and mating status of passively transported specimens.

The increased numbers of juvenile *C. lectularius* recorded as positive responders to a combination of airborne plus contact aggregation pheromone than to airborne aggregation pheromone alone in our bioassays (Fig. 9) can be attributed to the contact pheromone, which arrested responding insects and reduced further movement between shelters. We are currently investigating whether a suitable harborage in lieu of contact pheromone would be equally effective in eliciting aggregation responses in *C. lectularius*. If so, field testing could determine whether traps baited with airborne aggregation pheromone can detect the presence of *C. lectularius* in human dwellings with greater sensitivity than current methods.

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References

ADAMS, R. P. 1989. Identification of Essential Oils by Ion Trap Mass Spectroscopy. Academic, San Diego, CA.

ADOLF, S., POULET, S. A., and POHNERT, G. 2003. Synthesis and biological activity of α,β,γ,δ-unsaturated aldehydes from diatoms. *Tetrahedron* 59:3003–3008.

ALDRICH, J. R., OLIVER, J. E., TAGHIZADEH, T., FERREIRA, J. T. B., and LIEWEHR, D. 1999. Pheromones and colonization: reassessment of the milkweed bug migration model (Heteroptera: Lygaeidae: Lygaeinae). *Chemoecology* 9:63–71.

BENOIT, J. B., DEL GROSSO, N. A., YODER, J. A., and DENLINGER, D. L. 2007. Resistance to dehydration between bouts of blood feeding in the bed bug, *Cimex lectularius*, is enhanced by water conservation, aggregation, and quiescence. *Am. J. Trop. Med. Hyg.* 76:987–993.

BLUM, M. S. 1996. Semiochemical parsimony in the Arthropoda. *Annu. Rev. Entomol.* 41:353–374.

BOASE, C. 2001. Bedbugs: Back from the brink. *Pesticide Outlook* 12:159–162.

BORDEN, J. H. 1985. Aggregation pheromones, pp. 257–285, in G. A. KERKUT, and L. I. GILBERT (eds.). Comprehensive Insect Physiology Vol. 9 Pergamon, Oxford, GB.

BROWN, C. R., and BROWN, M. B. 2005. Between-group transmission dynamics of the swallow bug, *Oeciacus vicarius*. *J. Vector Ecol.* 30:137–143.

BYERS, J. A. 1992. Optimal fractionation and bioassay plans for isolation of synergistic chemicals: the subtractive-combination method. *J. Chem. Ecol.* 18:1603–1621.

BYERS, J. A. 2006. Pheromone component patterns of moth evolution revealed by computer analysis of the *Pherolist*. *J. Anim. Ecol.* 75:399–407.

CARAYON, J. 1966. Traumatic insemination and the paragenital system, pp. 81–166, in R. L. USINGER (ed.). Monograph of Cimicidae (Hemiptera–Heteroptera). Entomological Society of America, College Park, Maryland.

DOGGETT, S. L., GEARY, M. J., and USSELL, R. C. 2004. The resurgence of bed bugs in Australia: with notes on their ecology and control. *Environ. Health* 4:30–38.

HEISE, G. 1988. Zum Transport von Fledermauswanzen (Cimicidae) durch ihre Wirte. *Nyctalus* 2:469–473.

ISHIWATARI, T. 1974. Studies on the scent of stink bugs (Hemiptera: Pentatomidae) I. Alarm pheromone activity. *Appl. Entomol. Zool.* 9:153–158.

ISHIWATARI, T. 1976. Studies on the scent of stink bugs (Hemiptera: Pentatomidae) II. Aggregation pheromone activity. *Appl. Entomol. Zool.* 11:38–44.

JOHNSON, C. G. 1941. The ecology of the bed-bug, *Cimex lectularius* L., in Britain. *J. Hyg.* 41:345–461.

JUMEAN, Z., GRIES, R., UNRUH, T., ROWLAND, E., and GRIES, G. 2005. Identification of the larval aggregation pheromone of codling moth, *Cydia pomonella*. *J. Chem. Ecol.* 31:911–924.

KEMPER, H. 1936. Die Bettwanze und ihre Bekämpfung. *Z. Kleintierk. Pelztierk.* 12:1–107.

KHRIMIAN, A. 2005. The geometric isomers of methyl-2,4,6-decatrienoate, including pheromones of at least two species of stink bugs. *Tetrahedron* 61:3651–3657.

KRUEGER, L. 2000. Don't get bitten by the resurgence of bed bugs. *Pest Control* 68:58–64.

LEVINSON, H. Z., and BAR ILAN, A. R. 1971. Assembling and alerting scents produced by the bedbug, *Cimex lectularius* L. *Experientia* 27:102–103.

LEVINSON, H. Z., LEVINSON, A. R., and MASCHWITZ, U. 1974a. Action and composition of the alarm pheromone of the bedbug *Cimex lectularius* L. *Naturwissenschaften* 61:684–685.

LEVINSON, H. Z., LEVINSON, A. R., MÜLLER, B., and STEINBRECHT, R. A. 1974b. Structure of sensilla, olfactory perception, and behavior of the bedbug, *Cimex lectularius*, in response to its alarm pheromone. *J. Insect Physiol.* 20:1231–1248.

LOCKWOOD, J. A., and STORY, R. N. 1985. Bifunctional pheromone in the first instar of the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae): its characterization and interaction with other stimuli. *Ann. Entomol. Soc. Am.* 78:474–479.

LOCKWOOD, J. A., and STORY, R. N. 1986. Adaptive function of nymphal aggregation in the southern green stink bug, *Nezara viridula* (L.) (Hemiptera: Pentatomidae). *Environ. Entomol.* 15:739–749.

LORENZO, M. G., and LAZZARI, C. R. 1996. The spatial pattern of defecation in *Triatoma infestans* and the role of faeces as a chemical mark of the refuge. *J. Insect Physiol.* 42:903–907.

LORENZO FIGUEIRAS, A. N., KENIGSTEN, A., and LAZZARI, C. R. 1994. Aggregation in the haematophagous bug *Triatoma infestans*: Chemical signals and temporal pattern. *J. Insect Physiol.* 40:311–316.

LORENZO FIGUEIRAS, A. N., and LAZZARI, C. R. 1998. Aggregation in the haematophagous bug *Triatoma infestans*: a novel assembling factor. *Physiol. Entomol.* 23:33–37.

LOYE, J. E. 1985. The life history and ecology of the cliff swallow bug, *Oeciacus vicarius* (Hemiptera: Cimicidae). *CORSTOM Ser. Entomol. Méd. Parasitol.* 23:133–159.

MARQUES, F. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Female-produced sex pheromone of the predatory bug *Geocoris punctipes*. *J. Chem. Ecol.* 26:2843–2855.

MARX, R. 1955. Über die Wirtsfindung und die Bedeutung des artspezifischen Duftstoffes bei *Cimex lectularius* Linné. *Z. Parasitenk.* 17:41–72.

MELLANBY, K. 1939a. Fertilization and egg production in the bed-bug, *Cimex lectularius* L. *Parasitology* 31:193–199.

MELLANBY, K. 1939b. The physiology and activity of the bed-bug (*Cimex lectularius*) in a natural infestation. *Parasitology* 31:200–211.

MONTES, C., CUADRILLERO, C., and VILELLA, D. 2002. Maintenance of a laboratory colony of *Cimex lectularius* (Hemiptera: *Cimicidae*) using an artificial feeding technique. *J. Med. Entomol.* 39:675–679.

MORROW, E. H., and ARNQVIST, G. 2003. Costly traumatic insemination and a female counter-adaptation in bed bugs. *Proc. R. Soc. Lond. Biol. Sci.* 270:2377–2381.

PANAGIOTAKOPULU, E., and BUCKLAND, P. C. 1999. *Cimex lectularius* L., the common bed bug from Pharaonic Egypt. *Antiquity* 73:908–911.

REINHARDT, K., NAYLOR, R., and SIVA-JOTHY, M. T. 2003. Reducing a cost of traumatic insemination: female bedbugs evolve a unique organ. *Proc. R. Soc. Lond. Biol. Sci.* 270:2371–2375.

REINHARDT, K., and SIVA-JOTHY, M. T. 2007. Biology of the bed bugs (Cimicidae). *Annu. Rev. Entomol.* 52:351–374.

SCHOFIELD, C. J., and PATTERSON, J. W. 1977. Assembly pheromone of *Triatoma infestans* and *Rhodnius prolixus* nymphs. *J. Med. Entomol.* 13:727–734.

SILJANDER, E., PENMAN, D., HARLAN, H., and GRIES, G. 2007. Evidence for male- and juvenile-specific contact pheromones of the common bed bug *Cimex lectularius*. *Entomol. Exp. Appl.* 125:215–219.

STAPLES, J. K., KRALL, B. S., BARTELT, R. J., and WHITMAN, D. W. 2002. Chemical defense in the plant bug *Lopidea robiniae*. *J. Chem. Ecol.* 28:601–615.

STEPHENS, P. A., SUTHERLAND, W. J., and FRECKLETON, R. P. 1999. What is the Allee effect? *Oikos* 87:185–190.

STUTT, A. D., and SIVA-JOTHY, M. T. 2001. Traumatic insemination and sexual conflict in the bed bug *Cimex lectularius*. *Proc. Natl. Acad. Sci. U.S.A.* 98:5683–5687.

TAKÁCS, S., and GRIES, G. 2001. Communication ecology of webbing clothes moth: evidence for male-produced aggregation signal(s). *Can. Entomol.* 133:725–727.

USINGER, R. L. 1966. Monograph of Cimicidae (Hemiptera–Heteroptera). Entomological Society of America, College Park, Maryland.

VAN DEN DOOL, H., and KRATZ, P. D. 1963. A generalization of the retention index system including linear temperature-programmed gas-liquid partition chromatography. *J. Chromatogr.* 2:463–471.

WERTHEIM, B., VAN BAALEN, E. A., DICKE, M., and VET, L. E. M. 2005. Pheromone-mediated aggregation in nonsocial arthropods: an evolutionary ecological perspective. *Annu. Rev. Entomol.* 50:321–346.

ZAR, J. H. 1999. Biostatistical Analysis. Prentice-Hall, New Jersey.

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Sex Pheromone of the Plant Bug, *Phytocoris calli* Knight

Qing-He Zhang · Jeffrey R. Aldrich

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Abstract Female *Phytocoris calli* Knight produce a sex pheromone from metathoracic scent glands. The pheromone consists of hexyl acetate (HA; present in both sexes), with the female-specific compounds (*E*)-2-hexenyl acetate (E2HA), octyl acetate (OA), and (*E*)-2-octenyl acetate (E2OA). HA and E2OA are key components of the pheromone, since deletion of either ester from the blend resulted in a total suppression of conspecific male trap catches. However, the binary blend of HA and E2OA was only slightly attractive to males, and was significantly less active than the four-component blend. The two ternary blends, HA/OA/E2OA and HA/E2HA/E2OA, were each as attractive as the full four-component blend. Evidence from previous research on the pheromones of *Phytocoris* species suggests that the apparent chemical redundancy in the pheromone of *P. calli* may actually be involved in maintaining reproductive isolation from other sympatric species. The patterns observed for pheromones of the five *Phytocoris* species whose pheromones have been directly (*P. californicus*, *P. relativus*, *P. difficilis*, and *P. calli*) or indirectly (*P. breviusculus*) studied are discussed vis-à-vis the pheromone intractable species of *Lygus* and *Lygocoris* plant bugs.

Keywords Electroantennogram · Plant bug · *Lygus* · Infochemicals · Metathoracic scent gland · Heteroptera · Miridae

Q.-H. Zhang (✉)
Sterling International, Inc.,
3808 N. Sullivan Rd, Bldg 16P,
Spokane, WA 99216, USA
e-mail: qing-he@rescue.com

J. R. Aldrich
Invasive Insect Biocontrol & Behavior Laboratory, USDA-ARS,
BARC-West, B-007, 10300 Baltimore Ave.,
Beltsville, MD 20705, USA

Introduction

Plant bugs (Miridae) are the most speciose heteropteran family that includes some 10,000 described members. The mirid genus *Phytocoris* is among the largest of the plant bug genera, containing about 650 species (Schuh and Slater 1995), 200 of which occur in western North America (Stonedahl 1988). Many *Phytocoris* are predacious, with mottled color patterns that render them cryptic on the bark of trees they inhabit. Some species, however, are serious pests of fruit and nuts (Wheeler 2001). Despite the abundance and agricultural importance of plant bugs, only 10 or so sex pheromones of mirids have been identified (Millar 2005). Millar (2005), somewhat casually, noted that mirid pheromones fall into two groups: "The first (e.g., *Campylomma verbasci* and *Phytocoris* spp.) is characterized by pheromones that were straightforward to identify, whereas pheromones of the second group, including *Lygus* and *Lygocoris* spp., have proven to be remarkably intractable despite decades of effort by numerous research groups." Since Millar's review, Innocenzi et al. (2005) reported that females of the European tarnished plant bug, *Lygus rugulipennis* Poppius, attract males with a blend of *(E)*-4-oxo-2-hexenal and hexyl butyrate, with the key to synthetic pheromone activity being the slow release of the active compounds; at higher release rates, the *(E)*-4-oxo-2-hexenal/ hexyl butyrate blend did not attract bugs (Innocenzi et al. 2004). For rice leaf bug, *Trigonotylus caelestialium* (Kirkaldy) (Miridae), high pheromone concentrations were also inhibitory (Kakizaki and Sugie 2001).

The first sex pheromone identifications of *Phytocoris* spp. were for two western US species, *P. californicus* (Millar and Rice 1998) and *P. relativus* (Millar et al. 1997). Our elucidation (Zhang and Aldrich 2003a) of the pheromone of the eastern US species, *Phytocoris difficilis*, and

concomitant inferences to the pheromone composition of the sympatric species, *P. breviusculus*, by default make the sex pheromones of *Phytocoris* spp. the best known of all 1400 mirid genera (Schuh and Slater 1995).

The present pheromone investigation of a fifth member of the genus *Phytocoris* was undertaken after the fortuitous discovery of this species by one of us (Q-HZ) in residential Spokane, WA, USA. Although *P. calli* is apparently not an economically significant pest or predator, we undertook the study to expand our understanding of the pheromone systems of these insects, and perhaps, to obtain new information that may suggest fruitful approaches to deciphering the pheromones of species in the group of "intractable" mirids.

Methods and Materials

Adult Insects and Preparation of Extracts *Phytocoris calli* adults were collected from residential porch lights around 9:30 P.M.—midnight during the summer of 2007 in Spokane, WA, USA. All bugs were dissected within 10–20 hr of capture for extraction of the metathoracic scent glands (MSG) and subsequent electrophysiological and chemical analyses. MSGs from male and female *P. calli* were excised from CO_2-anesthetized bugs submerged in tap water, and the glands were extracted individually in 50 µl of methyl-*tert*-butyl ether. Extracts were kept at −20 °C until analysis.

Gas Chromatography-Electroantennogram Detector (GC-EAD) and GC-Mass Spectrometry (GC-MS) Analyses Mirid extracts were analyzed in the splitless mode with a Varian CP-3800 GC equipped with a polar column (HP-INNOWax; 30 m×0.53 mm×1.0 µm; Agilent Technologies), and a 1:1 effluent splitter that allowed simultaneous flame ionization and electroantennogram detection of separated compounds. Helium was used as the carrier gas, and the injector temperature was 220 °C. The column temperature was 50 °C for 1 min, rising to 240 °C at 10 °C min^{-1}, and then held for 10 min. The outlet for the electroantennogram detector (EAD) was held in a humidified air-stream flowing at 0.5 m sec^{-1} over the antennal preparation. A glass capillary indifferent electrode was filled with Beadle-Ephrussi Ringer solution (Zhang et al. 2000), grounded via a silver wire, and inserted into the open side of an excised mirid head. A similar recording electrode, connected to a high-impedance DC amplifier with automatic baseline drift compensation, was inserted over an antenna (the tip of the antenna was excised). Antennal signals were stored and analyzed on a computer equipped with a serial IDAC interface box and the program EAD ver. 2.5 (Syntech, Hilversum, The Netherlands). The MSG extracts were analyzed by GC-MS on an HP 6890 GC series coupled with an HP 5973 Mass Selective Detector by using the same type of GC column and conditions as described above. Compounds were identified by comparison of retention times and mass spectra with those of standards.

Chemical Standards The following authentic standards were obtained from commercial sources or were synthesized: 1-hexanol, (*E*)-2-hexenol, (*E*)-2-octenol, nonanal, hexyl acetate (HA), hexyl propanoate, octyl acetate (OA), and octyl butyrate (Aldrich Chemical Co., Milwaukee, WI, USA); (*E*)-2-octenyl acetate (E2OA), hexyl butyrate, (*E*)-2-hexenyl butyrate, (Bedoukian Research Inc., Danbury, CT, USA); heptyl acetate (Eastman Organics); and (*Z*)-3-octenyl acetate (J. G. Millar, Univ. California, Riverside). (*E*)-2-Hexenyl acetate (E2HA) was synthesized as described previously (Aldrich et al. 1997, 1999).

Field Trapping Two field-trapping experiments were carried out from August to September 2007 in a residential backyard with several apple, cherry, and other angiosperm trees that are common in residential Spokane, WA. Pherocon VI traps (Trécé Inc., Adair, OK, USA) with removable sticky inserts and baited with 10–80 µl of individual or mixed neat test compounds loaded onto gray rubber septa (5 mm sleeve-type, The West Co., Lititz, PA, USA) were used in the trials. Rubber septa were replaced every week. Traps were hung 1.5 m above the ground on a fence, ca. 5 m apart within each trap line. For each trapping experiment, one set of traps was deployed with their initial trap positions being random. The trap positions were systematically rotated after each visit, based on a procedure of Latin-square design (Byers 1991), so that traps appeared at least once per location. To minimize positional effects, mirid collections and trap rotations were carried out when ≥ 2 mirids were caught in a trap. Each replicate lasted several days to 1 wk, depending on mirid flight activity. Sticky inserts were taken to the laboratory for recording of species, gender, and number of bugs. Experiment 1 (August 7 to September 11, 2007) tested six treatments: a mixture of the EAD-active female MSG volatiles (4-compound "full blend"), subtraction of each EAD-active component from the full blend, and a blank. Experiment 2 (August 25 to September 24, 2007) was conducted to determine the potential activity of the two key EAD-active compounds, hexyl acetate, (*E*)-2-octenyl acetate, and their binary blend in comparison with the four-compound "full blend".

Statistical Analysis Trap catch data were transformed by log $(x+1)$ to fit the assumption of homogeneity of variance for analysis of variance (ANOVA). Means were compared by ANOVA followed by the Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test (SPSS 8.0 for Windows) at $\alpha = 0.05$ (Day and Quinn 1989).

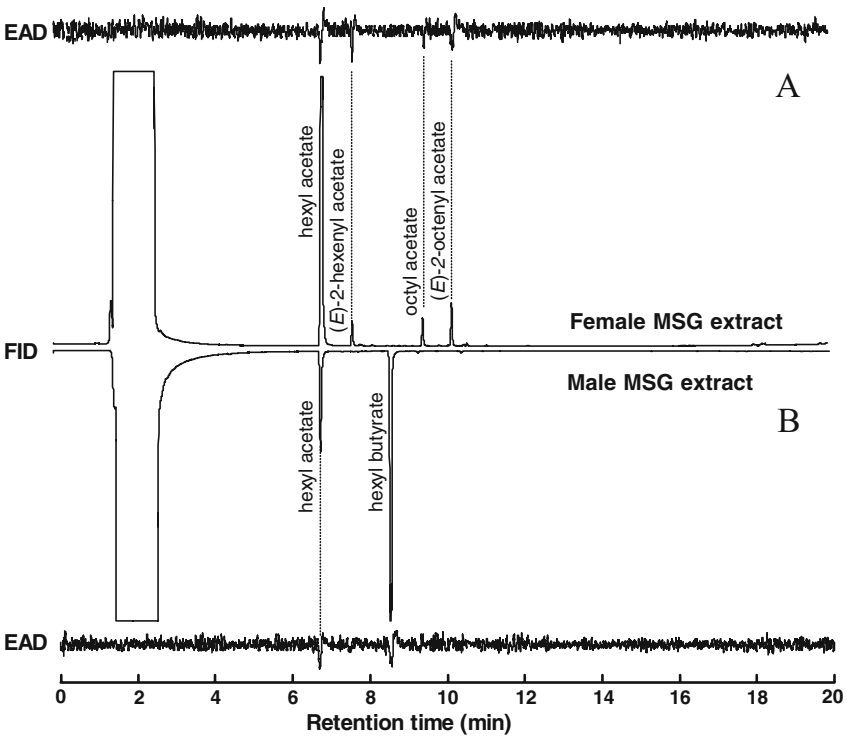

Fig. 1 Coupled flame ionization (FID) and electroantennogram (EAD) detection of *Phytocoris calli* male antennae to metathoracic scent gland (MSG) extracts of conspecific females (A) and males (B)

Results

GC-EAD and Chemical Identifications Antennae of *P. calli* males responded strongly to one major and three minor components from female MSG extracts. These compounds were identified by GC-MS as HA, E2HA, OA, and E2OA (in a ratio of ca. 20:1:1:1), respectively (Fig. 1A and Table 1). In male MSG extracts, there were only two major volatile components, HA and hexyl butyrate, each of which elicited strong responses from male antennae (Fig. 1B, Table 1). In addition to the EAD-active compounds, several other minor or trace amounts of chemicals also were

Table 1 Metathoracic scent gland components identified from *Phytocoris calli*

Chemical	Relative amount (%) ± SE		Male EAD activity
	Females (N=3)	Males (N=3)	
Hexyl acetate	**86.87±0.57**	**25.36±7.01**	***
***(E)*- 2-Hexeny acetate**	**3.16±0.37**	0.05±0.03	***
Hexyl propanoate		0.51±0.44	
1-Hexanol	0.81±0.16	0.19±0.08	
Heptyl acetate	0.05±0.03	0.02±0.02	
Nonanal		0.10±0.03	
(E)-2-Hexenol	0.28±0.21		
Hexyl butyrate	0.21±0.10	**73.05±7.56**	***
(E)- 2-Hexeny butyrate		0.46±0.05	
Octyl acetate	**3.58±0.41**		***
(Z)-3-Octenyl acetate	0.10±0.02		
***(E)*-2-Octenyl acetate**	**4.82±0.30**		***
(E)-2-Octenol	0.11±0.01		
Octyl butyrate		0.26±0.06	

Data for major and EAD-active compounds are in bold.

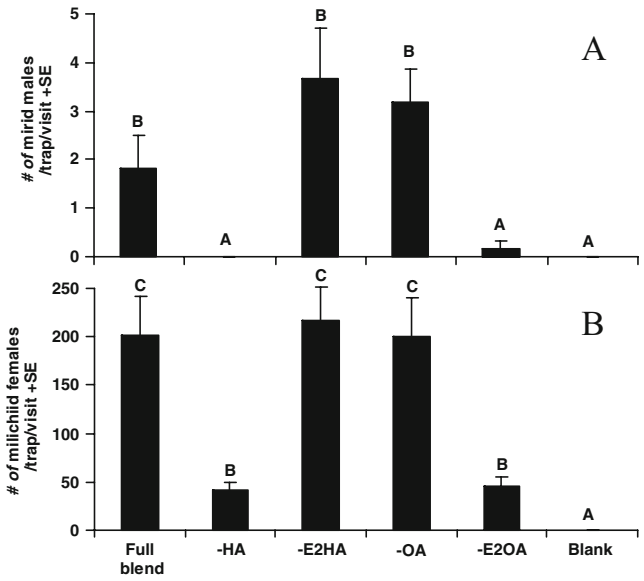

Fig. 2 Captures of *Phytocoris calli* males (A) and *Leptometopa latipes* females (B) in traps baited with full blend [HA (50 µl)/E2HA (10 µl)/OA (10 µl)/E2OA (10 µl)] or ternary blends of the four EAD-active female MSG volatiles (*HA* hexyl acetate, *E2HA* (*E*)-2-hexenyl acetate, *OA* octyl acetate, *E2OA* (*E*)-2-octenyl acetate). Means (N=6) followed by the same letter are not significantly different (P>0.05), ANOVA on log (x+1), followed by the Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test

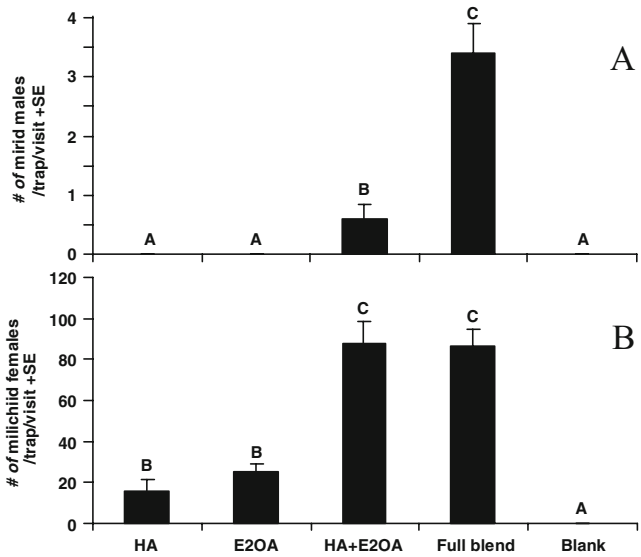

Fig. 3 Captures of *Phytocoris calli* males (A) and *Leptometopa latipes* females (B) in traps baited with hexyl acetate (50 µl), (*E*)-2-octenyl acetate (10 µl), and their binary blend (50 µl+10 µl) in comparison with the four-compound "full blend" (HA [50 µl]/E2HA [10 µl]/OA [10 µl]/E2OA [10 µl]). Means (N=5) followed by the same letter are not significantly different (P>0.05), ANOVA on log (x+1), followed by the Ryan–Einot–Gabriel–Welsh (REGW) multiple Q test

identified by GC-MS, including 1-hexanol and heptyl acetate from both sexes, (*E*)-2-hexenol, (*E*)-2-octenol, and (*Z*)-3-octenyl acetate from females, and hexyl propanoate, nonanal, (*E*)-2-hexenyl butyrate, and octyl butyrate from males (Table 1).

Field Experiments In experiment 1, traps baited with the blend of the four EAD-active components ("full blend") of female MSG volatiles (HA/E2HA/OA/E2OA) caught significant numbers of *P. calli* males (P<0.05, Fig. 2A). Subtraction of E2HA or OA from the four-component "full blend" had no effect on trap catches. However, deletion of HA or E2OA from the blend entirely eliminated catches of *P. calli* males (Fig. 2A), indicating that these two compounds may be key components of the pheromone. In experiment 2, traps baited with HA or E2OA alone caught a non-significant number of bugs, while traps baited with the binary blend caught some *P. calli* males, but significantly (>4 times) less in than traps baited with the four-component "full-blend" (Fig. 3A).

In addition to male bugs, large numbers of female milichiid flies (*Leptometopa latipes* Meigen) were captured in most traps. As for catches of mirid males, traps baited with the "full-blend" caught large numbers of *L. latipes* females, and deletion of E2HA or OA from the four-component "full blend" had no effect on the catches of flies. However, removal of HA or E2OA from the blend dramatically reduced trap catches of the milichiid females (Fig. 2B). Traps baited with HA or E2OA alone caught low numbers of milichiid females; however, the binary blend of these two compounds elicited much greater trap catch than did either compound individually (Fig. 3B). There was no difference in catches of milichiid females between traps baited with the binary blend or the four-compound "full-blend.".

Most mirids (males from traps or lights or females from lights) were collected or trapped during the first half of the scotophase (after sunset to midnight), whereas milichiids were captured exclusively during the daytime.

Discussion

Phytocoris sex pheromones, including that of *P. calli*, consist of HA, which is produced by both sexes, plus related female-specific esters (Millar 2005) (Table 2). Pheromone compounds are produced by adult females in the MSG (Zhang and Aldrich 2003a). Redundant pheromone esters in sympatric *Phytocoris* species (Millar et al. 1997; Millar and Rice 1998) and *P. calli* may help maintain reproductive isolation, although more research is needed to verify whether this possibility is correct or not.

Traps baited with HA and E2OA also were strongly attractive to the scavenging milichiid fly, *Leptometopa latipes* (Fig. 3B). Similar synergistic attraction between hexyl butyrate and *(E)*-2-hexenyl butyrate to *L. latipes* also was reported in a previous study of the tarnished plant bug, *Lygus lineolaris* (Zhang and Aldrich 2004). These results

Table 2 Known sex pheromone components (*), sex attractants (o), and an attraction-inhibitor (x) of *Phytocoris* mirids

Chemicals	*P. relativus*	*P. californicus*	*P. difficilis*	*P. breviusculus*	*P. calli*
Hexyl acetate	*	*	*	o	*
(*E*)-2-Hexenyl acetate			*	x	*
Octyl acetate					*
(*E*)-2-Octenyl acetate		*	*	o	*
(*E*)-2-Octenyl butyrate	*	x			
References	1	2	3	3	4

1 Millar et al., 1997; *2* Millar and Rice, 1998; *3* Zhang and Aldrich 2003a; *4* this paper.

suggest that milichiid females may use pheromonal compounds from plant bugs as kairomones to find freshly injured or dead bugs on which to feed.

The major volatiles from males, whether from aeration or MSG dissection, are almost identical among the five *Phytocoris* species studied, with hexyl butyrate being the dominant component (Millar et al. 1997; Millar and Rice 1998; Zhang and Aldrich 2003a, b) (Table 1). Hexyl butyrate elicited strong EAD responses from antennae of male *P. calli* (this paper), *P. difficilis*, and *P. breviusculus* (Zhang and Aldrich 2003a, b). For *P. difficilis*, we demonstrated that hexyl butyrate totally stopped attraction of males to the female-produced sex pheromone. We consider this signal to be a natural anti-sex pheromone, perhaps that repels other males from further mating attempts, or for precluding a male from remating with a female. A similar inhibitory effect of hexyl butyrate also has been reported in two other mirids, *Lygocoris pabulinus* (Groot et al. 2001) and *Lygus lineolaris* (Zhang et al. 2007). It remains to be determined whether hexyl butyrate is an anti-sex pheromone for *P. calli*. The phenomenon of a male-to-male inhibitory pheromone was reported long ago as one likely mode of action for hair pencil secretions of male Lepidoptera (Hirai et al. 1978).

Intractable and easily identified mirid pheromones are all female-produced, and apparently based on compounds (especially esters) biosynthesized in the MSG (Millar 2005). The MSG is characteristic of "true bugs" (Heteroptera) and is usually considered to be a defensive gland (Aldrich 1988). However, males or females of various seed bugs (Lygaeidae) (Aldrich et al. 1997, 1999; Marques et al. 2000) and broad-headed bugs (Alydidae) (Leal et al. 1995, 1996; Aldrich et al. 2000) release attractant pheromones from the MSG, while retaining the ability to release chemical irritants from the MSG when attacked by predators. In most bugs, the MSG consists of a pair of lateral accessory glands and a median reservoir in which the principal irritants (aldehydes and acids) are enzymatically derived from esters produced in the accessory glands (Aldrich et al. 1978). The sexual role of the MSG is evidently associated with compartmentalization of the MSG, such that the insects are able to release pheromone directly from the lateral accessory glands or accumulate defensive compounds in the median reservoir until needed for defense.

In mirids, species of *Phytocoris* and *Campylomma* seem to express the sexual manifestations of the MSG primarily, with little capacity to produce aldehydic or acidic allomones. At the other extreme is the aposematic mirid, *Lopidea robiniae* (Uhler), with a MSG secretion dominated by *(E)*-2-hexenal (Staples et al. 2002). Judging by the coexistence of *(E)*-4-*oxo*-2-hexenal with hexyl and *(E)*-2-hexenyl butyrates in the MSG of *Lygus* bugs (Aldrich et al. 1988; Innocenzi et al. 2004), the defensive and sexual roles of the MSG seem more entwined for *Lygus* bugs than is the case for *Phytocoris* spp., thus complicating the task of deciphering the sex pheromones.

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References

ALDRICH, J. R., BLSUM, M. S., HEFETZ, A., FALES, H. M., LLOYD, H. A., and ROLLER, P. 1978. Proteins in a nonvenomous defensive secretion: biosynthetic significance. *Science* 201:452–454.

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J. R., LUSBY, W. R., KOCHANSKY, J., HOFFMANN, M. P., WILSON, L. T., and ZALOM, F. G. 1988. *Lygus* bug pheromones vis-à-vis stink bugs., pp. 213–216, in Proc. Beltwide Cotton Prod. Res. Conf. National Cotton Council, Memphis TN, New Orleans, LA.

ALDRICH, J. R., LEAL, W. S., NISHIDA, R., KHRIMIAN, A. P., LEE, C.-J., and SAKURATANI, Y. 1997. Semiochemistry of aposematic seed bugs (Heteroptera: Lygaeidae). *Entomol. Exp. Appl.* 84:127–135.

ALDRICH, J. R., OLIVER, J. E., TAGHIZADEH, T., FERREIRA, J. T. B., and LIEWEHR, D. 1999. Pheromones and colonization: Reassessment of the milkweed bug migration model (Heteroptera: Lygaeidae: Lygaeinae). *Chemoecology* 9:63–71.

ALDRICH, J. R., ZHANG, A., and OLIVER, J. E. 2000. Attractant pheromone and allomone from the metathoracic scent gland of a broad-headed bug (Hemiptera: Alydidae). *Can. Entomol.* 132:915–923.

BYERS, J. A. 1991. BASIC algorithms for random sampling and treatment randomization. *Comput. Biol. Med.* 21:69–77.

DAY, R. W., and QUINN, G. P. 1989. Comparisons of treatments after an analysis of variance in ecology. *Ecol. Monographs* 59:433–463.

GROOT, A. T., DRIJFHOUT, F. P., HEIJBOER, A., VAN BEEK, T. A., and VISSER, J. H. 2001. Disruption of sexual communication in the mirid bug *Lygocoris pabulinus* by hexyl butanoate. *Agric. For. Entomol.* 3:49–55.

HIRAI, K., SHOREY, H. H., and GASTON, L. K. 1978. Competition among courting male moths: male to male inhibitory pheromone. *Science* 202:644–645.

INNOCENZI, P. J., HALL, D. R., MASUH, H., PHYTHIAN, S. J., CHITTAMARU, S., GUARINO, S., and CROSS, J. V. 2004. Investigation of long-range female sex pheromone of the European tarnished plant bug, *Lygus rugulipennis*: Chemical, electrophysiological, and field studies. *J. Chem. Ecol.* 30:1509–1529.

INNOCENZI, P. J., CROSS, J. V., HESKETH, H., and HALL, D. 2005. Attraction of male European tarnished plant bug, *Lygus rugulipennis* to components of the female sex pheromone in the field. *J. Chem. Ecol.* 31:1401–1413.

KAKIZAKI, M., and SUGIE, H. 2001. Identification of female sex pheromone of the rice leaf bug, *Trigonotylus caelestialium*. *J. Chem. Ecol*. 27:2447–2458.

LEAL, W. S., HIGUCHI, H., MIZUTANI, N., NAKAMORI, H., KADOSAWA, T., and ONO, M. 1995. Multifunctional communication in *Riptortus clavatus* (Heteroptera: Alydidae): Conspecific nymphs and egg parasitoid *Ooencyrtus nezarae* use the same adult attractant pheromone as chemical cue. *J. Chem. Ecol.* 21:973–985.

LEAL, W. S., UEDA, Y., and ONO, M. 1996. Attractant pheromone for male rice bug, *Leptocorisa chinensis*: Semiochemicals produced by both male and female. *J. Chem. Ecol.* 22:1429–1437.

MARQUES, F. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Female-produced sex pheromone of the predatory bug *Geocoris punctipes*. *J. Chem. Ecol.* 26:2843–2856.

MILLAR, J. G., RICE, R. E., and WANG, Q. 1997. Sex pheromone of the mirid bug *Phytocoris relativus*. *J. Chem. Ecol.* 23:1743–1754.

MILLAR, J. G., and RICE, R. E. 1998. Sex pheromone of the plant bug *Phytocoris californicus* (Heteroptera: Miridae). *J. Econ. Entomol.* 91:132–137.

MILLAR, J. G. 2005. Pheromones of true bugs. *Topics Curr. Chem.* 240:37–84.

SCHUH, R. T., and SLATER, J. A. 1995. True Bugs of the World (Hemiptera: Heteroptera): Classification and Natural History. Comstock Publ. Associates, Ithaca, New York.

STAPLES, J. K., KRALL, B. S., BARTELT, R. J., and WHITMAN, D. W. 2002. Chemical defense in the plant bug *Lopidea robiniae* (Uhler). *J. Chem. Ecol.* 28:601–615.

STONEDAHL, G. M. 1988. Revision of the mirine genus *Phytocoris* Fallén (Heteroptera: Miridae) for western North America. *Bull. Am. Mus. Nat. Hist.* 188:1–257.

WHEELER, A. G. J. 2001. Biology of the Plant Bugs (Hemiptera: Miridae): Pests, Predators, Opportunists. Cornell University Press, Ithaca.

ZHANG, Q.-H., SCHLYTER, F., and BIRGERSSON, G. 2000. Bark volatiles from nonhost angiosperm trees of spruce bark beetle, *Ips typographus* L. (Coleoptera: Scolytidae): Chemical and electrophysiological analysis. *Chemoecology* 10:69–80.

ZHANG, Q.-H., and ALDRICH, J. R. 2003a. Pheromones of milkweed bugs (Heteroptera: Lygaeidae) attract wayward plant bugs: *Phytocoris* mirid sex pheromone. *J. Chem. Ecol.* 29:1835–1851.

ZHANG, Q.-H., and ALDRICH, J. R. 2003b. Male-produced anti-sex pheromone in a plant bug. *Naturwissenschaften* 90:505–508.

ZHANG, Q.-H., and ALDRICH, J. R. 2004. Attraction of scavenging chloropid and milichiid flies (Diptera) to metathoracic scent gland compounds of plant bugs (Heteroptera: Miridae). *Environ. Entomol.* 33:12–20.

ZHANG, Q.-H., CHAUHAN, K. R., ZHANG, A., SNODGRASS, G. L., DICKENS, J. C., and ALDRICH, J. R. 2007. Antennal and behavioral responses of *Lygus lineolaris* (Palisot de Beauvois) (Heteroptera: Miridae) to metathoracic scent gland compounds. *J. Entomol. Sci.* 42:92–104.

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Interspecific Pheromone Plume Interference Among Sympatric Heliothine Moths: A Wind Tunnel Test Using Live, Calling Females

Jonathan P. Lelito · Andrew J. Myrick · Thomas C. Baker

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Abstract Three species of North American heliothine moths were used to determine the level at which interspecific female interference of male attraction to conspecific females occurs. We used live calling females of *Heliothis virescens*, *H. subflexa*, and *Helicoverpa zea*, as lures for conspecific males in a wind tunnel, and then placed heterospecific females on either side of the original species such that the plumes of the three females overlapped downwind. In nearly all combinations, in the presence of heterospecific females, fewer males flew upwind and contacted or courted the source than when only conspecific females were used in the same spatial arrangement. Males did not initiate upwind flight to solely heterospecific female arrangements. Our results show that the naturally emitted pheromone plumes from heterospecific females of these three species can interfere with the ability of females to attract conspecific males when multiple females are in close proximity. However, the fact that some males still located their calling, conspecific females attests to the ability of these male moths to discriminate point source odors by processing the conflicting information from interleaved strands of attractive and antagonistic odor filaments on a split-second basis.

Keywords *Heliothis* · *Helicoverpa* · Pheromone blend · Behavioral antagonist · *Z*11-16:Ald · *Z*9-16:Ald · *Z*11-16:OH · Z9-14:Ald · Flight behavior · Olfactory orientation

J. P. Lelito (✉) · A. J. Myrick · T. C. Baker
104 Chemical Ecology Laboratory, Center for Chemical Ecology, Department of Entomology, The Pennsylvania State University, University Park, PA 16802, USA
e-mail: jpl207@psu.edu

Introduction

The heliothine moths are a well-studied group of crop pests in North America. The two most economically important species are the tobacco budworm, *Heliothis virescens* Fabricius (Lepidoptera: Noctuidae), and the corn earworm, *Helicoverpa zea* Boddie. A third species, which is a pest of tomatillo in Mexico, is *Heliothis subflexa* Guenée. All three of these moths share the same major pheromone blend component, (*Z*)-11-hexadecenal (*Z*11-16:Ald), but differ in the other components of their respective blends. In *H. virescens*, the secondary component is (*Z*)-9-tetradecenal (*Z*9-14:Ald; Roelofs et al. 1974; Tumlinson et al. 1975; Klun et al. 1980b; Pope et al. 1982; Vetter and Baker 1983; Teal et al. 1986), while for *H. zea*, the secondary component is (*Z*)-9-hexadecenal (*Z*9-16:Ald; Klun et al. 1979, 1980a; Vetter and Baker 1984; Pope et al. 1984). Finally, in *H. subflexa*, secondary components include *Z*9-16:Ald, (*Z*)-11-hexadecen-1-ol (*Z*11-16:OH), and (*Z*)-11-hexadecenyl acetate (Z11-16:Ac; Teal et al. 1981; Klun et al. 1982; Heath et al. 1990, 1991; Vickers 2002; Groot et al. 2006).

The secondary components of one species of moth can act as behavioral antagonists if they are added to the sex pheromone blend of another species (Cardé et al. 1977; Löfstedt 1990, 1993; Löfstedt et al. 1991; Gries et al. 1996; Potting et al. 1999; Quero and Baker 1999), often most strongly where the species in question are sympatric and synchronic (Cardé et al. 1977; Guerin et al. 1984; Gemeno et al. 2000; McElfresh and Millar 1999, 2001; Gries et al. 2001; El-Sayed et al. 2003; Groot et al. 2007). This is expected as a result of selection against mating mistakes by males in areas where the species co-occur.

These antagonistic effects are well known in wind tunnel studies for the heliothine moths, mainly from adding the

heterospecific secondary components onto the same point source as the synthetic conspecific pheromone blend (Vickers et al. 1991; Vickers and Baker 1997; Fadamiro and Baker 1997; Fadamiro et al. 1999). Both Z11-16:OH and Z11-16:Ac from the *H. subflexa* blend antagonize the attraction of male *H. virescens* (Vetter and Baker 1983, 1984; Vickers and Baker 1997) and *H. zea* (Teal et al. 1984; Quero and Baker 1999; Quero et al. 2001). However, the antagonistic effects caused by the coemission of a heterospecific compound from the same point source does not necessarily mean that a confluent plume from a nearby heterospecific female would interfere with the attraction of a male to a conspecific female whose plume was being overlapped. Plumes are comprised of individual strands of odor interspersed with pockets of clean air, the strands having been sheared off at the source and later shredded into finer substrands by microturbulence (Murlis 1986).

Moths have an exceptionally good ability to fly upwind to and locate sources of their pheromone, even when sources of behaviorally antagonistic compounds are placed 10 cm away to create confluent plumes (Witzgall and Priesner 1985; Liu and Haynes 1994; Baker et al. 1998; Fadamiro et al. 1999). For instance, *H. virescens* males were able to discriminate strands of their two-component pheromone from strands of the antagonist Z11-16:Ac when the strands were experimentally generated to be separated by only 1 mm (Baker et al. 1998; Fadamiro et al. 1999). When the antagonist was coemitted in every strand along with the pheromone, however, attraction was negligible, and the suppression of attraction was apparent on a strand-by-strand basis in the behavior of flying males in both *H. virescens* (Vickers and Baker 1997) and *H. zea* (Quero et al. 2001).

Our goal in the current study was to examine the behavior of males in a wind tunnel in response to live, calling conspecific females in the close presence of live, calling heterospecific females. The experiments examine the effects of heterospecific blends that arrived downwind on flying males' antennae in naturally mixed or staggered strands. Using each of the six species pairs possible, we tested the hypothesis that the sex pheromone plumes from heterospecific live heliothine moths may interfere with the attraction of males to their conspecific females when the time-averaged plumes are known to overlap. However, when sufficient proportions of heterospecific strands might arrive asynchronously on the antenna with conspecific strands, we hypothesized that low levels of attraction to conspecific females should still occur.

Methods and Materials

Insects Moths of the three species used in this experiment were maintained over many generations on a modified pinto bean diet (*H. zea* and *H. virescens*; Shorey and Hale 1965) and a corn–soy diet (*H. subflexa*; Vickers 2002). Single larvae were placed into individual diet-filled cups, removed at pupation, and sexed under a dissecting scope. Sexed pupae of each species were placed into screen cages lined with paper toweling and provided with a 10% sucrose solution for adults to feed on upon eclosion. Each day, any live, properly formed adults of either sex were removed and placed into a separate cage, sorted by species. Male and female moths of the three species were kept in separate Percival environmental chambers (Percival Scientific, Boone, IA, USA) at 29–30°C and 50% or higher relative humidity, under a reversed light cycle, 16:8-h light/dark, with the scotophase starting at 9:30 A.M. Dead and malformed moths were discarded daily, and the sucrose–water dispenser was refilled. The entire screen cage was sprayed twice daily (just before lights-off and just after lights-on) with distilled water. All male moths were between 3 and 6 d of age, and all female moths were between 2 and 6 d of age when utilized for these experiments.

Behavioral Assays The wind tunnel, with a width of 1.2 m, a length of 2.8 m, and a height of 1 m at its peak (a bowed shape based on Miller and Roelofs 1978), was set to a wind speed of 60 cm s^{-1}, the room darkened to 2.96 lx (2.20 lx of red light and 0.76 lx of white light) emanating from eight overhead incandescent light sources (four red, four white) connected to a variable transformer. Just before the onset of the scotophase, 15 male moths of each of the three species, in individual 6 by 6-cm metal screen cages, were placed into the wind tunnel itself. At the same time, at least 10 female moths of each species were placed into individual metal screen cages, in an adjacent fume hood in the darkened wind tunnel room to prevent the males from being exposed to pheromone prior to flight. All flights were conducted in the last 4 hr of the scotophase, giving the moths several hours to adjust to the conditions in the wind tunnel room.

Control flights were performed each day to judge the response level of males of each species to conspecific females under our wind tunnel conditions. Three calling females of the same species were placed in individual metal screen cylinders (4 cm diameter×6 cm height) on a metal screen pedestal 35 cm above the wind tunnel floor in a triangular arrangement with the apex of the three-female triangle facing upwind (Fig. 1). The separation between the two downwind cages was only 2–3 cm, and thus the maximum distance that plumes from the calling females (depending on where they were situated when calling) would be separated by was 4 cm. Females were placed into the wind stream and allowed to acclimate for up to 10 min, by which time they had resumed calling. Once all three

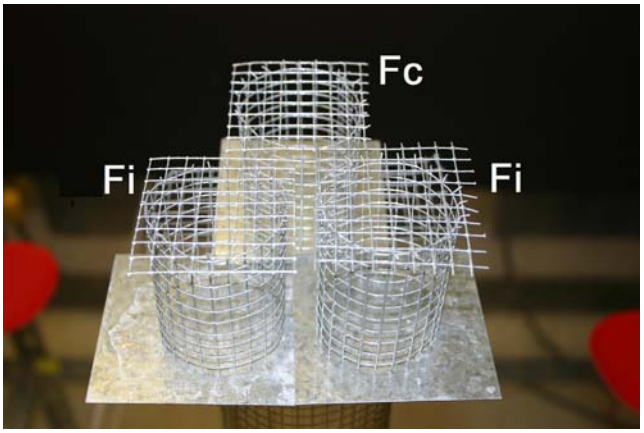

Fig. 1 Arrangement of female cages in the control and experimental wind tunnel experiments conditions. *Fc* indicates the conspecific female position. *Fi* indicates the interference female positions for the experimental treatments; during control flights for each species, all three positions are occupied by conspecific females. The direction of air movement in the wind tunnel is from *top* to *bottom* in the image

females were visibly calling, males were released in the center of the wind tunnel by hanging their cage, open end upwind, on a pedestal 35 cm high, 1 m directly downwind from the females. Each male was given up to 2 min to respond, after which nonresponders' cages were removed, while responding males were captured and removed with a net. Between each male test, the females were observed, and in the few instances when they were no longer visibly calling, they were replaced with another individual from the fume hood; the new females were given time to resume calling if the move from the hood to the wind tunnel had disturbed them. Males of a given species were also tested against three females of each of the other two species. Each individual male was exposed only to one of these treatments and then discarded.

For each male, we scored his progress toward completion of the entire flight sequence: "no flight," "wing fanning," "flight," "casting," "locked on plume," "half the distance to source," "10 cm from source," "contact source," and "hair pencils and courtship." The temperature at the beginning and end of the flight period was averaged to yield the temperature recorded for that day.

After the control flights were complete, experimental flights were performed. These were conducted in a manner similar to the control flights, except that the apical female in the triangular arrangement was a conspecific of the male being tested, and the other two cages held individual heterospecific females of the same species (Fig. 1). The same protocol of observing the females in a calling posture and replacement of noncalling ones was used. Males again were given 2 min to respond. Experimental flights were conducted such that individual females of both "interfering" species were assessed; that is, male *H. subflexa* were tested to *H. subflexa* females under interference from two *H. virescens* and subsequently from two *H. zea* females. As before, an individual male of a given species only was exposed to one experimental treatment and not used again.

For simplicity, treatments will hereafter be referred to by three-letter combinations. For example, the array of three *H. subflexa* females would be abbreviated as "SSS," while an array comprised of one *H. subflexa* female in the presence of two *H. zea* females would be abbreviated "ZSZ," because this annotation most closely reflects the spatial arrangement of the species of females on the platform.

Plume Strand Analyses The degree to which plume strands of females were or were not coincident in their arrival downwind was measured by using cages of calling females placed in the positions on the platform as described above. We used calling females of *Trichoplusia ni* and *H. virescens* and the Quadroprobe four-antenna biosensor (Park et al. 2002) on which two *T. ni* male antennae and two *H. virescens* antennae were placed on the four-channel simultaneous electroantennogram (EAG) probe. The probe was situated in the wind tunnel 1 m downwind of the calling females where males had been released earlier in the behavioral experiments. The system for odor classification, using a computer algorithm (Myrick et al. 2005; Hetling et al. 2006), was first trained to classify the plume strands from calling *T. ni* and *H. virescens* females, as well as a synthetic mixture of 10 μg of each of the two major components of both species emitted from a filter paper with greater than 95% accuracy for all training odors. *T. ni* and *H. virescens* females were used due to the need to discriminate the major pheromone components of females so closely placed together in upwind cages. Threshold for detection "events" (EAGs) from plume strands was set at 50 μV. Readings from the Quadroprobe were then taken in the confluent plumes from calling *T. ni* and *H. virescens* females from 1 m downwind using the caged female configurations described above (Fig. 1). EAGs from the calling females of the three heliothine moth species used in behavioral experiments could not be placed in different classes due to the predominant EAG responses resulting from the major component of all three species, Z11-16:Ald. A total of 83s (195 strands), 33s (85 strands), and 33s (135 strands) was used for the Quadroprobe algorithm training sessions in the plumes from *H. virescens* females, *T. ni* females, and the synthetic mixture, respectively.

Statistical Analyses A Fisher's exact test was used to compare each category of male response among female treatments within each species of male assessed (Fisher 1922). Chi-square contingency tests (χ^2) were used to examine the difference in response rates between contacting

the source and extending hair pencils for each species of male, under each treatment (Steel and Torrie 1960).

Results

Males of all three species exhibited a significant reduction in at least one of their upwind flight attraction behaviors when two heterospecific females were positioned next to their own female, compared to when three conspecific females were present (Figs. 2, 3, 4, 5, 6, and 7). No males of any species responded with attraction-related behaviors to an array of three heterospecific females; it is important to note that none even "locked on" to the plumes of heterospecific females (Figs. 2, 3, 4, 5, 6, and 7).

The presence of either *H. subflexa* or *H. zea* females on either side of a *H. virescens* female significantly reduced the upwind flight behavior of the *H. virescens* males, starting with the "locking on" stage (Figs. 2 and 3). At subsequent stages of upwind flight, reductions caused by the heterospecific females were not as severe, and several of the *H. virescens* males were able to land at the cage of the centrally located, conspecific female (Figs. 2 and 3).

It is interesting to note that the effects of heterospecific females seemed to carry over to the courtship stage after males had landed, even though the pheromone components they were exposed to near the cage were more likely to be only conspecific. Seventy-one percent of *H. virescens* males that contacted the conspecific female array also exhibited the hair pencil courtship display, whereas in the ZVZ and SVS treatments, the courtship responses were significantly lower, 44.4% and 37.5%, respectively ($p<0.05$; Chi-square 2×2 test of independence).

Similarly, the presence of either *H. virescens* or *H. zea* females significantly reduced the upwind flight behavior of *H. subflexa* males to a conspecific, starting downwind at the "locking on" to the plume stage (Figs. 4 and 5). Several of the *H. subflexa* males were able to land at the cage of the centrally located conspecific female (Figs. 4 and 5).

The effects of heterospecific females around *H. subflexa* females seemed to carry over to the courtship stage. For *H. subflexa* males flying to the SSS treatment, 41.2% of those that contacted the source also exhibited the hair pencil courtship display. However, in response to the VSV and ZSZ treatments, the percentages of males that had contacted the source that also exhibited courtship was significantly lower, 0% and 20%, respectively ($p<0.05$; Chi-square 2×2 test of independence).

H. zea males did not respond as well to conspecific females as did *H. virescens* and *H. subflexa*, but their responses to either of the heterospecific female controls (SSS and ZZZ) were significantly lower (Figs. 6 and 7). The presence of heterospecific females on either side of a *H. zea* female significantly reduced the upwind flight behavior of the *H. zea* males, with the interference from *H. subflexa* females (Fig. 6) being greater than that of *H. virescens* females (Fig. 7), in that, in the latter case, only "locking on" to the plume stage was affected. *H. subflexa* females interfered with male *H. zea* attraction to *H. zea* females at every stage. For *H. zea*, such a low percentage of males landed on their conspecific female cages that no analysis of the subsequent hair pencil displays of these landed males would be meaningful.

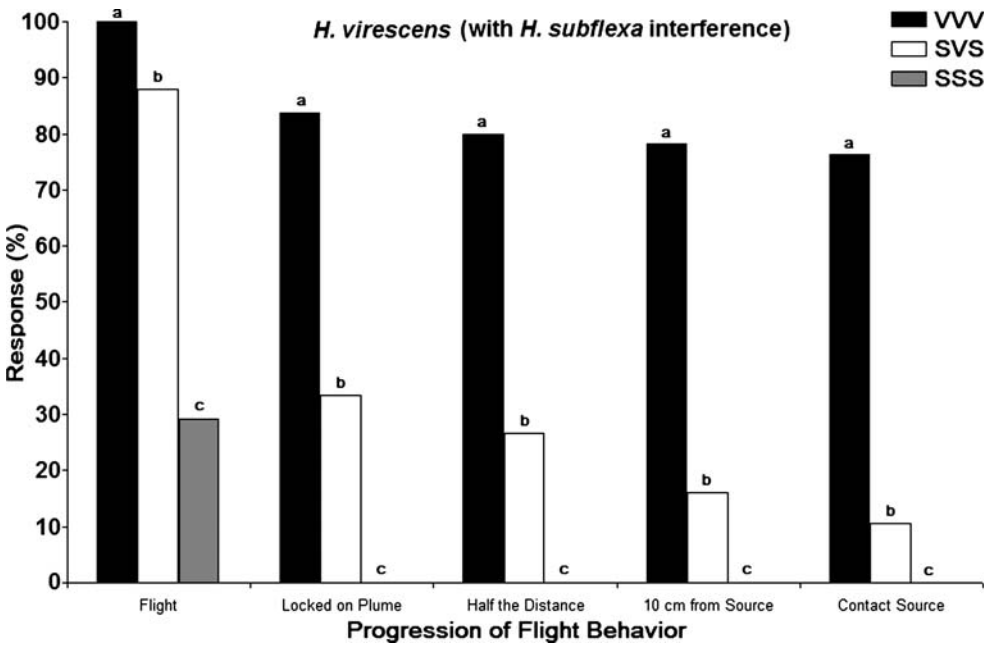

Fig. 2 Responses of male *H. virescens* to conspecific (*VVV*) and heterospecific (*VSV* and *SSS*) treatments involving *H. subflexa* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

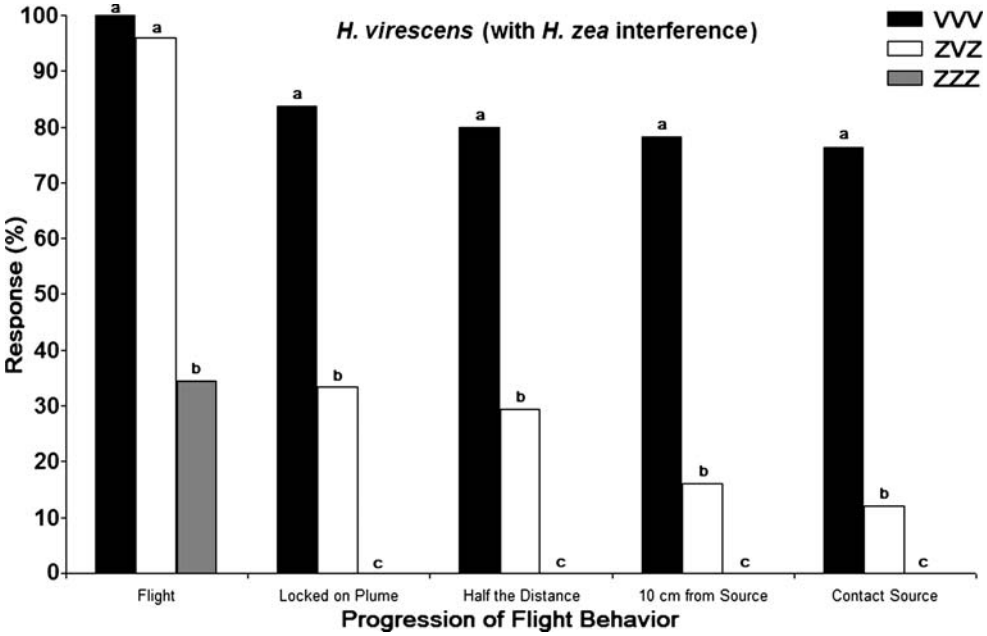

Fig. 3 Responses of male *H. virescens* to conspecific (*VVV*) and heterospecific (*VZV* and *ZZZ*) treatments involving *H. zea* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

The degree of coincident arrival of plume strands, as measured by the Quadroprobe four-antenna system using two heterospecific, calling females, was substantial. The fact that females of the two species were placed so closely together in their cages on the platform perhaps makes this not so surprising. Strands classified as "mixture" comprised 670 out of the 1,525 strands arriving on the probe (43.9%) for the VTV (*H. virescens*/*T. ni*/*H. virescens*) placement. Strands classified as *T. ni* comprised 600 out of 1,525 strands (39.3%), and those identified as *H. virescens* were 255 out of 1,525 (16.7%). For the TVT arrangement, 285 out of 625 strands (45.6%) were classified as "mixture," 270 (43.2%) were classified as *T. ni*, and 70 (11.2%) were classified by the algorithm as *H. virescens*.

Discussion

Despite years of studies on the behavioral antagonism caused by the addition of synthetic heterospecific heliothine pheromone components to the synthetic sex pheromone blends of other heliothine species, there has been a relative lack of information about the degree of antagonism caused by actual overlapping plumes emitted by heterospecific

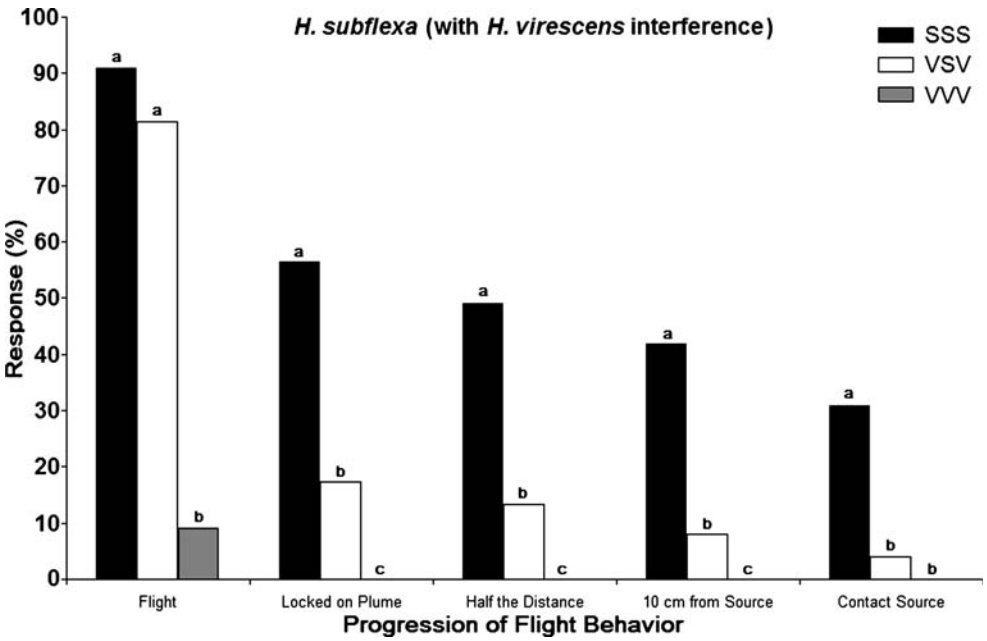

Fig. 4 Responses of male *H. subflexa* to conspecific (*SSS*) and heterospecific (*VSV* and *VVV*) treatments involving *H. virescens* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

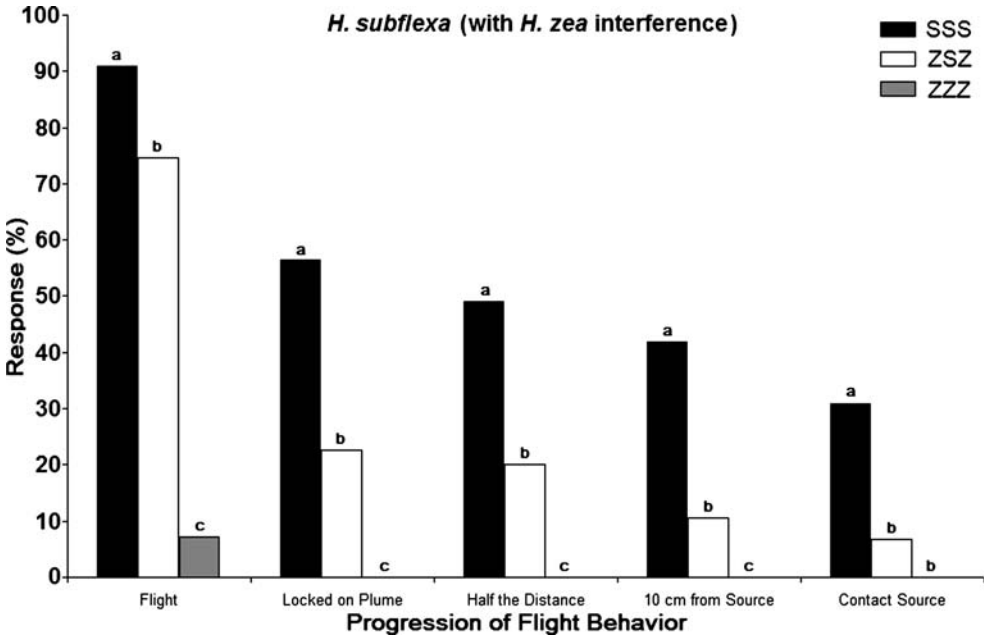

Fig. 5 Responses of male *H. subflexa* to conspecific (*SSS*) and heterospecific (*ZSZ* and *ZZZ*) treatments involving *H. zea* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

heliothine females. The question of naturally emitted female–female sex pheromone plume interactions relates directly to the possible selection pressures that may have occurred over evolutionary time. Practical issues of sex pheromone monitoring trap specificity, not the evolutionary forces that determine species-specific communication channels, were the initial driving force behind the identification of these field-trapping heliothine blends.

Our results using live, calling females of these three heliothine species show that there is no cross-attraction whatsoever between males of one species and females of the other two species. This is in agreement with field-trapping studies that used synthetic blends, as well as those that used live females, as lures in field-trapping tests (Haile et al. 1973; Sparks et al. 1979a, b; Klun et al. 1979, 1982; Carpenter et al. 1984; Lopez and Witz 1988). The interfering effects of a confluence of plumes in previous field-trapping tests using cages of calling females could not be verified due to the uncertainties produced by the ever-shifting wind fields under these conditions. In the current experiments, these uncertainties were eliminated.

It had been inferred from studies that used synthetic lures that male *H. subflexa* will not fly upwind to females of either *H. virescens* or *H. zea* because *H. virescens*

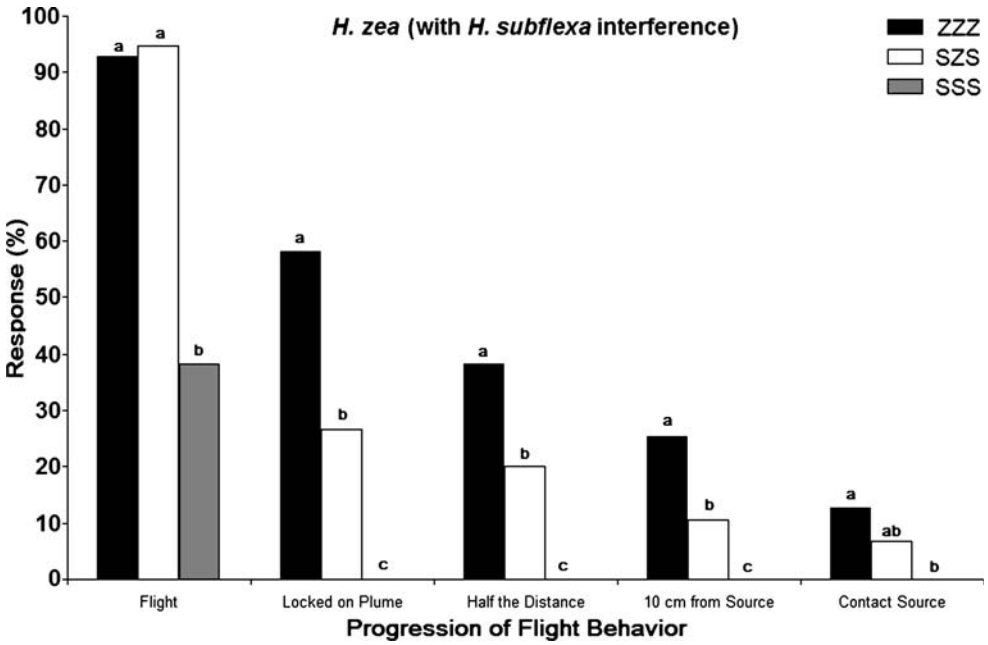

Fig. 6 Responses of male *H. zea* to conspecific (*ZZZ*) and heterospecific (*SZS* and *SSS*) treatments involving *H. subflexa* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

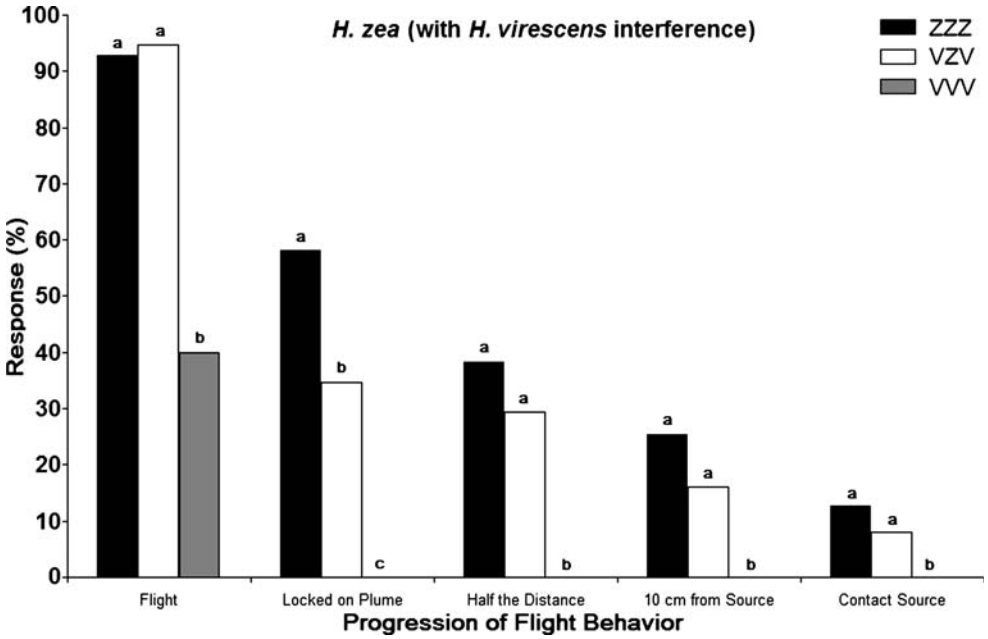

Fig. 7 Responses of male *H. zea* to conspecific (*ZZZ*) and heterospecific (*VZV* and *VVV*) treatments involving *H. virescens* females. Within each behavioral category, *bars having no letters* in common are significantly different from each other

females emit trace amounts, at best, of Z9-16:Ald and Z11-16:OH, and *H. zea* females do not emit Z11-16:OH (Klun et al. 1979; Pope et al. 1984; Heath et al. 1990; Vickers et al. 1991). Our results support these suppositions, showing no cross-attraction of *H. subflexa* males to females of the other two species.

It had been assumed that neither *H. virescens* nor *H. zea* males will fly upwind to *H. subflexa* females because they emit Z11-16:OH, which is antagonistic to males of both these species (Klun et al. 1982; Teal et al. 1984; Vickers and Baker 1997; Quero and Baker 1999). It had also been assumed that *H. virescens* males would not be attracted to *H. zea* females because they do not emit the essential component Z9-14:Ald, and *H. zea* males would not be attracted to *H. virescens* females because the amount of Z9-14:Ald they emit relative to Z11-16:Ald acts as a behavioral antagonist. Field-trapping tests that used live, calling females (Haile et al. 1973; Sparks et al. 1979a, b) had supported these ideas, and our results confirm them.

We have further shown that the plume strands from heterospecific heliothine females contain sufficient amounts of the above-mentioned antagonistic compounds to interfere with upwind flight and source location of conspecific females by males when the plume strands are intertwined. This had been conjectured in past studies (Vickers et al. 1991; Vickers 2002), based on the potential antagonism imposed by the known heterospecific antagonist compounds applied to single sources, but it had not been rigorously tested before by using live, calling females.

To determine the precise effects when heterospecific females are in close proximity, we measured the percentages of plume strands that might be registered on heliothine male antennae downwind from the release point as "mixture" (coincident strands from two females) or else as the "pure" blend of either species. We found that nearly 50% of the strands in two configurations, VTV and TVT, were classified as "mixture" from the plumes of calling females placed in such close proximity. We suggest that this tendency toward "mixture" strands is responsible for the interspecific interference in male attraction we document here. This, in combination with the presence of antagonists in the noncoincident odor strands, reduces attraction of a given species of male moth to conspecifics. Nevertheless, sufficient numbers of strands of pure pheromone reach the males' olfactory systems to allow a significant percentage to locate their conspecific females in this closely spaced array.

Two unexpected instances of interference occurred, and they were unexpected because there are no known antagonists involved that can explain the reduced levels of upwind flight. First, *H. virescens* male attraction was reduced by the presence of *H. zea* plumes, the only explanation based on known behavior-modifying compounds for these species being that the ratio of Z9-14:Ald to Z11-16:Ald would be diluted due to the lack of emission of Z9-14:Ald by *H. zea* females. Second, the reduction in upwind flight by *H. subflexa* males in the presence of either *H. virescens* or *H. zea* females was also unexpected due to the lack of any known antagonism in *H. subflexa* males to any compounds emitted by the females of these two species. Barring the possibility of antagonism from other coemitted compounds from the females of either of these species, the only other explanation for reduced attraction of *H. subflexa* males would be that a ratio shift caused by overemission of some compounds by the other species would register suboptimally in the *H. subflexa* central

nervous system (Vickers et al. 1991; Vickers 2002), as suggested previously by Klun et al. (1982).

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References

BAKER, T. C., FADAMIRO, H. Y., and COSSÉ, A. A. 1998. Moth uses fine tuning for odor resolution. *Nature* 393:350.

CARDÉ, R. T., CARDÉ, A. M., HILL, A. S., and ROELOFS, W. L. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling species *Archips argyrospilus* and *A. mortuanus* and other sympatric tortricine moths (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 3:71–84.

CARPENTER, J. E., PAIR, S. D., and SPARKS, A. N. 1984. Trapping of different Noctuid moth species by one trap baited with two lures. *J. Ga. Entomol. Soc.* 19:120–124.

EL-SAYED, A. M., DELISLE, J., DE LURY, N., GUT, L. J., JUDD, G. J. R., LEGRAND, S., REISSIG, W. H., ROELOFS, W. L., UNELIUS, C. R., and TRIMBLE, R. M. 2003. Geographic variation in pheromone chemistry, antennal electrophysiology, and pheromone-mediated trap catches of North American populations of the oblique banded leafroller. *Environ. Entomol.* 32:470–476.

FADAMIRO, H. Y., and BAKER, T. C. 1997. *Helicoverpa zea* males (Lepidoptera: Noctuidae) respond to the intermittent fine structure of their sex pheromone plume and an antagonist in a flight tunnel. *Physiol. Entomol.* 22:316–324.

FADAMIRO, H. Y., COSSÉ, A. A., and BAKER, T. C. 1999. Fine-scale resolution of finely spaced pheromone and antagonist filaments by flying male *Helicoverpa zea*. *J. Comp. Physiol. A* 185:131–141.

FISHER, R. A. 1922. On the interpretation of c^2 from contingency tables, and the calculation of P. *J. R. Stat. Soc.* 85:87–94.

GEMENO, C., LUTFALLAH, A. F., and HAYNES, K. F. 2000. Pheromone blend variation and cross-attraction among populations of the black cutworm moth (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 93:1322–1328.

GRIES, G., GRIES, R., KHASKIN, G., SLESSOR, K. N., GRANT, G. G., LISKA, J., and KAPITOLA, P. 1996. Specificity of nun and gypsy moth sexual communication through multiple-component pheromone blends. *Naturwissenschaften* 83:382–385.

GRIES, G., SCHAEFER, P. W., GRIES, R., LISKA, J., and GOTOH, T. 2001. Reproductive character displacement in *Lymantria monacha* from Northern Japan? *J. Chem. Ecol.* 27:1163–1176.

GROOT, A. T., SANTANGELO, R. G., RICCI, E., BROWNIE, C., GOULD, F., and SCHAL, C. 2007. Differential attraction of *Heliothis subflexa* males to synthetic pheromone lures in Eastern US and Western Mexico. *J. Chem. Ecol.* 33:353–368.

GROOT, A. T., HOROVITZ, J. L., HAMILTON, J., SANTANGELO, R. G., SCHAL, C., and GOULD, F. 2006. Experimental evidence for interspecific directional selection on moth pheromone communication. *Proc. Natl. Acad. Sci. USA* 103:5858–5863.

GUERIN, P. M., BALTENSWEILER, W., ARN, H., and BUSER, H. R. 1984. Host race pheromone polymorphism in the larch budmoth. *Experientia* 40:892–894.

HAILE, D. G., SNOW, J. W., and GOODENOUGH, J. L. 1973. Reduced capture of tobacco budworm and corn earworm males in electric grid traps baited simultaneously with virgin females of both species. *J. Econ. Entomol.* 66:739–740.

HEATH, R. R., MITCHELL, E. R., and CIBRIAN-TOVAR, J. 1990. Effect of release rate and ratio of (Z)-11-hexadecen-1-ol from synthetic pheromone blends on trap capture of *Heliothis subflexa* (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 16:1259–1268.

HEATH, R. R., MCLAUGHLIN, J. R., PROSHOLD, F., and TEAL, P. E. A. 1991. Periodicity of female sex pheromone titer and release in *Heliothis subflexa* and *H. virescens* (Lepidoptera: Noctuidae). *Ann. Entomol. Soc. Am.* 84:182–189.

HETLING, J. R., MYRICK, A. J., PARK, K. C., and BAKER, T. C. 2006. Hybrid olfactory biosensor using multichannel electroantennogram: design and application, pp. 243–265, in M. Akay (ed.). Handbook of Neural Engineering. Brain–Computer Interface, vol. II. Wiley, New York.

KLUN, J. A., PLIMMER, J. R., and BIERL-LEONHARDT, B. A. 1979. Trace chemicals: essence of sexual communication systems in *Heliothis* species. *Science* 204:1328–1330.

KLUN, J. A., PLIMMER, J. R., BIERL-LEONHARDT, B. A., SPARKS, A. N., PRIMIANI, M., CHAPMAN, O. L., LEE, G. H., and LEPONE, G. 1980a. Sex pheromone chemistry of female corn earworm moth, *Heliothis zea*. *J. Chem. Ecol.* 6:165–175.

KLUN, J. A., BIERL-LEONHARDT, B. A., PLIMMER, J. R., SPARKS, A. N., PRIMIANI, M., CHAPMAN, O. L., LEPONE, G., and LEE, G. H. 1980b. Sex pheromone chemistry of female tobacco budworm moth, *Heliothis virescens*. *J. Chem. Ecol.* 6:177–183.

KLUN, J. A., LEONHARDT, B. A., LOPEZ, J. D., and LACHANCE, L. E. 1982. Female *Heliothis subflexa* (Lepidoptera, Noctuidae) sex pheromone-chemistry and congeneric comparisons. *Environ. Entomol.* 11:1084–1090.

LIU, Y.-B., and HAYNES, K. F. 1994. Evolution of behavioral responses to sex pheromone in mutant laboratory colonies of *Trichoplusia ni*. *J. Chem. Ecol.* 20:231–238.

LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C. 1993. Moth pheromone genetics and evolution. *Philos. Trans. R. Soc. Lond., B Biol. Sci.* 340:167–177.

LÖFSTEDT, C., HERREBOUT, W. M., and MENKEN, S. B. J. 1991. Sex pheromones and their potential role in evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* 2:20–208.

LOPEZ, J. D., and WITZ, J. A. 1988. Influence of *Heliothis virescens* sex pheromone dispensers on captures of *H. zea* males in pheromone traps relative to distance and wind direction. *J. Chem. Ecol.* 14:265–276.

MILLER, J. R., and ROELOFS, W. L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MCELFRESH, J. S., and MILLAR, J. C. 1999. Geographic variation in sex pheromone blend of *Hemileuca electra* from Southern California. *J. Chem. Ecol.* 25:2505–2525.

MCELFRESH, J. S., and MILLAR, J. C. 2001. Geographic variation in the pheromone system of the saturniid moth *Hemileuca eglanterina*. *Ecology* 82:3505–3518.

MURLIS, J. 1986. The structure of odor plumes, pp. 27–38, in T. L. Payne, C. E. J. Kennedy, and M. C. Birch (eds.). Mechanisms in Insect OlfactionClarendon, Oxford.

MYRICK, A. J., BAKER, T. C., PARK, K.-C., and HETLING, J. R. 2005. Bioelectronic artificial nose using four-channel moth antenna biopotential recordings. pp. 313–316, in Proceedings of the 2nd International IEEE EMBS Conference on Neural Engineering.

PARK, K. C., OCHIENG, S. A., ZHU., and BAKER, T. C. 2002. Odor discrimination using insect electroantennogram responses from an insect antennal array. *Chem. Senses* 27:343–352.

POPE, M. M., GASTON, L. K., and BAKER, T. C. 1982. Composition, quantification, and periodicity of sex pheromone gland volatiles

from individual *Heliothis virescens* females. *J. Chem. Ecol.* 8:1043–1055.

POPE, M. M., GASTON, L. K., and BAKER, T. C. 1984. Composition, quantification, and periodicity of sex pheromone volatiles from individual *Heliothis zea* females. *J. Insect Physiol.* 30:943–945.

POTTING, R. P. J., LÖSEL, P. M., and SCHERKENBECK, J. 1999. Spatial discrimination of pheromones and behavioural antagonists by the tortricid moths *Cydia pomonella* and *Adoxophyes orana*. *J. Comp. Physiol. A* 185:419–425.

QUERO, C., and BAKER, T. C. 1999. Antagonistic effect of (Z)-11-Hexadecen-1-ol on the pheromone-mediated flight of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). *J. Insect Behav.* 12:701–710.

QUERO, C., FADAMIRO, H. Y., and BAKER, T. C. 2001. Responses of male *Helicoverpa zea* to single pulses of sex pheromone and behavioural antagonist. *Physiol. Entomol.* 26:106–115.

ROELOFS, W. L., HILL, A. S., CARDÉ, R. T., and BAKER, T. C. 1974. Two sex pheromone components of the tobacco budworm moth, *Heliothis virescens*. *Life Sci.* 14:1555–1562.

SHOREY, H. H., and HALE, R. L. 1965. Mass-rearing of the larvae of nine Noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

SPARKS, A. N., CARPENTER, J. E., KLUN, J. A., and MULLINIX, B. G. 1979a. Field responses of male *Heliothis zea* (Boddie) to pheromonal stimuli and trap design. *J. Ga. Entomol. Soc.* 14:318–325.

SPARKS, A. N., RAULSTON, J. R., LINGREN, P. D., CARPENTER, J. E., KLUN, J. A., and MULLINIX, B. G. 1979b. Field response of male *Heliothis virescens* to pheromonal stimuli and traps. *Bull. Entomol. Soc. Am.* 25:268–274.

STEEL, R. G. D., and TORRIE, J. H. 1960. Principles and Procedures of Statistics. McGraw Hill, New York.

TEAL, P. E. A., HEATH, R. R., TUMLINSON, J. H., and MCLAUGHLIN, J. R. 1981. Identification of sex pheromone of *Heliothis subflexa* (Gn.) (Lepidoptera: Noctuidae) and field trapping studies using different blends of components. *J. Chem. Ecol.* 7:1011–1022.

TEAL, P. E. A., TUMLINSON, J. H., MCLAUGHLIN, J. R., HEATH, R. R., and RUSH, R. A. 1984. (Z)-11-Hexadecen-1-ol: a behavioral modifying chemical present in the pheromone gland of female *Heliothis zea* (Lepidoptera: Noctuidae). *Can. Entomol.* 116:777–779.

TEAL, P. E. A., TUMLINSON., and HEATH, R. R. 1986. Chemical and behavioral analyses of volatile sex pheromone components released by calling *Heliothis virescens* (F.) females (Lepidoptera: Noctuidae). *J. Chem. Ecol.* 12:107–125.

TUMLINSON, J. H., HENDRICKS, D. E., MITCHELL, E. R., DOOLITTLE, R. E., and BRENNEN, M. M. 1975. Isolation, identification, and synthesis of the sex pheromone of the tobacco budworm. *J. Chem. Ecol.* 1:203–214.

VETTER, R. S., and BAKER, T. C. 1983. Behavioral responses of male *Heliothis virescens* in a sustained flight-tunnel to combinations of seven compounds identified from female glands. *J. Chem. Ecol.* 9:747–759.

VETTER, R. S., and BAKER, T. C. 1984. Behavioral responses of male *Helicoverpa zea* moths in a sustained flight-tunnel to combinations of four compounds identified from female sex pheromone gland. *J. Chem. Ecol.* 10:193–202.

VICKERS, N. J. 2002. Defining a synthetic blend attractive to male *Heliothis subflexa* under wind tunnel conditions. *J. Chem. Ecol.* 28:1255–1267.

VICKERS, N. J., and BAKER, T. C. 1997. Chemical communication in heliothine moths. VII. Correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol. A* 180:523–536.

VICKERS, N. J., CHRISTENSEN, T. A., MUSTAPARTA, H., and BAKER, T. C. 1991. Chemical communication in heliothine moths: flight behavior of male *Helicoverpa zea* and *Heliothis virescens* in response to varying ratios of intra- and interspecific sex pheromone components. *J. Comp. Physiol. A* 169:275–280.

WITZGALL, P., and PRIESNER, E. 1985. Are sex attractant traps a valid approach to determine dispersal in *Coleophora laricella*? *Z. Angew. Entomol.* 100:360–367.

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Adults and Nymphs Do Not Smell the Same: The Different Defensive Compounds of the Giant Mesquite Bug (*Thasus neocalifornicus*: Coreidae)

Kathleen L. Prudic · Koji Noge · Judith X. Becerra

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Abstract Heteropteran insects often protect themselves from predators with noxious or toxic compounds, especially when these insects occur in aggregations. The predators of heteropteran insects change from small insect predators to large avian predators over time. Thus, a chemical that is deterrent to one type of predator at one point in time may not be deterrent to another type of predator at another point in time. Additionally, these predator deterrent compounds may be used for other functions such as alarm signaling to other conspecifics. Defensive secretion compounds from the adult and the nymph giant mesquite bug (*Thasus neocalifornicus*: Coreidae) were isolated and identified by gas chromatography–mass spectrometry and NMR. The predominant compounds isolated from the nymph mesquite bugs during a simulated predator encounter were (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal. In adults, the major compounds released during a simulated predator encounter were hexyl acetate, hexanal, and hexanol. Results from predator bioassays suggest the nymph compounds are more effective at deterring an insect predator than the adult compounds. By using behavioral bioassays, we determined the role of each individual compound in signaling to other mesquite bugs. The presence of the nymph secretion near a usually compact nymph aggregation caused nymph mesquite bugs to disperse but did not affect adults. Conversely, the presence of the adult secretion caused the usually loose adult aggregation to disperse, but it did not affect nymph aggregation. The compounds that elicited nymph behavioral responses were (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while those that elicited adult behavioral responses were hexyl acetate and hexanal. The differences between the chemical composition of nymph and adult defensive secretions and alarm behavior are possibly due to differences in predator guilds.

Keywords Aggregation · Carbonyl defense · Coreidae · Defensive chemistry · Warning coloration

K. L. Prudic · K. Noge · J. X. Becerra (✉)
Department of Entomology, University of Arizona,
Tucson, AZ 85721, USA
e-mail: jxbecerra@ag.arizona.edu

K. L. Prudic
Ecology and Evolutionary Biology, Yale University,
New Haven, CT 06520, USA

Introduction

Many species of warning colored insects have evolved chemical defenses that deter predatory attack and increase insect survival (Blum 1981; Eisner et al. 2007). However, the identification of which chemical is important in conferring functional benefits is difficult since insects often contain many compounds, and chemical mixtures may change in both space and time. The predators' behavioral response also varies with the identity of the predator (Eisner 1970; Eisner et al. 2007). Complications arise when compounds have multiple functions such as deterring predators and alerting conspecifics to predator attack (Eisner 1970; Eisner et al. 2007). Despite these complications and difficulties, however, an understanding of how these compounds function and to whom they are signaling is imperative in understanding adaptive significance and evolutionary history.

Heteropteran insects are known for their chemical defenses and gregarious behavior (Aldrich 1988; Millar 2005). Typically, the defensive secretions are mixtures of aliphatic compounds, most with carbon chains of C_4, C_6,

and C_8 (Aldrich and Yonke 1975; Aldrich 1988; Millar 2005). These compounds are thought to be more effective at deterring arthropod predators than birds or mammals (Eisner 1970). They may function not only as toxins and repellents to predators, but also as alarm pheromones to conspecifics, thereby alerting and dispersing threatened aggregations (Calam and Youdeowei 1968, Aldrich and Blum 1978; Blum 1981; Leal et al. 1994). However, it is often unclear which chemicals are defensive against predators, which elicit a dispersal behavior among conspecifics, and whether those chemical identities change through different life stages of insects.

The genus *Thasus* consists of neotropical and neosubtropical coreid bugs that range from the American southwest to northern South America. This genus contains some of the largest species of Heteroptera with some reaching over 50 mm in length (Brailovsky et al. 1994). Giant mesquite bugs (*Thasus neocalifornicus*: Coreidae) are among the largest heteropterans of the desert southwest USA. These insects appear to have different visual defensive strategies between the nymph and adult life stage. Adults are large (~40 mm), cryptic, and occur in small, loose aggregations, while nymphs are smaller (~5–30 mm), highly conspicuous, and occur in larger, compact aggregations (Olsen 2004). Both adults and nymphs occur simultaneously on the same host, and both life stages emit a visible secretion when disturbed. They are specialists on mesquite tress (*Prosopis* spp.: Fabaceae), and are frequently seen in southeastern Arizona, especially around Tucson (Forbes and Schaefer 2003). Nymphs are seen more often because of their conspicuous feeding aggregations on the young petioles or seed pods of mesquite (Olsen 2004). They are vividly colored red and white (Fig. 1). Adult giant mesquite bugs are colored dark olive with dark red and black legs (Fig. 1). Their size probably protects them from many insect predators, but they are attacked by lizards and birds (Olsen 2004). Both nymphs and adults emit a foul-smelling odor when disturbed, and they appear to disperse as observed in other closely related species (Aldrich and Blum 1978). Because of their different visual defensive strategies and potential predators, we investigated whether nymph and adult giant mesquite bugs contained different defensive compounds and whether those compounds elicited different behavioral responses in nymphs and adults.

By using gas chromatography–mass spectrometry (GC–MS) and ^{1}H and ^{13}C NMR, we investigated whether nymph and adult giant mesquite bugs emit different chemical compounds when disturbed. Once we identified the compounds, we used behavioral assays to determine: (1) if nymph chemicals were better at deterring insect predators than adult chemicals; and (2) if nymph and adult chemicals elicited similar or different escape behaviors in nymphs and adults.

Methods and Materials

Insect Collection Adults and third instar nymphs were collected off host plant, Arizona mesquite trees (*Prosopis*

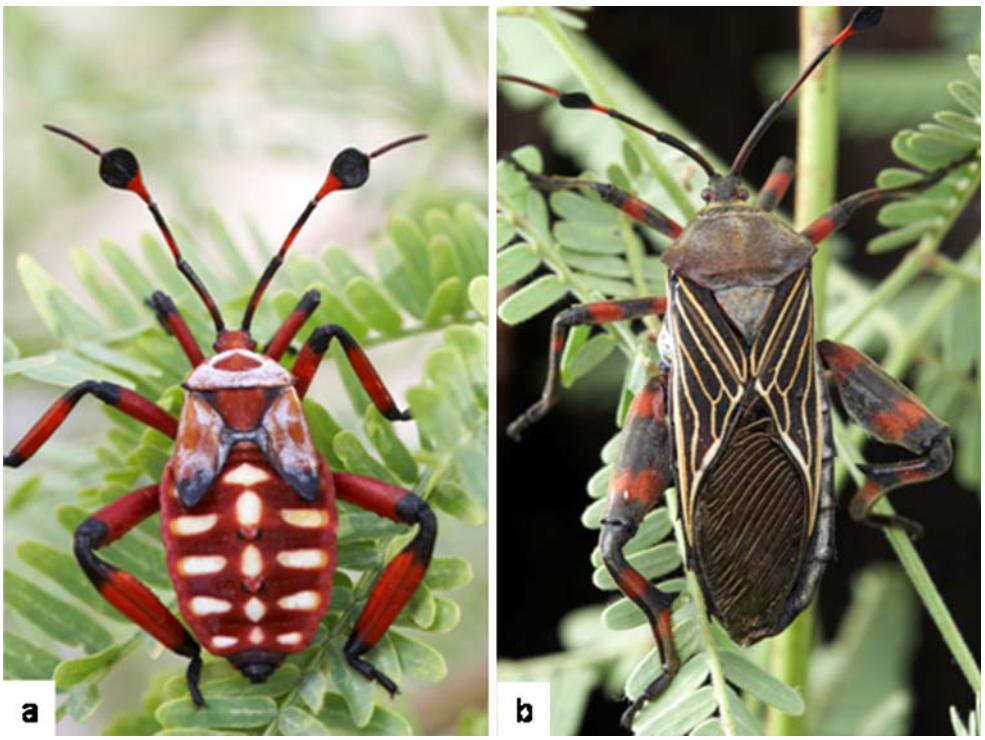

Fig. 1 Photo of **a** nymph and **b** adult giant mesquite bug, *Thasus neocalifornicus*, courtesy of Alex Wild

veluntina: Fabaceae) on June 30th, 2007 from two locations in Tucson, Pima County, AZ, USA: The University of Arizona campus (32.23°N, 110.95°W) and the Tucson Botanical Gardens (32.24°N, 110.91°W). Giant mesquite bugs were kept in the lab and fed Arizona mesquite leaves and pods for the period of experimentation.

Preparation of Extracts To collect the secretion emitted during a predation event, we pinned either an adult or a nymph (instar 3 or 4) onto a glass plate with forceps and collected the secretion from the abdomen on filter paper. The filter paper was extracted in 1 ml hexane for 5 min, and the extract was analyzed by GC and GC–MS.

To confirm our results, we also extracted whole insects. Nymphs (instar 4 or 5) were dipped into hexane (5 ml) for 3–5 min, and the extract was analyzed by GC and GC–MS. Adults were extracted with 10 ml hexane and analyzed in the same way. For identification of 4-oxo-(*E*)-2-hexenal, eight nymphs were extracted with hexane for 3 h. The extract was concentrated and then applied onto a SiO_2 column (300 mg), and successively eluted with 3 ml each of hexane, 3, 4, 10, and 50% ethyl acetate in hexane. 4-Oxo-(*E*)-2-hexenal (2.4 mg) was eluted purely in the 10% ethyl acetate fraction.

Chemical Analyses and Identification Gas chromatography (GC) analysis was performed with an Agilent 6890N GC with a flame ionization detector, by using an HP-5MS capillary column (Agilent Technologies, 30 m×0.25 mm i.d., 0.25 μm film thickness) with helium carrier gas at 2.0 ml/min in splitless mode. The oven temperature was programmed to change from 50°C (3-min holding) to 300°C at 10°C/min and then held for 5 min. The injector temperature was maintained at 200°C, and the detector temperature at 300°C. The relative percentages of each component were calculated based on the peak area by flame ionization detector (FID).

GC–Mass Spectrometry (GC–MS) analysis was performed with the Agilent 6890 N GC linked to an Agilent 5975B operated at 70 eV with an HP-5MS capillary column (Agilent Technologies, 30 m×0.32 mm i.d., 0.25 μm in film thickness) under the same analytical conditions described above, except the velocity of helium carrier gas was 1.2 ml/min.

All compounds except for 4-oxo-(*E*)-2-hexenal were identified by comparing their GC retention times and mass spectra with those of authentic standards. (*E*)-2-Hexenal, hexyl acetate, hexanol, and hexanal standards were purchased from Sigma Chemical Co. (USA). 4-Oxo-(*E*)-2-hexenal was synthesized by a one-step reaction described in Moreira and Millar (2005) and verified by GC–MS. The structure of the 4-oxo-(*E*)-2-hexenal was confirmed by ^{1}H and ^{13}C NMR spectra by using the purified natural compound. ^{1}H and ^{13}C NMR spectra were acquired on a Bruker DRX-500 spectrometer (^{1}H at 500 MHz and ^{13}C at 125 MHz) with a Bruker Dual ($^{13}C/^{1}H$) 5-mm probe at a sample temperature of 25°C in a $CDCl_3$ solution with tetramethylsilane (TMS) as an internal standard.

Mesquite Bug Behavioral Assays Mesquite bugs were organized into groups of five individuals and placed into separate arenas measuring 25 by 35 cm. Each group was composed of either third instar nymphs or adults (in total: three groups per life stage). Within each group, individuals were labeled one through five. During each trial, a single focal individual was observed for behavioral changes. The identity of the focal individual rotated among replicates. Volatiles were introduced into the arena via a piece of cotton with 3 μl of compound. The treatment compounds were distilled water (control treatment), nymph secretion collected from the abdomen, adult secretion collected from the abdomen, 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenal, hexyl acetate, hexanol, hexanal, 4-oxo-(*E*)-2-hexenal+(*E*)-2-hexenal, and hexyl acetate+hexanol+hexanal (N=15 observations/treatment). We measured the change in distance (cm) to the volatile source, change in number of neighbors in 5-cm radius, and change in distance to the nearest bug neighbor (0–43 cm) before and 2 min after the volatile(s) was introduced into the arena. All data were evaluated for normality and homoscedasticity. Count data were square root transformed to normalize. Data were analyzed with one way ANOVA by comparing control response to treatment response with JMP-In 5.0 (SAS Institute 2002). Bonferroni alpha level corrections were made to reduce type I errors.

Insect Predator Behavioral Assays California mantids, (*Stagmomantis californica*: Mantidae), were hand reared from oothecae collected on the University of Arizona campus. They were tested during their 4th instar and averaged 40 mm in length. A mantid was fed a maintenance diet of two crickets per day. In addition, for the first experiment, each mantid was fed a cricket the 1st day and a giant mesquite bug nymph the next (N=5). Cricket and nymph were paired based on weight. We recorded whether the mantid oriented, attacked, and ate each prey type. For the second experiment, crickets were treated with 2 μl of either distilled water (control treatment), or nymph secretion, adult secretion, 4-oxo-(*E*)-2-hexenal, (*E*)-2-hexenal, hexyl acetate, hexanol, or hexanal. These crickets were fed to another set of 4th instar mantids. We recorded whether the mantid oriented, attacked, and ate each prey type. Each mantid experienced one water treatment and one other treatment (N=5 per treatment, N=35 across all treatments). All data were evaluated for normality and homoscedasticity. Data were analyzed with a log-linear model by using JMP-In 5.0 (SAS Institute, 2002).

Results

Mesquite Bug Chemical Profiles Giant mesquite bug nymphs emitted (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while adults emitted hexyl acetate, hexanal, and hexanol when disturbed. The two life stages had no compounds in common. The same pattern of compounds was also observed in the whole body extraction. Female and male adults differed slightly in the relative amounts of each compound, but these differences were not significant.

The mass and NMR spectra of purified 4-oxo-(*E*)-2-hexenal are summarized as follows: MS *m*/*z* (%): 112 (M+, 15), 84 (14), 83 (100), 57 (13), 55 (49); ^{1}H NMR ($CDCl_3$): δ 9.77 (1H, d, J=7.2 Hz), 6.86 (1H, d, J=16.3 Hz), 6.77 (1H, dd, J=16.3, 7.2 Hz), 2.72 (2H, q, J=7.2 Hz), 1.15 (3H, t, J=7.2 Hz); and ^{13}C NMR ($CDCl_3$): δ 200.31, 193.34, 144.69, 137.29, 34.52, 7.54. The total amount of 4-oxo-(*E*)-2-hexenal was 2.4 mg from eight nymphs for NMR analysis. The NMR spectra of the purified compounds were consistent with published spectra (Moreira and Millar 2005).

Mesquite Bug Behavioral Responses There was no effect of arena on mesquite bug behavior ($F_{1,5}$=1.22, P=0.23). Therefore, data were pooled across arena groups within a life stage. As compared to distilled water, nymph giant mesquite bugs disperse more in the presence of their own defensive compounds. Response by nymphs to adult secretion or any of the individual adult compounds did not differ from their response to water (Fig. 2; P>0.05). The presence of nymph secretion caused nymphs to travel farther away from the chemical source ($F_{1,14}$=5.33, P=0.012), to have fewer nymphs in a 5 cm radius ($F_{1,14}$=6.52, P<0.01), and to be farther away from the nearest giant mesquite bug ($F_{1,14}$=9.74, P<0.01). (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal alone elicited bugs to travel away from the source ($F_{1,14}$=3.73, P=0.012 and $F_{1,14}$=12.34, P<0.01, respectively), but a mixture of these two compounds was

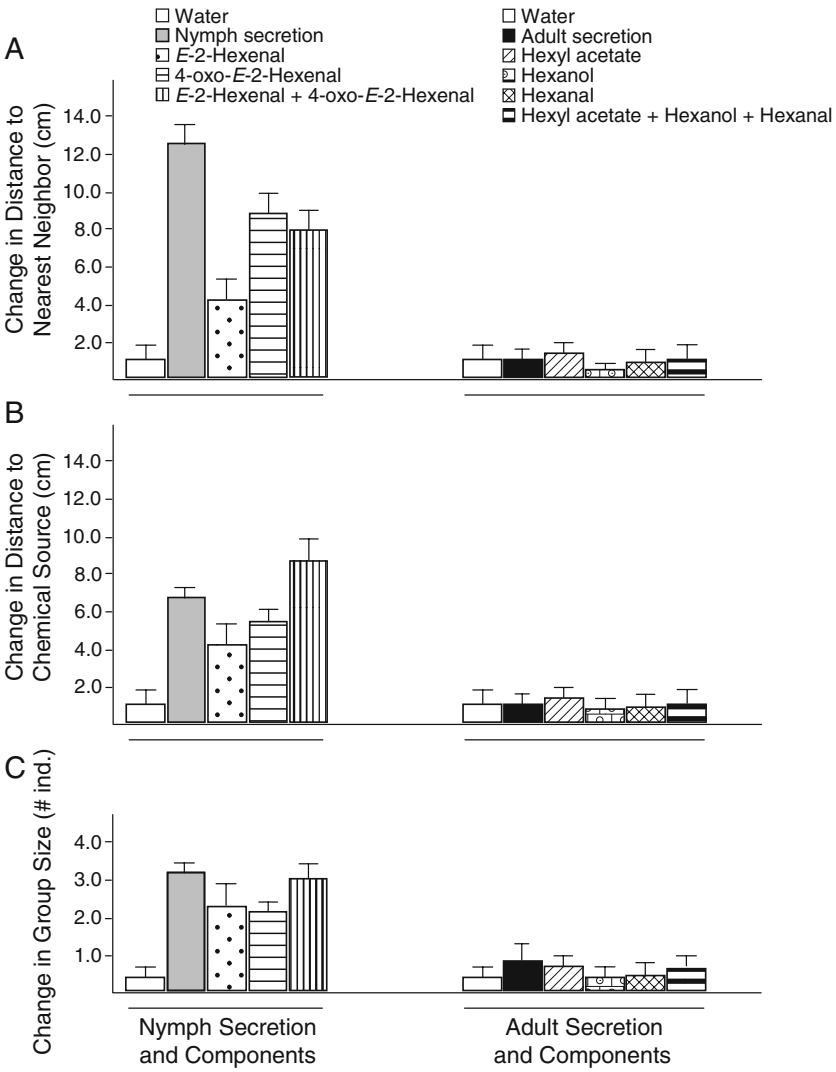

Fig. 2 Nymph behavioral responses to water (the control treatment), nymph secretion, adult secretion, and various authentic compounds from each secretion. We measured several variables: **a** change in distance to nearest neighbor, **b** change in distance to chemical source, and **c** change in aggregation group size after the treatment compound had been in the arena for 2 min. Means+SE shown (N=15 bugs per treatment)

almost as effective as the actual nymph secretion ($F_{1,14}$= 8.63, P<0.01). The same pattern was found also in both change in distance to nearest neighbor ($F_{1,14}$=7.90, P< 0.01) and change in group size ($F_{1,14}$=8.84, P<0.01).

Compared to distilled water, adult giant mesquite bugs also dispersed more in the presence of their own defensive compounds (Fig. 3). The presence of the adult secretion resulted in fewer adults nymphs in a 5 cm radius ($F_{1,14}$= 5.60, P=0.01) and farther away from the nearest adult bug ($F_{1,14}$=4.22, P=0.03). Adults also tended on average to travel farther away from the adult secretion source, but the difference was not significant after the Bonferroni correction ($F_{1,14}$=2.03, P>0.05). Hexyl acetate and hexanal alone elicited dispersal behavioral responses (P<0.02 for all comparisons), but hexanol did not (P>0.05 for all comparisons). A mixture of all three compounds was almost as effective as the actual adult secretion in changing the distance to source ($F_{1,14}$=3.04, P=0.05) and a change in group size ($F_{1,14}$=6.29, P=0.01). None of the behavioral responses by adults to nymph secretion or to any of the individual nymph compounds differed from the response to distilled water, the control treatment (P>0.05 for all comparisons).

Insect Predator Responses Compared to crickets, nymph mesquite bugs were not as palatable to mantids (Fig. 4). Untreated crickets were always oriented on, attacked, and eaten by mantids, while giant mesquite bug nymphs were oriented and attacked by mantids, but often released before consumption ($F_{1,9}$=8.46, P=0.004). Crickets treated with nymph secretions were more unpalatable to mantid predators than crickets treated with adult secretions ($F_{1,9}$=12.47, P=0.003). This effect was best explained by the presence of 4-oxo-(*E*)-2-hexenal ($F_{1,9}$=13.86, P=0.002). This compound caused four of the five mantids to die after attacking those crickets even though the crickets were not consumed.

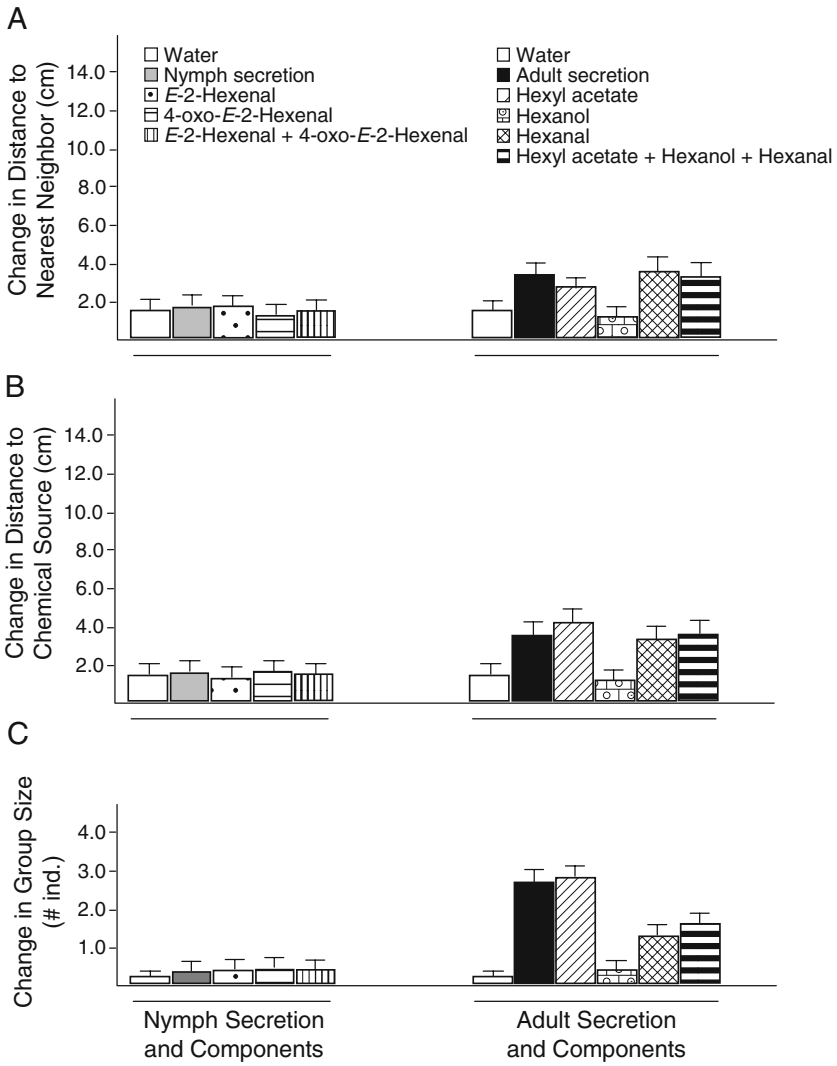

Fig. 3 Adult behavioral responses to water (the control treatment), nymph secretion, adult secretion, and various authentic compounds from each secretion. We measured several variables: **a** change in distance to nearest neighbor, **b** change in distance to chemical source, and **c** change in aggregation group size after the treatment compound had been in the arena for 2 min. Means+SE shown (N=15 bugs per treatment)

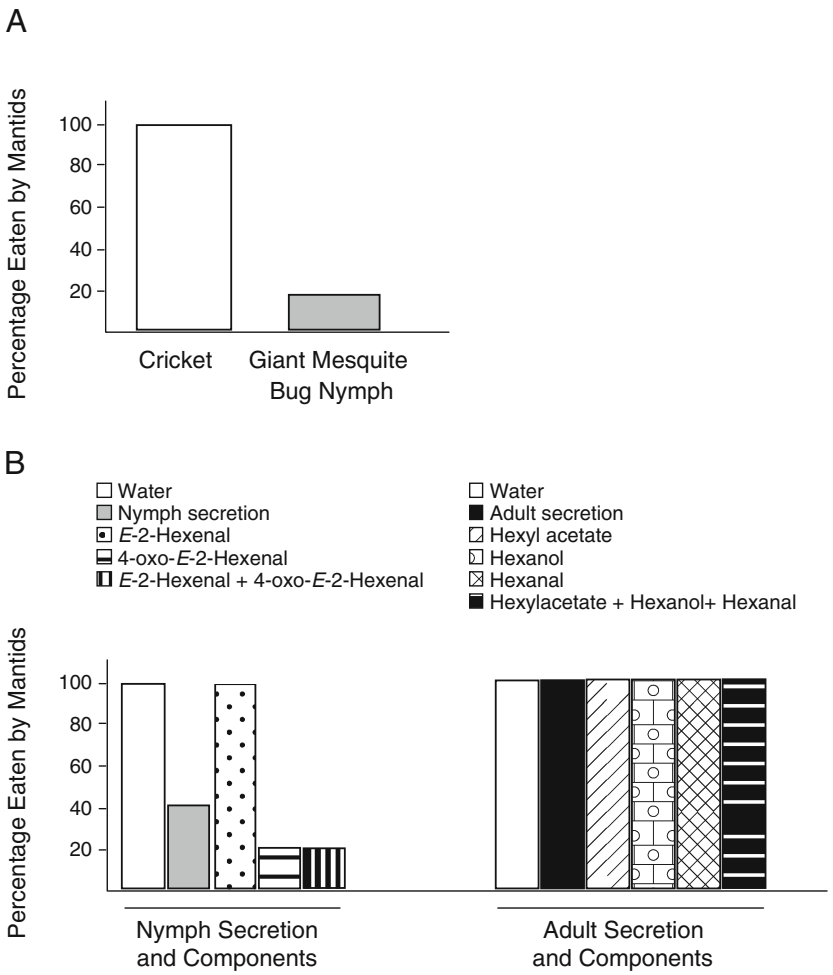

Fig. 4 Praying mantid responses to **a** nymph giant mesquite bug and **b** crickets treated with nymph secretion, adult secretion, and individual chemical components of the two secretions (N=5 mantids per treatment)

Crickets painted with adult bug secretion and all other compounds not listed above were always consumed by the mantids (P>0.05 for all comparisons; Fig. 4).

Discussion

Our chemical analyses revealed that giant mesquite bugs (*T. neocalifornicus*) nymphs and adults emit different compounds when disturbed. Nymphs emit (*E*)-2-hexenal and 4-oxo-(*E*)-2-hexenal, while adults emit hexyl acetate, hexanal, and hexanol. These results were confirmed with whole-body extracts of both nymphs and adults. All compounds are found in other heteropteran and even coreid species, and are produced in the metathoracic gland from fatty acids acquired from the host plant (Blum 1981; Aldrich 1988; Leal et al. 1994; Millar 2005). Chemical differences between life stages have been shown in other coreid species (Prestwich 1976; Aldrich 1988; Leal et al. 1994), but this is the first time it has been documented in the genus *Thasus*.

Our predator behavioral experiments demonstrated that nymphs were chemically protected from insect predators while adults were not (Fig. 4). California mantids failed to consume nymphs and crickets treated with nymph secretion, but did consume crickets treated with adult secretion. We attempted to test the mantids' responses to adult giant mesquite bugs, but mantids failed to orient or attack adults. Mantids did not seem to perceive adult mesquite bugs as potential prey, perhaps because of their size. However, further behavioral experiments are needed to test this interpretation. Of all compounds, 4-oxo-(*E*)-2-hexenal was particularly deterrent and toxic to mantids. Mantids often died after exposure to nymph secretion and to 4-oxo-(*E*)-2-hexenal even without consuming prey. Tarantulas (*Eurypelma* sp.) have also been shown to die after exposure to the vapors of the giant mesquite bug nymph secretion (De La Torre-Bueno and Ambrose 1936). 4-Oxo-(*E*)-2-hexenal is known for its mutagenic and cytotoxic qualities by reacting with deoxyguanosine (Kasai et al. 2005); however, this is, we believe, the first time it has been evaluated as a source of chemical defense against predators. Relative to 4-oxo-(*E*)-2-hexenal, the other

compounds did not deter mantid prey consumption. The lack of response by adult mesquite bugs to nymph alarm pheromones fits with the cryptic defensive strategy of adults. Differences in both chemical composition and behavior between life stages of giant mesquite bugs and the predator responses to these bugs and their compounds support the idea that the nymph compounds are particularly effective at deterring arthropod predators (Eisner 1970). Further research will explore the adaptive significance of this pattern and how concentration differences may affect predator response. The compounds may no longer be relevant defensive compounds in an adult giant mesquite bug because the predator species have changed.

Our mesquite bug behavioral experiments indicated that the secretions can be also used as an alarm signal to other conspecifics in the same life stage (Figs. 2 and 3). Nymphs dispersed from their aggregation when nymph secretions, (*E*)-2-hexenal or 4-oxo-(*E*)-2-hexenal, were placed in the arena. However, nymphs failed to disperse when they were exposed to adult secretion or any of the individual compounds found in the adult secretion. Adults followed a similar pattern of dispersing after experiencing adult secretion, hexyl acetate, or hexanal. Hexanol did not elicit a behavioral response by either nymphs or adults. Future research, however, should address the question whether behavioral responses change when concentrations of secretion compounds change. These compounds are known alarm pheromones in other heteropteran species (Aldrich 1988; Millar 2005). It is not unusual that the two life stages respond differently to the signals (Aldrich 1988), although there are examples of nymphs and adults responding to each other's alarm pheromones (e.g., *Leptoglossus zonatus*, Leal et al. 1994).

The described alarm pheromones also may be particularly important in the evolution of warning coloration and gregariousness in nymphs of the giant mesquite bug. Warning coloration is thought to facilitate evolution of gregariousness (Sillen-Tullberg 1988, 1993; Tullberg and Hunter 1995). Yet, there is still a high individual cost of being attacked after a group is discovered by a predator, especially when the group is small (Gamberale and Tullberg 1998). One way to mitigate this cost is for the individuals under attack to disperse after a predator encounter and reform another group at a different location. Future research should focus on the question if the alarm pheromone compounds found in the nymphs of giant mesquite bugs function in this way.

The giant mesquite bug is a predominant insect in the southeastern Arizona, yet little is currently known about its ecology and evolution. Here, we have described some aspects of its chemical ecology and behavioral responses. We have shown that a single compound, 4-oxo-(*E*)-2-hexenal, functions both as a deterrent to predators and an alarm pheromone to conspecifics. Because of these multiple functions it may be a particularly interesting compound to study other members of the genus *Thasus*. We have also shown that the alarm pheromones in giant mesquite bugs are composed of multiple components and are different between nymphs and adults. The threat of predation is not communicated from one life stage to another. The adaptive reason explaining the need for multiple components in an alarm signal remains unexplored in this group.

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References

ALDRICH, J. R. 1988. Chemical ecology of the Heteroptera. *Annu. Rev. Entomol.* 33:211–238.

ALDRICH, J. R., and BLUM, M. S. 1978. Aposematic aggregation of a bug (Hemiptera: Coreidae): The defensive display and formation of aggregations. *Biotropica* 10:58–61.

ALDRICH, J. R., and YONKE, T. R. 1975. Natural products of abdominal and metathoracic scent glands of Coreid bugs. *Ann. Entomol. Soc.* 68:955–960.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. p. 562. Academic, New York.

BRAILOVSKY, H., SCHAEFER, C. W., BARRERA, E., and PACKAUSKAS, R. J. 1994. A revision of the genus *Thasus* (Hemiptera: Coreidae: Coreinae: Nematopodini). *J. New York Entomol. Soc.* 102:318–343.

CALAM, D. H., and YOUDEOWEI, A. 1968. Identification and functions of secretion from the posterior scent gland of fifth instar larva of the bug *Dysdercus intermedius*. *J. Insect Physiol.* 14:1147–1158.

DE LA TORRE-BUENO, R. T., and AMBROSE, W. G. 1936. Effects of the protective vapors of the Coreid bug *Thasus gigas* on a tarantula (*Eurypelma* sp.). *Bull. Brook. Entomol. Soc* 31:184.

EISNER, T. 1970. Chemical defense against predation in arthropods, pp. 157–217, in E. Sondheimer, and J. B. Simeone (eds.). Chemical EcologyAcademic, New York.

EISNER, T., EISNER, M., and SIEGLER, M. 2007. Secret weapons: Defenses of insects, spiders, scorpions, and other many-legged creatures. Belknap, Harvard University, Boston, MA.

FORBES, G., and SCHAEFER, C. W. 2003. Further notes on the genus *Thasus* (Hemiptera: Heteroptera: Coreidae). *J. New York Entomol. Soc.* 111:235–241.

GAMBERALE, G., and TULLBERG, B. S. 1998. Apsosematism and gregariousness: the combined effect of group size and coloration on signal repellence. *Proc. Roy. Soc. B.* 265:889–894.

KASAI, H., MAEKAWA, M., KAWAI, K., HACHISUKA, K., TAKAHASI, Y., NAKAMURA, H., SAWA, R., MATSUI, S., and MATSUDA, T. 2005. 4-Oxo-2-hexenal, a mutagen formed by 3-fat peroxidation, causes DNA adduct formation in mouse organs. *Indust. Health* 43:699–701.

LEAL, W. S., PANIZZI, A. R., and NIVA, C. C. 1994. Alarm pheromone system of leaf-footed bug *Leptolossus zonatus* (Heteroptera: Coreidae). *J. Chem. Ecol.* 20:1209–1215.

MIILAR, J. G. 2005. Pheromones of true bugs. *Topics Curr. Chem.* 240:37–84.

MOREIRA, J. A., and MILLAR, J. G. 2005. Short and simple synthesis of 4-oxo-(*E*)-2-hexenal and homologs: Pheromone components and defensive compounds of Hemiptera. *J. Chem. Ecol* 31:965–968.

OLSEN, C. E. 2004. 50 Common Insects of the Southwest. Western National Parks Association, Tucson, AZ.

PRESTWICH, G. D. 1976. Composition of the scents of eight east African Hemipterans. Nymph–adult chemical polymorphism in Coreids. *Ann. Entomol. Soc.* 69:812–814.

SAS INSTITUTE INC. 2002. JMP-In Statistical Exploration Software. Version 5. Cary, NC.

SILLÉN-TULLBERG, B. 1988. Evolution of gregariousness in aposematic butterfly larvae: a phylogenetic analysis. *Evolution* 42: 293–305.

SILLÉN-TULLBERG, B. 1993. The effect of biased inclusion of taxa on the correlation between discrete characters in phylogenetic trees. *Evolution* 47:1182–1191.

TULLBERG, B. S., and HUNTER, A. F. 1995. Evolution of larval gregariousness in relation to repellent defenses and warning coloration in tree-feeding Macrolepidoptera: a phylogenetic analysis based on independent contrasts. *Biol. J. Linn. Soc.* 57:253–276.

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An Unexpected Mixture of Substances in the Defensive Secretions of the Tubuliferan Thrips, *Callococcithrips fuscipennis* (Moulton)

Gunther Tschuch · Peter Lindemann · Gerald Moritz

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Abstract Adults and larvae of the thrips *Callococcithrips fuscipennis* (Moulton) (Thysanoptera: Tubulifera: Phlaeothripidae) live in the sticky wax masses of adult females of the felt scale insect *Callococcus acaciae* (Maskell) (Sternorrhyncha: Coccoidea: Eriococcidae). The scale is sessile and feeds on *Kunzea* shrubs (Myrtales: Myrtaceae). If stressed, the thrips produce droplets of secretions. The mixture contains pentadecane, tridecane, two monoterpenoids, hexadecyl butanoate, and smaller amounts of 15 other esters of long-chain unbranched alcohols identified as acetates, butanoates, hexanoates, and octanoates. The monoterpenoids are dolichodial, an iridoid, and an unknown substance with a mass spectrum very similar to that of anisomorphal and peruphasmal, diastereomers of dolichodial, but with a different retention time. Iridoids, butanoates, hexanoates, and octanoates have not been previously identified in Thysanoptera.

Keywords *Callococcus acaciae* · Dolichodial · Hexadecyl butanoate · Thysanoptera · Phlaeothripidae · Eriococcidae · Coccoidea · Alkanes · Iridoids · Acetates · Butanoates · Hexanoates · Octanoates

Introduction

During the last three decades, there have been more than 30 papers dealing with secretions of thrips, especially of the family Phlaeothripidae (Moritz 2006). Several thrips species, when they are disturbed, raise and lower their abdomen with a drop of fluid on the abdominal tip (Lewis 1973). The secretory reservoir seems to be the hindgut, but the gland cells have not yet been found (Howard et al. 1983, 1987). Some of the complete secretions, as well as single components, have been found to be ant repellents (Howard et al. 1983; Blum 1991; Blum et al. 1992; Tschuch et al. 2005).

The present paper deals with *Callococcithrips fuscipennis* (Thysanoptera: Phlaeothripinae), originally described as *Rhynchothrips* by Moulton (1968, pp. 97–98). The species subsequentially was associated with the genus *Teuchothrips* (Mound and Houston 1987), but this assignment was controversial[1] and changed recently to *Callococcithrips* (Mound and Wells 2007). *C. fuscipennis* are frequently found near Canberra (Australia) inside the wax masses of the adult female felt scale insects *Callococcus acaciae* (Maskell) (Coccoidea: Eriococcidae)[2], which are sessile on *Kunzea ericoides* (Myrtaceae) shrubs. Because of limited knowledge of *C. fuscipennis* biology, it remains unclear whether they are predators of *C. acaciae*, or feed on other items such as plant, scale secretions, scale feces, or other insects living in the wax. Predation by phlaeothripid thrips on scale insects is not unlikely, especially on larvae and eggs, and is well known on some species of armored (Diaspididae, Palmer and Mound 1990) and soft scales (Coccidae, Pfeiffer 1997).

G. Tschuch (✉) · G. Moritz
Developmental Biology, Institute of Biology,
Martin-Luther-University Halle-Wittenberg,
06099 Halle, Germany
e-mail: gunther.tschuch@zoologie.uni-halle.de

P. Lindemann
Pharmaceutical Biology, Institute of Pharmacy,
Martin-Luther-University Halle-Wittenberg,
06099 Halle, Germany

[1] Moulton (1968) described another *Teuchothrips fuscipennis* in the same 1968 paper on pp. 100–101. This species was found to be a synonym for *Heligmothrips erinaceus* (Karny) (Mound 1970).

[2] Originally, the species was described by Maskell (1893) as *Sphaerococcus acaciae* and was subsequently combined with the genus *Callococcus* by Morrison and Morrison (1927). *Callococcus* was first placed into the family Asterolecaniidae, but is now a genus of the family Eriococcidae (Miller et al. 1998).

C. acaciae was originally named according to the host-plant "*Acacia* spec." by Maskell (1893), but this is likely to be a mistake. The host plants are species of the genera *Kunzea* (Myrtaceae) (personal communication, Penelope J. Gullan, University of California, Davis). Banks et al. (1976) collected *C. acaciae* from *Kunzea* and from *Leptospermum* (Myrtaceae). The wax masses of *C. acaciae* are unique, measuring around 10 mm in diameter and containing a sticky tangle of filaments. The stickiness of the wax is caused by a special surface coating and is the topic of a recent investigation (Tschuch et al. 2006)[3]. There are strong indications that *C. acaciae*, together with *C. pulchellus* (Maskell), arose from a gall-inducing Myrtaceae-feeding ancestor and re-evolved its protective cover (Cook and Gullan 2004). Other closely related species are still gall-formers on myrtaceans. In contrast to the wax of other scale insects, ants avoid contact with *C. acaciae* wax (Mound and Wells 2007). Therefore, it is likely that *C. fuscipennis* may have little need for a defensive secretion against predators, even though one seems to be produced. This suggests that these secretions may have additional biological functions. The objective of this study was to determine the composition of *C. fuscipennis* secretion.

Methods and Materials

Sample Collection Small branches and twigs of *K. ericoides* with wax masses of *C. acaciae* were collected in Canberra (Australia) early in March 2006. The samples were stored and transported at room temperature. After 2 weeks, the wax masses were dissected with caution to get undisturbed adult *C. fuscipennis* with a high amount of secretion. The adult thrips were placed on a glass slide and stimulated mechanically to produce droplets, which were collected with a fine glass capillary tube (Minicaps 5 µl, Hirschmann Laborgeräte). The tube was then rinsed with distilled methanol (Aldrich, 49,429-1). For chemical analysis, droplets were collected from ten thrips and investigated within 2 h.

Chemical Analysis Through coupled gas chromatography/mass spectrometry analyses, samples were dissolved in a small amount (50–100 µl) of methanol, and 5 µl was injected into an HP 5890GC linked to a 5972MSD (Hewlett-Packard) that was operated in electron impact mode at 70 eV. Separations were carried out with an HP5-MS column (30 m×0.25 mm ID; stationary phase: 0.25 µm). The carrier gas was helium (1 ml/min), the injector temperature was set to 300°C, and the detector temperature to 280°C. Column temperature was maintained at 100°C following the injection, and then linearly increased to 300°C at a rate of 7°C/min. The NIST database, literature data, and the software AMDIS (NIST, version 2.63) and NIST MS Search were used to analyze mass spectra and to compare retention times. For comparison with authentic compounds, the following substances and mixtures were used: tridecane (Aldrich, T57401), pentadecane (Aldrich, P3406), hexadecyl acetate (Sigma, P0260), octadecyl acetate (Sigma, S5003), purified secretions from stick insects *Anisomorpha buprestoides* (Stoll) (Pseudophasmidae; Dossey et al. 2006; Zhang et al. 2007; obtained from Aaron T. Dossey, Gainsville, FL, USA, one sample with only anisomorphal and another containing anisomorphal, peruphasmal, and trace dolichodial, dissolved in $CDCl_3$ and verified by NMR), and crude methanol extracts (1 h at 25°C) from chopped leaves of "cat thyme" germander *Teucrium marum* (Lamiaceae) (plant obtained from M. Hoffmann, Halle, and bought from a local florist), containing mainly dolichodial and teucrein.

In addition, hexadecyl and octadecyl butanoate, synthesized from authentic alcohols (Aldrich W25,540-8 and 258768) were used as standards. For comparison of the retention indices of iridoids, tetradecane (Fluka, 87140) was used as a standard.

Results

Most of the components of the defensive secretions were saturated alkanes (Fig. 1, *1* and *4*), monoterpenoids (*2* and *3*), and esters of saturated long-chain alcohols and short-chain acids (*5* to *20*; the chromatogram shows tailing, but the small number of animals available and small sample amount made it impossible to purify the sample, to use another solvent, or to repeat the investigation). The two monoterpenoids showed mass spectra characteristic of iridoids with base peaks at *m*/*z* 81 and further diagnostic ions at *m*/*z* 67, 95, and 109. The main component (*4*) was identified as pentadecane, and the first eluting component (*1*) as tridecane by comparison with authentic compounds.

Substance *2* produced a mass spectrum very similar to that of dolichodial (2-formyl-3-methyl-α-methylidenecyclopentaneacetaldehyde) in the NIST 98 database. Retention time and mass spectral comparisons with authentic dolichodial from *T. marum* and in a secretion from *A. buprestoides* confirmed the identification. It was not possible to determine the enantiomeric composition of dolichodial.

The mass spectrum of *3* was very similar to that of anisomorphal from *A. buprestoides* (the C2-epimer of

[3] In that paper the coccid species was incorrectly named as *Callococcus banksiae* instead of *C. acaciae*.

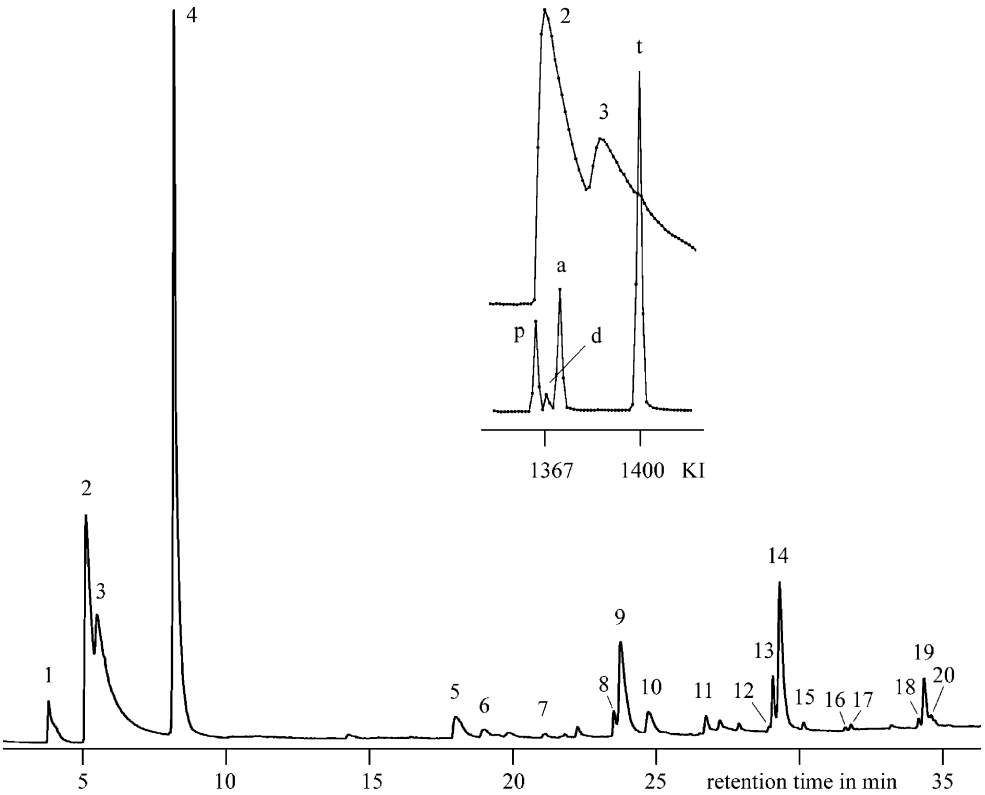

Fig. 1 Total ion gas chromatogram of the secretions of ten adult *Callococcithrips. fuscipennis* (*1* tridecane, *2* dolichodial, *3* unknown, *4* pentadecane, *5* to *20* esters, see Table 1; *X*-axis—retention time). *Insertion* Detail of the chromatogram (*upper graph*) in comparison to the chromatogram of a mixture (*lower graph*) containing peruphasmal (*p*), dolichodial (*d*), anisomorphal (*a*), and tetradecane (*t*); *X*-axis—Kovat's index

dolichodial) and peruphasmal from *P. schultei* (another diastereomer of dolichodial; see Dossey et al. 2006 and supporting information therein). As compared to dolichodial, the mass spectra of all three substances show the same small differences, including the lack of the molecular ion (M^+ 166) but the presence of $(M\text{-}1)^+$ (165, 3%), and differences in the relative intensities of the signals at *m/z* 133 and 137 compared with *m/z* 138 (Fig. 2). The retention index of *3* was distinctly different from those of the known stereoisomers of dolichodial (Fig. 1, insertion). Compared with dolichodial (KI=1367), the Kovat's index (KI) on a DB-5 column was lower for peruphasmal (KI=1,361: Δ KI=−6), higher for anisomorphal (KI=1,373: Δ KI=+6), and highest for 3 (KI=1,386: Δ KI=+19).

The mass spectra of many of the substances that eluted after pentadecane (Fig. 1, *5* to *20*) are not included in common databases. They show a relatively high abundance of diagnostic fragments with *m/z* values of 61, 89, 117, and 145, characteristic of esters of long-chain aliphatic alcohols with short fatty acids. A McLafferty-type rearrangement of

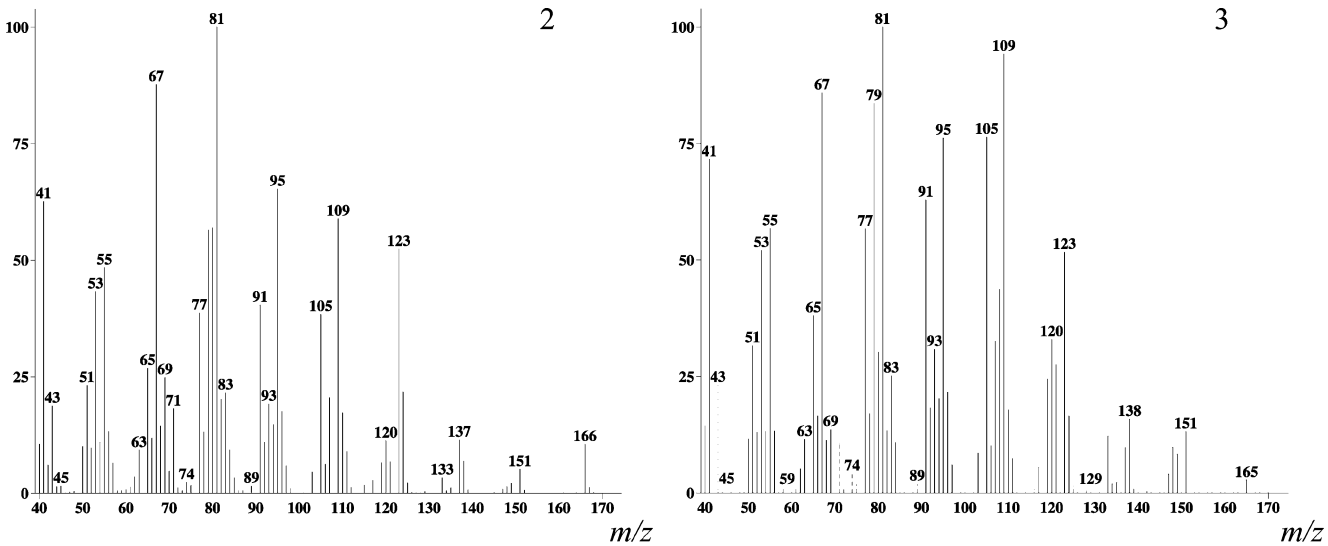

Fig. 2 Mass spectra of substances *2* and *3*. Spectrum of *2* is identical to the mass spectrum of dolichodial, while spectrum *3* is more similar to those of anisomorphal (*a*) and peruphasmal (*p*; see Fig. 1)

such esters, taking place at the alcohol side produces the respective acid and an alkene corresponding to the alcohol (Francke et al. 2000). Whereas the acid gives rise to a signal representing the protonated form (Table 1, $(A+1)^+$), the alkene fragment and a corresponding signal, produced by loss of the ethylene from the alkene are clearly visible ($(M-A)^+$ and $(M-A-C_2H_4)^+$). Another diagnostic fragment of esters of long-chain alcohols with short fatty acids is produced upon α-cleavage at the acid side, i.e., loss of the alkyl chain of the acid $(M-(A-COOH))^+$.

Apart from the most abundant ester, hexadecyl butanoate (Fig. 1, 14), almost all possible combinations of C12 … C16 and C18 alcohols with acetic, butyric, hexanoic, and octanoic acids in the mass range from 256 to 340 were found (*M* in Table 1). The esters with odd-numbered alcohols had low abundances. From that group, only traces of tridecyl butanoate (7), pentadecyl butanoate (11), and pentadecyl hexanoate (17) could be identified clearly.

The acetates, butanoates, and the hexanoates had no branch points in either the acid or alcohol parts. Besides the fragments mentioned above, the only signals between *A* and *M* were from the consistent low-mass ion series 69, 83, 97, …, indicating that there were no branch points in the alcohol portion. Branches in the acid portion of hexanoate isomers would cause cleavages before and behind the branch point, generating noticeable peaks of >1% abundance if the loss is greater than a methyl group (McLafferty and Tureček 1993). For instance, 3-methylpentanoates have diagnostic peaks at *M*-28 (loss of an ethyl group) and at *M*-57 (loss of a butyl group). In all possible branched isomers of hexanoates, there would be at least one loss causing a fragment ion other than the diagnostic ion mentioned above. However, with these considerations it is not possible to distinguish between the unbranched butanoates and the branched 2-methylpropanoates, because the abundance for the loss of a methyl group is too low, and the loss of a propyl group is observable as well in the unbranched butanoates. Here, the characteristic ions at *m/z* 60 and 61 are helpful. They appear in all butanoates and longer acid esters (other than methyl esters) if the acid's α-carbon is not substituted (McLafferty and Tureček 1993), as a result of a consecutive rearrangement of two hydrogen atoms. Peaks at *m/z* 60 and 61 with abundances of 5–12% both occur in all butanoates listed in Table 1 (*5*, *7*, *9*, *11*, *14*, and *20*). Thus, the butanoates also are not branched. Two acetates (*10* and *15*) and two butanoates (*14* and *20*) were compared with authentic substances and yielded matching retention times and mass spectra. Unfortunately, the abundances of the octanoates (*12*, *16*, and *18*) are too low to exclude all possible branch points for these substances.

Because wax particles were sometimes found to be stuck on the thrips cuticle, these could have contaminated the collected secretions. However, neither compounds known from the *C. acaciae* wax (Tschuch et al. 2006) nor any from the scale host-plant *K. ericoides* (Christoph 2001) were detected in the thrips extracts.

Table 1 Esters found in the defensive secretion of *Callococcithrips fuscipennis* and (in brackets) relative abundances of their diagnostic fragment ions $(A+1)^+$, $(M-A-C_2H_4)^+$, $(M-A)^+$, and $(M-(A-COOH))^+$

Number (Fig. 1)	Ester name	$(A+1)^+$	$(M-A-C_2H_4)^+$	$(M-A)^+$	$(M-(A-COOH))^+$	Kovat's index
5	C12 butanoate	89 (100)	140 (6)	168 (7)	213 (2)	1,778
6	C14 acetate	61 (35)	168 (3)	196 (2)	n/a	1,811
7	C13 butanoate	89 (100)	154 (6)	182 (5)	227 (2)	1,886
8	C12 hexanoate	117 (100)	140 (8)	168 (12)	213 (2)	1,968
9	C14 butanoate	89 (100)	168 (3)	196 (4)	241 (2)	1,975
10	C16 acetate*	61 (45)	196 (5)	224 (2)	n/a	2,009
11	C15 butanoate	89 (100)	182 (3)	210 (3)	255 (1)	2,083
12	(C12 octanoate)	145 (51)	140 (4)	168 (11)	213 (7)	2,165
13	C14 hexanoate	117 (100)	168 (3)	196 (7)	241 (3)	2,170
14	*C16 butanoate**	*89 (100)*	*196 (1)*	*224 (3)*	*269 (2)*	*2,179*
15	C18 acetate*	61 (34)	224 (2)	252 (2)	n/a	2,210
16	(C13 octanoate)	145 (3)	154 (3)	182 (3)	227 (2)	2,263
17	C15 hexanoate	117 (100)	182 (1)	210 (4)	255 (2)	2,272
18	(C14 octanoate)	145 (21)	168 (3)	196 (2)	241 (1)	2,359
19	C16 hexanoate	117 (100)	196 (2)	224 (4)	269 (2)	2,366
20	C18 butanoate*	89 (100)	224 (2)	252 (2)	297 (2)	2,375

The ester with the highest abundance is hexadecyl butanoate (italics). The substances marked by an asterisk were confirmed by comparison with authentic substances. The identifications of the three octanoates (in brackets) are likely, but, because of the low abundances, it is not possible to exclude the existence of branch points completely (see text).
n/a Fragment ion not occurring in acetates, *A* mass of acid, *M* mass of molecule

Discussion

Iridoid monoterpenes have never been reported before from Thysanoptera. However, dolichodial and its stereoisomers are well known from some species of ants (Dolichoderinae: Cavill and Hinterberger 1960; Cavill et al. 1976), from two species of stick insects (Pseudophasmatidae: Meinwald et al. 1962; Dossey et al. 2006), from larvae of several saw flies (Tenthredinidae: Boeve et al. 1984, 1992), from rove beetles (Staphylinidae: Huth and Dettner 1990), and from the plant *T. marum* (Pagnoni et al. 1976). Compound 3 very likely is a third stereoisomer of dolichodial, but not anisomorphal and peruphasmal. This is supported by the fact that the mass spectra of other known dolichodial isomers, like chrysomelidial and plagiodial, show big mass spectral and/or retention time differences between each other, and the spectra are different from the well-known stereoisomers mentioned above, as well as from substance 3 (Meinwald et al. 1977; Meinwald and Jones 1978; Sugawara et al. 1979).

In previously reported bioassays, dolichodial repelled insects, such as ants, flies, cockroaches (Eisner et al. 2000), and termites (Cornelius et al. 1995). Observations on *A. buprestoides* showed that iridoids also may repel vertebrates such as birds (Eisner 1965). In *T. marum*, dolichodial also was suggested to be an insect repellent because no insects are known to feed on the plant (Eisner et al. 2000). Other iridoid dialdehydes such as chrysomelidial and plagiodial, found in the secretions of larvae of leaf beetles (Chrysomelidae), also act as defensive substances (Meinwald et al. 1977; Sugawara et al. 1979; Pasteels et al. 1982). Thus, the two iridoid dialdehydes found in *C. fuscipennis*, dolichodial and the unknown compound 3, may have a defensive function.

The function of the esters in *C. fuscipennis* secretion is uncertain. Hexadecyl butanoate has been found in arthropods only in Dufour's gland secretions or cephalic extracts of a bumblebee (Tengö et al. 1991) and some other bee species from the families Melittidae (Tengö and Bergström 1976) and Apidae (Batra and Hefetz 1979; Francke et al. 2000). In *C. fuscipennis* secretion, hexadecyl butanoate possibly acts in a manner analogous to that of the acetates found in many other thrips species (Suzuki et al. 2004). For example, in *Gynaikothrips ficorum*, the secretions contain mainly hexadecyl acetate dissolved in pentadecane (Howard et al. 1987). Both substances are repellents against ants, and they work better as a mixture. Acetates may help to spread secretions and to wet sensory organs of predators with long-lasting coatings (see discussion in Tschuch et al. 2005). The use of butanoates instead of acetates may be advantageous because of the lower polarity, thus causing a better interaction with the cuticular lipids of the aggressor. This is a topic of further investigations.

The iridoids in the secretion of *C. fuscipennis* could serve a defensive function once the thrips are outside of the wax masses of scale insects, or against insects entering or living inside the wax masses as predators or parasitoids. Additionally, the larval defensive secretion in *Frankliniella occidentalis* (Thripidae), the most studied of all the thrips secretions (Teerling et al. 1993; MacDonald et al. 2002), has several other functions besides defense. Hence, the secretion in *C. fuscipennis* could also have other functions, which might at least partly explain why the species produces such a secretion despite an apparent lack of need for one due to its protective wax mass habitat. The protective nature of these insects' habitat, along with the chemical complexity of the secretions, suggests that these secretions may indeed have functions besides defense.

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References

BANKS, H. J., CAMERON, D. W., EDMONDS, J. S., and RAVERTY, W. D. 1976. Chemistry of the Coccoidea. III Isolation of a bianthrone glucoside from *Callococcus acaciae* and of ceroalbolinic acid from *Cryptes baccatum* (Hemiptera: Insecta). *Aust. J. Chem.* 29:2225–2230.

BATRA, S. W. T., and HEFETZ, A. 1979. Chemistry of the cephalic and Dufour's secretions of *Melissodes* bees. *Ann. Ent. Soc. Am.* 72:514–515.

BLUM, M. S. 1991. Chemical ecology of the Thysanoptera, pp. 95–112, in B. L. Parker, M. Skinner, and T. Lewis (eds.). Towards Understanding Thysanoptera. USDA Forest Service General Technical Report NE-147.

BLUM, M. S., FOOTIT, R., and FALES, H. M. 1992. Defensive chemistry and function of the anal exudate of the thrips *Haplothrips leucanthemi*. *Comp. Biochem. Physiol. C* 102:209–211.

BOEVE, J.-L., BRAEKMAN, J. C., DALOZE, D., HOUART, M., and PASTEELS, J. M. 1984. Defensive secretions of Nematinae larvae (Symphyta—Tenthredinidae). *Experientia* 40:546–547.

BOEVE, J.-L., DETTNER, K., FRANCKE, W., MEYER, H., and PASTEELS, J. M. 1992. The secretion of the ventral glands in *Nematus* sawfly larvae. *Biochem. Syst. Ecol.* 20:107–111.

CAVILL, G. W. K., and HINTERBERGER, H. 1960. The chemistry of ants. IV. Terpenoid constituents of some *Dolichoderus* and *Iridomyrmex* species. *Aust. J. Chem.* 13:514–519.

CAVILL, G. W. K., HOUGHTON, E., MCDONALD, F. J., and WILLIAMS, P. J. 1976. Isolation and characterization of dolichodial and

related compounds from the Argentine ant, *Iridomyrmex humilis*. *Insect Biochem.* 6:483–490.

CHRISTOPH, F. 2001. Chemische Zusammensetzung und antimikrobielle Eigenschaften der Teebaumöle der Gattungen *Kunzea*, *Leptospermum* und *Melaleuca* unter besonderer Berücksichtigung von Handelsölen. Dissertation, Universität Hamburg.

COOK, L. G., and GULLAN, P. J. 2004. The gall-inducing habit has evolved multiple times among the eriococcid scale insects (Sternorrhyncha: Coccoidea: Eriococcidae). *Biol. J. Linn. Soc.* 83:441–452.

CORNELIUS, M. L., GRACE, J. K., FORD, P. W., and DAVIDSON, B. S. 1995. Toxicity and repellency of semiochemicals extracted from a dolichoderine ant (Hymenoptera: Formicidae) to the formosan subterranean termite (Isoptera: Rhinotermitidae). *Environ. Entomol.* 24:1263–1268.

DOSSEY, A. T., WALSE, S. S., ROCCA, J. R., and EDISON, A. S. 2006. Single-insect NMR: A new tool to probe chemical biodiversity. *ACS Chemical Biology* 1:511–514 and supporting information (DOI 10.1021/cb600318u; http://pubs.acs.org/subscribe/journals/acbcct/suppinfo/cb600318u/cb600318usi060919_000000.doc).

EISNER, T. 1965. Defensive spray of a phasmid insect. *Science* 148:966–968.

EISNER, T., EISNER, M., ANESHANSLEY, D. J., WU, C.-L., and MEINWALD, J. 2000. Chemical defense of the mint plant, *Teucrium marum*. *Chemoecology* 10:211–216.

FRANCKE, W., LÜBKE, G., SCHRÖDER, W., RECKZIEGEL, A., IMPERATRIZ-FONSECA, V., KLEINERT, A., ENGELS, E., HARTFELDER, K., RADTKE, R., and ENGELS, W. 2000. Identification of oxygen containing volatiles in cephalic secretions of workers of Brazilian stingless bees. *J. Braz. Chem. Soc.* 11:562–571.

HOWARD, D. F., BLUM, M. S., and FALES, H. M. 1983. Defense in thrips: forbidding fruitiness of a lactone. *Science* 220:335–336.

HOWARD, D. F., BLUM, M. S., JONES, T. H., FALES, H. M., and TOMALSKI, M. D. 1987. Defensive function and chemistry of the anal exudate of the Cuban laurel thrips *Gynaikothrips ficorum* (Marchal). *Phytophaga* 1:163–170.

HUTH, A., and DETTNER, K. 1990. Defense chemicals from abdominal glands of 13 rove beetle species of subtribe Staphylinina (Coleoptera: Staphylinidae, Staphylininae). *J. Chem. Ecol.* 16:2691–2711.

LEWIS, T. 1973. Thrips, Their Biology, Ecology and Economic Importance. p. 348. Academic, New York.

MACDONALD, K. M., HAMILTON, J. G. C., JACOBSON, R., and KIRK, W. D. J. 2002. Effects of alarm pheromone on landing and take-off by adult western flower thrips. *Ent. Exp. Appl.* 103:279–282.

MASKELL, W. M. 1893/1892. Art. XXVI.—Further coccid notes: with descriptions of new species from Australia, India, Sandwich Islands, Demerara, and South Pacific. *Trans. Proc. N. Z. Inst.* 25:201–252.

MCLAFFERTY, F. W., and TUREČEK, F. 1993. Interpretation of Mass Spectra. 4th edn.University Science Books, Sausalito, CA.

MEINWALD, J., and JONES, T. H. 1978. Synthesis and stereochemistry of chrysomelidial and plagiolactone. *J. Am. Chem. Soc.* 100:1883–1886.

MEINWALD, J., CHADHA, M. S., HURST, J. J., and EISNER, T. 1962. Defense mechanisms of arthropods—IX. Anisomorphal, the secretion of a phasmid insect. *Tetrahedron Lett.* 1962:29–33.

MEINWALD, J., JONES, T. H., EISNER, T., and HICKS, K. 1977. New methylcyclopentanoid terpenes from larval defensive secretion of chrysomelid beetle (*Plagiodera versicolora*). *Proc. Natl. Acad. Sci. U. S. A.* 74:2189–2193.

MILLER, D. R., GULLAN, P. J., and WILLIAMS, D. J. 1998. Family placement of species previously included in the scale insect genus *Sphaerococcus* Maskell (Hemiptera: Coccoidea). *Proc. Entomol. Soc. Wash.* 100:286–305.

MORITZ, G. 2006. Thripse: Fransenflügler, Thysanoptera. p. 384. Westarp-Wissenschaften, Hohenwarsleben.

MORRISON, H., and MORRISON, E. 1927. The Maskell species of scale insects of the subfamily Asterolecaniinae. *Proc. U. S. Natl. Mus.* 71:Art. 171–67.

MOULTON, D. 1968. New Thysanoptera from Australia. *Proc. Calif. Acad. Sci. Fourth Series* 36:93–124.

MOUND, L. A. 1970. Convoluted maxillary stylets and the systematics of some phlaethripinae Thysanoptera from *Casuarina* trees in Australia. *Austr. J. Zool.* 18:439–463.

MOUND, L. A., and HOUSTON, K. J. 1987. An annotated check-list of Thysanoptera from Australia. *Occas. Pap. Syst. Entomol.* 4:1–28.

MOUND, L. A., and WELLS, A. 2007. A new genus for an Australian thrips (Thysanoptera, Phlaethripinae) presumed predatory on a waxy eriococcid (Hemiptera, Coccoidea). *Zootaxa* 1645:57–61.

PAGNONI, U. M., PINETTI, A., TRAVE, R., and GARANTI, L. 1976. Monoterpenes of *Teucrium marum*. *Aust. J. Chem.* 29:1375–1381.

PALMER, J. M., and MOUND, L. A. 1990. 2.2.5 Thysanoptera, pp. 67–76, in D. Rosen (ed.). Armored Scale Insects—Their Biology, Natural Enemies and Control. Vol. 4BElsevier, Amsterdam.

PASTEELS, J. M., BRAEKMAN, J. C., DALOZE, D., and OTTINGER, R. 1982. Chemical defense in chrysomelid larvae and adults. *Tetrahedron* 38:1891–1897.

PFEIFFER, D. G. 1997. 3.3.8 Deciduous fruit trees, pp. 293–322, in Y. Ben-Dov, and C. J. Hodgson (eds.). Soft Scale Insects—Their Biology, Natural Enemies and Control. Vol. 7BElsevier, Amsterdam.

SUGAWARA, F., MATSUDA, K., KOBAYASHI, A., and YAMASHITA, K. 1979. Defensive secretion of chrysomelid larvae *Linaeidea aenea* Linné and *Plagiodera versicolora* Baly. *J. Chem. Ecol.* 5:929–934.

SUZUKI, T., HAGA, K., TSUTSUMI, T., and MATSUYAMA, S. 2004. Analysis of anal secretions from phlaeothripinae thrips. *J. Chem. Ecol.* 30:409–423.

TEERLING, C. R., PIERCE, H. D., BORDEN, J. H., and GILLESPIE, D. R. 1993. Identification and bioactivity of alarm pheromone in the western flower thrips, *Frankliniella occidentalis*. *J. Chem. Ecol.* 19:681–697.

TENGÖ, J., and BERGSTRÖM, G. 1976. Odor correspondence between *Melitta* females and males of their nest parasite *Nomada flavopicta* K. (Hymenoptera, Apidae). *J. Chem. Ecol.* 2:57–65.

TENGÖ, J., HEFEZ, A., BERTSCH, A., SCHMITT, U., LÜBKE, B., and FRANCKE, W. 1991. Species specificity and complexity of Dufour's gland secretion of bumble bees. *Comp. Biochem. Physiol.* 99B:641–646.

TSCHUCH, G., LINDEMANN, P., NIESEN, A., CSUK, R., and MORITZ, G. 2005. A novel long chained acetate in the defensive secretion of thrips. *J. Chem. Ecol.* 31:1555–1565.

TSCHUCH, G., LINDEMANN, P., RETTICH, W., MOUND,, L. A., and MORITZ, G. 2006. Designer-Lipide auf dem Wachs einer Schildlaus (Coccoidea, Eriococcidae). *Mitt. Dtsch. Ges. Allg. Angew. Entomol.* 15:143–146.

ZHANG, F., DOSSEY, A. T., ZACHARIAH, C., EDISON, A. S., and BRÜSCHWEILER, R. 2007. Strategy for automated analysis of dynamic metabolic mixtures by NMR. Application to an insect venom. *Anal. Chem.* 79:7748–7752.

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Oxidation of Ingested Phenolics in the Tree-Feeding Caterpillar *Orgyia leucostigma* Depends on Foliar Chemical Composition

Raymond Barbehenn · Quentin Weir · Juha-Pekka Salminen

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Abstract Tannins are believed to function as antiherbivore defenses, in part, by acting as prooxidants. However, at the high pH found in the midguts of caterpillars, the oxidative activities of different types of tannins vary tremendously: ellagitannins >> galloyl glucoses > condensed tannins. Ingested ascorbate is utilized by caterpillars to minimize phenolic oxidation in the midgut. Thus, leaves that contain higher levels of reactive tannins and lower levels of ascorbate were hypothesized to produce higher levels of phenolic oxidation in caterpillars. We tested this hypothesis with eight species of deciduous trees by measuring their foliar phenolic and ascorbate compositions and measuring the semiquinone radical (oxidized phenolic) levels formed in caterpillars that ingested each species. When the generalist caterpillars of *Orgyia leucostigma* (Lymantriidae) fed on the leaves of tree species in which condensed tannins were predominant (i.e., *Populus tremuloides*, *P. deltoides*, and *Ostrya virginiana*), semiquinone radical levels were low or entirely absent from the midgut contents. In contrast, species that contained higher levels of ellagitannins (or galloyl rhamnoses; i.e., *Quercus alba*, *Acer rubrum*, and *A. saccharum*) produced the highest levels of semiquinone radicals in *O. leucostigma*. Low molecular weight phenolics contributed relatively little to the overall oxidative activities of tree leaves compared with reactive tannins. Ascorbate levels were lowest in the species that also contained the highest levels of oxidatively active tannins, potentially exacerbating phenolic oxidation in the gut lumen. We conclude that the tannin compositions of tree leaves largely determine the effectiveness of foliar phenolics as oxidative defenses against caterpillars such as *O. leucostigma*.

Keywords Oxidative stress · Phenolic oxidation · Tannin · Semiquinone radical · Caterpillar · Herbivore · Tree chemical defense

R. Barbehenn (✉) · Q. Weir
Departments of Molecular,
Cellular and Developmental Biology and Ecology and
Evolutionary Biology, University of Michigan,
Ann Arbor, MI 48109-1048, USA
e-mail: rvb@umich.edu

J.-P. Salminen
Laboratory of Organic Chemistry and Chemical Biology,
Department of Chemistry, University of Turku,
20014 Turku, Finland

Introduction

Phenolics are ubiquitous chemical defenses in leaves, playing protective roles against a wide variety of biotic agents and abiotic stresses. Tannins are the predominant phenolics produced in tree leaves, often reaching levels of 5–20% dry weight (DW) in temperate deciduous species. Whereas tannins were long believed to act as protein-binding agents that reduced the digestive efficiencies of insect herbivores, protein binding *in vivo* is now known to be precluded by high pH and/or surfactants generated from lipid digestion (Martin et al. 1987). Instead, as defenses against caterpillars, tannins can act as anti-feedants or have toxic or anti-nutritive effects. Because tannins are not absorbed, their post-ingestive effects are thought to result from their oxidation in the midgut lumen (Barbehenn 2001; Barbehenn et al. 2005). The oxidation of phenolics produces semiquinone radicals and other reactive oxygen species. If levels of these products overwhelm the antioxidant defenses in the midgut lumen or tissues, oxidative stress ensues. The oxidative damage to a wide variety of biological molecules (including nutrients, cell membrane

components, and DNA) that occurs from oxidative stress is believed to contribute to reduced fitness in insect herbivores (Summers and Felton 1994; Bi and Felton 1995; Bi et al. 1997).

Ascorbate is the main low molecular weight (MW) antioxidant found in the gut fluids and tissues of leaf-feeding insects (Felton and Duffey 1992; Barbehenn et al. 2001, 2003a). However, antioxidant defense systems that utilize ascorbate can be overwhelmed when insects feed on leaves that contain either large amounts or highly reactive types of tannins (Barbehenn et al. 2005). The most reactive types of tannins in the high pH conditions found in caterpillar midguts are ellagitannins, followed by galloyl glycosides (or "gallotannins") and, distantly, by condensed tannins (Barbehenn et al. 2006b; Moilanen and Salminen 2008). Whether this pattern of tannin structure and reactivity also occurs in the complex chemical mixtures found in the midgut fluids of leaf-feeding herbivores has been examined with only one pair of tree species. *Acer saccharum* (containing relatively high levels of ellagitannins) produced high levels of semiquinone radicals in the midgut contents of caterpillars, while *Quercus rubra* (primarily containing condensed tannins) produced low levels of semiquinone radicals in caterpillars (Barbehenn et al. 2005, 2006a). In this study, the association between foliar chemical composition and phenolic oxidation was examined in a diverse group of tree species when eaten by the generalist caterpillar *Orgyia leucostigma* (Lymantriidae). The caterpillars of *O. leucostigma* are highly polyphagous and include the wide variety of tree species in this study in their diet (Baker 1972; Wagner 2005). Phenolics and ascorbate were quantified in the leaves of eight North American deciduous trees: *Populus tremuloides* (quaking aspen), *P. deltoides* (cottonwood), *Ostrya virginiana* (ironwood), *Carya glabra* (bitternut hickory), *Q. rubra* (red oak), *Q. alba* (white oak), *A. rubrum* (red maple), and *A. saccharum* (sugar maple). Phenolic oxidation in the midgut fluids of *O. leucostigma* was compared between tree species by measuring levels of semiquinone radicals with electron paramagnetic resonance (EPR) spectrometry. EPR spectrometry permits the sensitive and specific detection of free radicals in the complex chemical environment of biological samples (Rosen et al. 1999; Barbehenn et al. 2005). This study tested the hypothesis that the proportion of oxidatively active tannins to ascorbate in tree leaves is positively associated with levels of phenolic oxidation in caterpillars.

Methods and Materials

Tree Leaf Chemistry Six trees of each species were tagged at ten sites, primarily in parks and woodlands in Ann Arbor, MI, USA. Trees of each species were sampled from at least three different sites. A mixture of wild and planted trees of *Q. rubra* and the *Acer* species were used. All other trees were growing wild. Trunk diameters at breast height were measured to estimate the following tree sizes (mean [centimeters] ± SE): *P. tremuloides*, 14.5±2.0; *P. deltoides*, 39.6±4.5; *O. virginiana*, 13.6±2.6; *C. glabra*, 59.5±24.0; *Q. rubra*, 32.6±4.6; *Q. alba*, 39.6±11.0; *A. rubrum*, 19.4±4.5; and *A. saccharum*, 27.7±7.7. Branch tips were cut from the sunny sides of trees at a height of 2–4 m and immediately placed in flasks of water. Leaves from the same collection used for feeding *O. leucostigma* were kept intact on twigs in containers of water overnight in an incubator to control for potential changes in foliar chemistry during the feeding period. Leaf midribs were removed with a razor blade, and the remaining leaf lamina were weighed. To measure ascorbic acid, leaves were ground in liquid nitrogen, extracted in 5% metaphosphoric acid (containing 1 mM ethylenediaminetetraacetic acid), and stored at −80°C until analyzed with high-performance liquid chromatography (HPLC; Barbehenn 2003). To measure phenolics, leaves were frozen (−80°C), lyophilized, and ground. Phenolic analyses were performed in Turku, Finland with HPLC coupled with a diode array detector and mass spectrometer, as described previously (Salminen et al. 1999; Barbehenn et al. 2006a). Hydrolyzable tannins were quantified in pentagalloyl glucose equivalents (280 nm), flavonoids in quercetin equivalents (349 nm), chlorogenic acids in chlorogenic acid equivalents (315 nm), coumaroylquinic acids in coumaric acid equivalents (315 nm), and the remaining phenolics (other than condensed tannins) as gallic acid equivalents (280 nm). In addition, total condensed tannins were estimated with the acid–butanol assay (Ossipova et al. 2001), with purified birch leaf condensed tannins as a standard. The chemical compositions of the leaves fed to larvae during a second experiment with multiple tree species were also analyzed as described above.

Free Radicals in Caterpillars Eggs of *O. leucostigma* were obtained from the Canadian Forest Pest Management Institute (Sault Ste. Marie, Ontario). Larvae were reared until the final instar in an incubator (23°C, 16:8 h, light/dark) on an artificial diet, as described previously (Barbehenn et al. 2005). Larvae were randomly assigned to feed on the leaves of one of two tree species examined at one of four dates: *Acer* species on July 4, 2006, *Quercus* species on July 7, *Populus* species on July 11, and *O. virginiana* and *C. glabra* on July 14. In the first experiments, a single tree from each species was used, providing seven to nine insect replicates on each foliar chemical profile. To demonstrate that results for each species tested were not limited by the use of a single tree per species, tree leaves from five of the tree species were fed to separate groups of *O. leucostigma*

on the same day (July 15), using trees from each species different from those used in the first set of experiments. Confirmatory EPR measurements were made on two to three larvae per tree species. *Q. rubra* and *A. saccharum* showed similar results in the first set of experiments as were found previously (Barbehenn et al. 2005; unpublished data), and these species were not reexamined. Leaves were surface sterilized in a dilute bleach solution (2.5 ml/l; 10 min) and rinsed in water (10 min). Twigs containing clusters of leaves were kept in tubes of water to maintain leaf turgor. Groups of final-instars were placed on the leaves of each species in separate ventilated plastic boxes (30×20×10 cm) and placed in an incubator (23°C, 16:8 h, light/dark). Leaves of each species were readily eaten, although a lower amount of feeding damage was observed on *Q. alba*. Fresh leaves were provided on two consecutive days, using the same tree from each species. A collection of leaves from each tree was made on its second day for chemical analysis, as described above. On the third day of feeding, larvae were individually chilled (−20°C, 6 min), and their midguts were dissected. Midgut fluids were extracted in 300 µl of low-oxygen pH 10 carbonate buffer (70 mM, containing 10% dimethyl sulfoxide; Barbehenn et al. 2005).

Semiquinone and ascorbyl radicals were quantified with EPR spectrometry, as described previously (Barbehenn et al. 2006b). Standard solutions of the stable free radical, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; Aldrich Chemical Co.) were run to confirm that the method was consistent through time and to provide a free radical standard for converting double integrals of spectra to a concentration basis (nanomolar). Radical concentrations are expressed as TEMPO radical equivalents. Radical concentrations in midgut fluid volumes were calculated by using a value of 90% water in the midgut contents (Barbehenn et al. 2003a). In some cases, EPR spectra were either too weak to integrate accurately (e.g., negative double integral values) or produced double integral values that were overestimated because the baseline regions surrounding the signal contributed (incorrectly) to the overall double integral values. In these cases, WinEPR software (Bruker Instruments, Billerica, MA, USA) was used to correct each spectrum as follows. The first derivative spectrum was baseline corrected by using the baseline area on either side of the signal region. The corrected first derivative spectrum was then integrated. The resulting absorption spectrum was again baseline corrected by using the baseline areas on either side of the signal region. This produced a signal region flanked by flat baseline regions abutting a *Y*-axis value of zero. The corrected double integral value was then determined. For spectra that contained signals from both semiquinone and ascorbyl radicals, an additional calculation was made to separate the two component signals. The ascorbyl radical double integral value was determined from a regression of the peak heights of ascorbyl radical absorption spectra (downfield peaks) vs. the double integral values of these spectra. The semiquinone portion of the mixed spectrum was then calculated as the difference between the total radical and the ascorbyl radical double integral values.

To test the possibility that the unusual EPR spectra from caterpillars on *C. glabra* might have been caused by the reduction of quinones by ascorbate, EPR was used to examine juglone (Sigma Chemical Co.), a common quinone from species of Juglandaceae. Juglone (0.9 mM final concentration) was run alone and in mixtures with ascorbic acid (0.4–0.8 mM final concentration). The same parameters and experimental conditions for EPR were used as described above.

The relative contributions of low MW phenolics to the semiquinone levels measured in the midgut fluid *O. leucostigma* was estimated by using representative compounds from each of the groups of phenolic compounds measured: gallic acid, *p*-coumaric acid, chlorogenic acid, catechin, rutin, and its aglycone quercetin (Sigma Chemical Co.). In addition, salicortin, a "phenolic glycoside" purified from *P. tremuloides*, was examined (Lindroth et al. 1987). All compounds were solubilized in 70% ethanol, prepared from nitrogen-purged solvents, with the exception of rutin, which was solubilized in 90% methanol. Semiquinone concentrations formed from each phenolic were measured at three phenolic concentrations (ranging between 22–648 µg/ml; N=3 independent preparations/phenolic). The slopes of the regressions of semiquinone concentration on phenolic concentration normalize semiquinone radical concentrations for the effect of phenolic concentration (Barbehenn et al. 2006b). Two scans of each sample were run by using parameters and other experimental conditions identical to those described for examining gut fluid extracts.

Statistical Analysis Foliar tannins, low MW phenolics and ascorbic acid were compared among tree species with one-way analysis of variance (Wilkinson 2000). Post hoc comparisons among means were made with a Bonferroni adjustment of α for unplanned comparisons. EPR spectra from *O. leucostigma* were not significantly different among trees (experiments) within species in terms of semiquinone concentration (P>0.414; see Fig. 3a) or in line shape. Therefore, EPR data were pooled within tree species, providing results from two trees for most species and sample sizes of 9–12 replicate larvae per tree species. Larvae were fed leaves from only one tree from *C. glabra*, thus limiting generalization from these results because of pseudoreplication. No replication of *A. saccharum* and *Q. rubrum* trees was done after obtaining results that confirmed previous results from these species (Barbehenn et al. 2005; unpub-

Table 1 Phenolic and ascorbate composition of eight North American deciduous tree species

Species	Ellagitannins	Galloyl glycosides	Condensed tannins	Low MW phenolics	Total phenolics[a]	Ascorbic acid
Populus tremuloides	0±0a	0±0	5.5±0.9c	2.4±0.2cd	7.1±0.9a	0.77±0.08c
Populus deltoides	0±0a	0±0	1.6±1.2ab	3.6±0.4e	5.3±0.3a	0.45±0.04b
Ostrya virginiana	0±0a	0±0	5.2±0.5c	2.4±0.1cd	7.6±0.5a	0.46±0.03b
Carya glabra	1.7±0.2b	0±0	3.1±0.7abc	1.0±0.1ab	5.8±0.7a	1.18±0.12d
Quercus rubra	0.2±0.06a	0±0	4.0±0.5bc	2.0±0.1bc	5.3±0.5a	0.34±0.04ab
Quercus alba	4.2±0.4c	0±0	0.8±0.7ab	0.5±0.1a	5.8±0.6a	0.32±0.03ab
Acer rubrum	0±0a	17.3±1.1	0.7±0.2a	1.1±0.1c	19.1±1.3b	0.23±0.02a
Acer saccharum	7.5±0.2d	0.4±0.06	2.0±0.6abc	2.6±0.2b	12.9±0.7b	0.27±0.02a

Non-overlapping letters designate significantly different means ($P<0.05$).
Data are presented as percent DW (mean±SE). $N=6$ trees/species.
[a] Total phenolics was calculated as the sum of all individually measured phenolics.

lished data). Free radical levels in *O. leucostigma* (replicates) were compared among tree species with Mann–Whitney *U* tests, with a Bonferroni adjustment for unplanned comparisons (Wilkinson 2000). This experimental design was intended to examine the main hypothesis that caterpillar gut biochemistry varies among trees with specific foliar phenolic profiles. Associations between foliar chemistry in individual trees fed to *O. leucostigma* and semiquinone radical levels in the midgut fluids of each larva were examined with Pearson correlations (SAS 2003).

Results

Tannins comprised the bulk of the phenolic compounds in most of the tree species (Table 1, Fig. 1). *P. tremuloides*, *P. deltoides*, *O. virginiana*, and *Q. rubra* contained little or no ellagitannins or galloyl glycosides. By contrast, *Q. alba*, *A. saccharum*, and *C. glabra* contained substantial amounts of ellagitannins. *A. rubrum* and *A. saccharum* were the only species that contained galloyl glycosides; *A. rubrum* contained large amounts of digalloyl rhamnose and lower levels of tri-, tetra- and pentagalloyl rhamnose (unpublished data), whereas *A. saccharum* primarily contained small amounts of pentagalloyl glucose. Condensed tannins represented a major fraction (30–70%) of the phenolics in five of the species (*P. tremuloides*, *P. deltoides*, *O. virginiana*, *C. glabra*, and *Q. rubra*). By comparison, in three of the most oxidatively active species (*Q. alba*, *A. rubrum*, and *A. saccharum*), condensed tannin was a minor fraction (4–16%) of the phenolics (Fig. 1).

Low MW phenolics were present in similar, small amounts (0.5–2.6% DW) in most species (Table 2), comprising between 6% and 41% of the total phenolics (Fig. 1; excluding ascorbic acid). However, in *P. deltoides*, low MW phenolics were both at higher absolute levels (3.6% DW) and comprised a major fraction of the total phenolics (69%). Chlorogenic acid (and its derivatives) and flavonoid glycosides were the main types of low MW phenolics in each species (Table 2). While chlorogenic acid had relatively high oxidative activity, the oxidative activity of rutin (a representative flavonoid glycoside) was low (Table 3). The aglycone of rutin, quercetin, had substan-

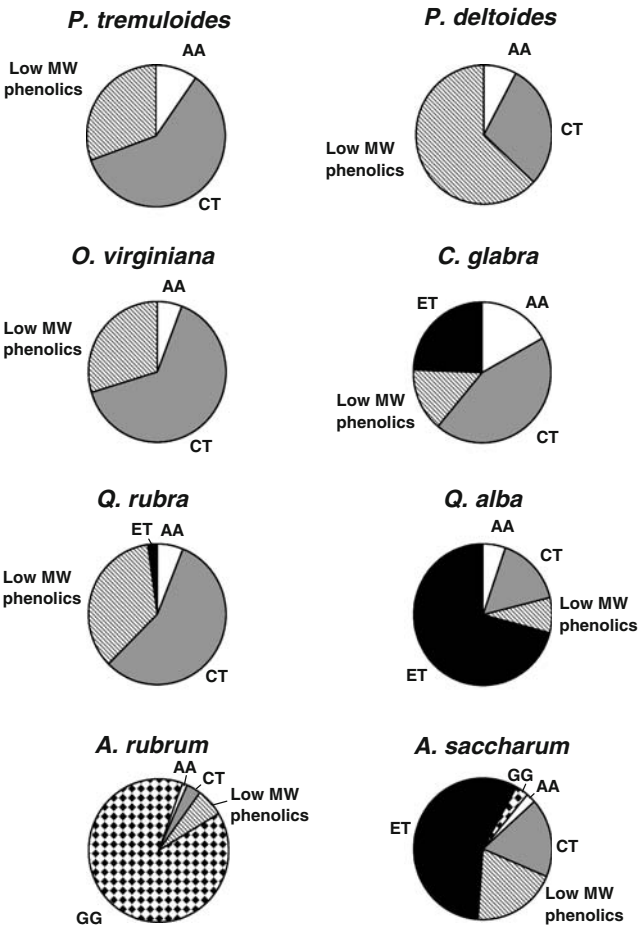

Fig. 1 Proportions of phenolics and ascorbate in the leaves of eight tree species. Average levels from six trees per species are presented. *ET* ellagitannin, *CT* condensed tannin, *GG* galloyl glycoside, *MW* molecular weight, *AA* ascorbic acid

Table 2 Low MW phenolic composition of eight North American deciduous tree species

Species	Gallic acid	Coumaryl quinic acid derivatives	Chlorogenic acid derivatives	Catechin	Flavonoid glycosides	Other low MW phenolics
Populus tremuloides	0±0	0.04±0.01	0.38±0.06bc	0.14±0.02b	1.48±0.08b	0.40±0.10
Populus deltoides	0±0	0±0	1.50±0.10e	0.04±0.01a	1.82±0.12b	0.24±0.03
Ostrya virginiana	0±0	0.35±0.05	0.52±0.07c	0.28±0.03c	1.30±0.16b	0±0
Carya glabra	0.01±0.003	0.02±0.01	0.13±0.02ab	0.04±0.01a	0.81±0.10a	0±0
Quercus rubra	0±0	0±0	1.10±0.10d	0.03±0.01a	0.71±0.04a	0.12±0.04
Quercus alba	0±0	0±0	0.05±0.02a	0±0a	0.45±0.04a	0±0
Acer rubrum	0.45±0.09	0±0	0±0a	0±0a	0.68±0.01a	0±0
Acer saccharum	0.02±0.003	0±0	0.96±0.08d	0.04±0.02a	1.36±0.14b	0.06±0.01

Data are presented as percent DW (mean±SE). N=6 trees/species. Derivatives include parent compounds (coumaryl quinic acid or chlorogenic acid). Non-overlapping letters designate significantly different means (P<0.05).

tially higher activity than the glycoside, but aglycones were below detectable levels in the tree leaves. The oxidative activities of catechin, rutin, and *p*-coumaric acid appeared to be negligible. Although gallic acid was highly active, it was present only in small amounts in *A. rubrum*. Salicortin produced no EPR spectrum. Based on these representative examples and the low foliar concentrations of low MW phenolics, their contributions to the semiquinone radical levels observed in the midgut contents of caterpillars appeared to be small.

Ascorbate levels varied widely between tree species: *C. glabra* and *P. tremuloides* ≫ *P. deltoides* and *O. virginiana* > *Q. rubra* and *Q. alba* > *A. rubrum* and *A. saccharum* (Table 1). For comparison with results provided on a fresh weight basis, ascorbate levels ranged from 6.0 μmol/g in *A. rubrum* to 25.6 μmol/g in *C. glabra*.

In the midgut contents of *O. leucostigma* that fed on tree species containing low levels of oxidatively active phenolics (i.e., *P. tremuloides*, *P. deltoides*, and *O. virginiana*), semiquinone radicals were absent or at low levels (Fig. 2; Table 4). In larvae on *P. tremuloides* and *P. deltoides*, only ascorbyl radicals (benign) were present. In larvae on *Q. rubra* and *O. virginiana*, EPR spectra were consistent with the line shapes of condensed tannin radicals. By contrast, species with substantial levels of ellagitannins, or galloyl glycosides, and low levels of ascorbic acid (i.e., *Q. alba*, *A. saccharum*, and *A. rubrum*) produced high levels of semiquinone radicals (Figs. 1 and 2). These EPR spectra were

Table 3 Semiquinone radical concentrations from representative low MW phenolics

Phenolic compound	Semiquinone concentration[a] [nM/(μg phenolic/ml)]
p-Coumaric acid	0.00±0.00a
Rutin	0.50±0.03a
Catechin	0.98±0.05a
Quercetin	4.19±0.38b
Chlorogenic acid	11.59±0.43c
Gallic acid	28.22±1.11d

Measurements were made with EPR spectrometry in pH 10 buffer (anaerobic).
Non-overlapping letters designate significantly different means (P<0.05).
[a] Data are presented as mean±SE, with N=3 (except rutin N=2).

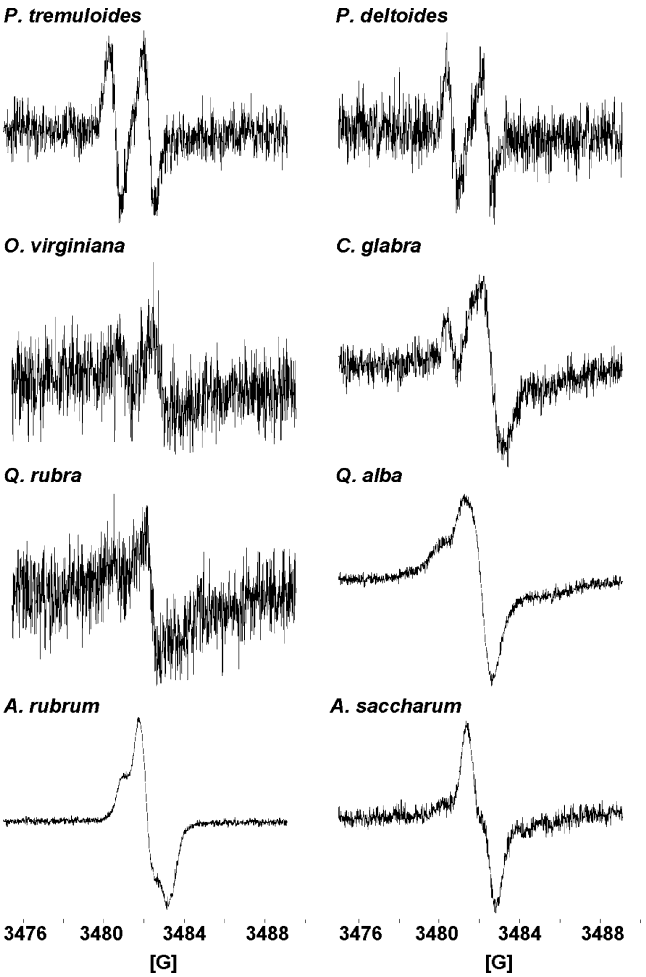

Fig. 2 Representative first derivative EPR spectra of midgut fluid extracts from *O. leucostigma* larvae on the leaves of eight tree species. An indication of the relative scale of each spectrum (in arbitrary *Y*-axis units) is given by the constant background noise level

Table 4 Free radical concentrations in the midgut fluids of *O. leucostigma* larvae after feeding on eight North American deciduous tree species

Species	Ascorbyl radical (nM)	Semiquinone radical (nM)	Number
Populus tremuloides	1098±66d	372±103a	9
Populus deltoides	667±69c	310±87a	9
Ostrya virginiana	369±69b	903±169b	12
Carya glabra	950±147d	5395±1060b	8
Quercus rubra	375±122bc	4459±1010b	9
Quercus alba	0±0a	45,450±5206d	11
Acer rubrum	0±0a	49,083±6568d	10
Acer saccharum	0±0a	15,452±1583c	9

Data are presented as mean±SE. *N* = larvae/tree species
Non-overlapping letters designate significantly different means ($P<0.05$).

similar to those of hydrolyzable tannins (Barbehenn et al. 2006b). EPR spectra from larvae on *C. glabra* were unique, showing substantial levels of both ascorbyl and semiquinone radicals (Fig. 2, Table 4). Similar "hybrid" EPR spectra were produced in pH 10 reaction mixtures that contained juglone and ascorbic acid (data not shown).

To quantify the association between foliar chemistry and levels of semiquinone radicals in *O. leucostigma*, correlations between these measures were plotted. The resulting correlations provided some support for the hypothesis that the proportion of oxidatively active tannins to ascorbate is positively associated with semiquinone radical levels. The correlation between total phenolics/ascorbate and semiquinone radical levels was relatively weak, in part, because species that contained high proportions of condensed tannins produced little or no measurable semiquinone radicals ($r=0.61$, $P<0.001$; Fig. 3a). The correlation between oxidatively active tannins/ascorbate and semiquinone radical levels appears to improve the correlation because weakly active species are positioned near the origin ($r=0.68$, $P<0.001$; Fig. 3b). However, *Q. alba* remained an exception to the pattern presented by the other species. The correlation between the percentage of oxidatively active tannins and semiquinone radical levels fits *Q. alba* and most other species closely ($r=0.78$, $P<0.001$; Fig. 3c). It is noteworthy that negative correlations were observed between ascorbate and semiquinone radical levels ($r=-0.62$; $P<0.001$) and between condensed tannins and semiquinone radical levels ($r=-0.53$; $P<0.001$).

Discussion

A major goal of this study was to examine the association between foliar chemistry (phenolics and ascorbic acid) and phenolic oxidation in the midgut contents of caterpillars. The percentage of the total phenolics composed of oxidatively active tannins (ellagitannins and galloyl glycosides) was strongly correlated with oxidative stress in *O. leucostigma*. A broad range of oxidative activities in tree species could be distinguished based on the levels of semiquinone radicals they produced in *O. leucostigma*: (1) species that produced a large percentage of oxidatively active tannins (*Q. alba*, *A. rubrum*, and *A. saccharum*), (2) intermediate species (*C. glabra* and *Q. rubra*), and (3) species that produced a small percentage of oxidatively active tannins (*P. tremuloides*, *P. deltoides*, and *O. virginiana*). Although only one insect species was examined, *O. leucostigma* is relatively phenolic tolerant (Barbehenn et al. 2005), and higher levels of oxidative stress would be expected in many less tannin-tolerant species.

As expected, ascorbate levels were negatively associated with phenolic oxidation in *O. leucostigma*: Low ascorbate levels were generally present in oxidatively active species, and high ascorbate levels were commonly present in species with low oxidative activities. Together with the analyses of foliar phenolics, these results generally support the hypothesis that the proportion of oxidatively active phenolics to ascorbate is associated with phenolic oxidation in caterpillars. However, *Q. alba* was an exception to this pattern. *Q. alba* produced higher levels of semiquinone radicals than expected, given its ellagitannin and ascorbate levels. One potential reason for this exception is the presence of highly reactive types of ellagitannins in *Q. alba*, including vescalagin, castalagin, cocciferin D_2, and castavaloninic acid (Moilanen and Salminen 2008). These ellagitannins are based on an acyclic glucose, whereas the main ellagitannins of the other species in this study (i.e., *Q. rubra*, *A. saccharum*, and *C. glabra*) have glucopyranose backbones (Salminen, unpublished data). Secondly, *Q. alba* contained low levels of condensed tannins, both in absolute terms and relative to the levels of the oxidatively active phenolics present (Table 1; Fig. 1). Because condensed tannins can decrease the rate of oxidation of more reactive tannins (Barbehenn et al. 2006a), a low percentage of condensed tannins means that the more reactive tannins can

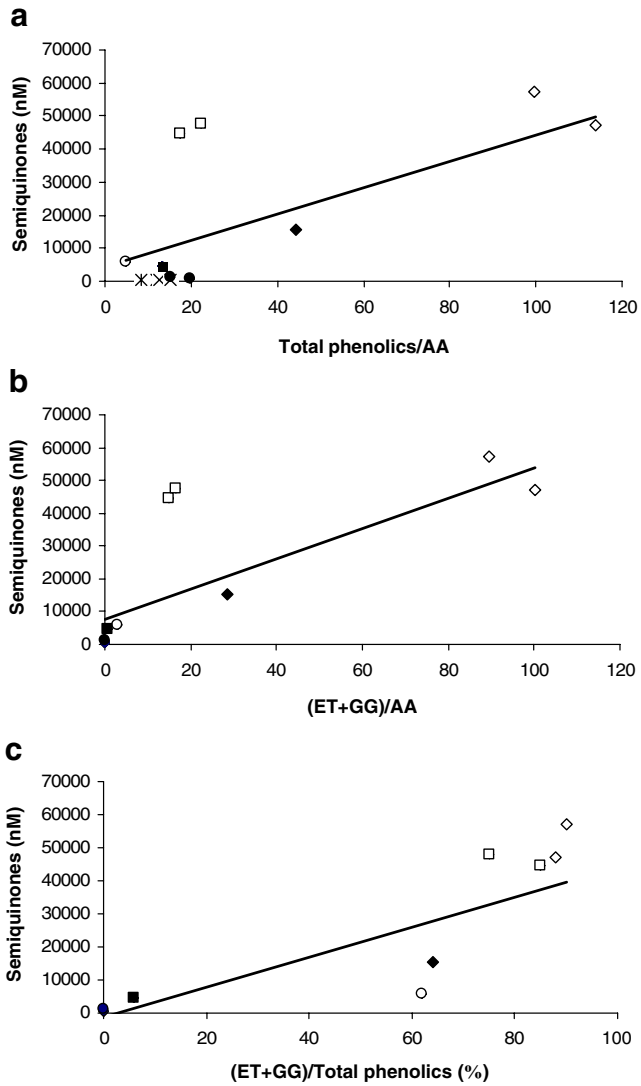

Fig. 3 Correlations between the foliar chemistry of ingested tree leaves and semiquinone radical levels in the midgut contents of final-instar *O. leucostigma*. Pearson correlation coefficients were calculated with individual data ($P<0.001$ for each correlation), but means, from each experiment, are plotted for clarity. *Total phenolics* is the sum of all individually measured phenolics. *Filled square Q. rubra*, *open square Q. alba*, *filled diamond A. saccharum*, *open diamond A. rubrum*, *open circle C. glabra*, *filled circle O. virginiana*, *ex mark P. deltoides*, *asterisk P. tremuloides*. Where not visible, data points are overlapping

oxidize near their maximum rates. This may explain the improved correlation between the percentage of oxidatively active phenolics and semiquinone radicals in *O. leucostigma* (Fig. 3c). The correlation in Fig. 3c is also a useful improvement over previous work, in which oxidative stress was associated with the proportion of total phenolics to ascorbic acid (Barbehenn et al. 2003b, 2005).

Tannins, especially ellagitannins and galloyl glycosides, were the primary sources of oxidative stress in the midgut contents of caterpillars in this study. This was due both to their intrinsic reactivity and to the high levels of tannins produced in oxidatively active leaves. Low MW phenolics were present in much lower levels than tannins in oxidatively active tree species, and many of these compounds have relatively low oxidative activities in the low-oxygen, high pH conditions of caterpillar midguts. Even in *P. deltoides*, which contained the highest levels of chlorogenic acid (1.5% DW), no measurable levels of semiquinone radicals were produced in the midguts of *O. leucostigma*. Although only one representative flavonoid and flavonoid glycoside was examined in this study, the low rates of browning of a wide range of these phenolics compared with ellagitannins support the generality of the EPR results on quercetin and rutin (Salminen, unpublished data). At sufficiently high levels, low MW phenolics could play other roles as chemical defenses in tree leaves. Unlike tannins, these phenolics can be absorbed from the midgut contents (Summers and Felton 1994; Barbehenn 2001). Therefore, low MW phenolics, and the quinones that they form, might produce oxidative stress and/or toxicity in insect tissues (Gant et al. 1988; Summers and Felton 1994; but see Johnson and Felton 2001). Other potential toxins in the trees in this study include alkaloids in species of Aceraceae, quinones in the Juglandaceae (e.g., *C. glabra*), and salicylates in the Salicaceae (Barbosa and Krischik 1987; Lindroth and Peterson 1988; Hemming and Lindroth 1995; Thiboldeaux et al. 1998).

Salicylates have often been called phenolic glycosides, and it has been suggested that products of their metabolism act as prooxidants in the midgut lumen (Clausen et al. 1989; Ruuhola et al. 2001). We were unable to detect the formation of measurable levels of semiquinone radicals in *O. leucostigma* that consumed the leaves of two salicylate-producing species, *P. tremuloides* and *P. deltoides*. In addition, salicortin produced no measurable semiquinone radicals and had a negligible rate of browning at pH 10 (Barbehenn, unpublished data). Previous work has noted the difficulty of detecting the oxidation of phenolics from *P. tremuloides* leaf tissues (Haruta et al. 2001), and work on the chemical fate of ingested salicylates from *Salix* species found little, if any, catechol (an oxidizable salicylate metabolite) in the frass of *Operophtera brumata* (Ruuhola et al. 2001). Together, these observations suggest that the chemical defenses of trees in the Salicaceae function most effectively as antifeedants and toxins in caterpillars.

We suspected that quinones in *C. glabra*, in combination with high levels of ascorbate, produced the unique EPR spectra observed in *O. leucostigma*. These spectra indicated the presence of both high levels of semiquinone and ascorbyl radicals (Table 4; Fig. 2). Mixtures of juglone and ascorbic acid produced EPR line shapes that were similar to those in *O. leucostigma* on *C. glabra*, suggesting that the chemical reduction of quinones by ascorbate could produce large concentrations of semiquinone and ascorbyl

radicals simultaneously. Only a single tree of *C. glabra* was examined, greatly limiting the extent to which results on this species can be interpreted. It is noteworthy, therefore, that similar "hybrid" EPR spectra were also observed when *O. leucostigma* larvae fed on another species of hickory (*C. ovata*) (Barbehenn, unpublished data). Foliar quinone levels were not measured in this study, and further work is needed to determine their potential impact on oxidative stress in caterpillars via redox cycling in the gut contents or tissues.

The phenolic composition of *A. rubrum* was unusual among the trees examined, containing large amounts of rhamnose-based galloyl glycosides (primarily digalloyl rhamnose). Previous work on *A. rubrum* foliar chemistry identified the presence of 1-*O*-galloyl-α-L-rhamnose (Abou-Zaid and Nozzolillo 1999), but found that the major phenolic component was ethyl *m*-digallate (Abou-Zaid et al. 2001). The complete absence of the latter compound in our analyses, and the presence of much higher levels of galloyl rhamnosides, lead us to question whether ethyl *m*-digallate could have been produced during the 2-day extraction period in ethanol that was used in previous studies. For example, methanol is known to cleave the *m*-depside bonds of digalloyl groups, producing methyl gallate (Hofmann and Gross 1990).

The results of this study suggest that trees such as *O. virginiana*, *Populus* species, and possibly *Q. rubra* might be superior host plants for generalist caterpillars. These tree species contain low levels of oxidatively active phenolics and produced little phenolic oxidation in the midgut lumen when ingested by *O. leucostigma*. Some field observations are consistent with this expectation: *Q. rubra* and *O. virginiana* are among the top host plant species for *Lymantria dispar* (Lymantriidae) (Liebhold et al. 1995), and *Populus* species are excellent host plants for *Malacosoma disstria* (Lasiocampidae) in its northern range (Parry and Goyer 2004). By comparison, *A. saccharum* and *A. rubrum* are relatively poor host plants for *L. dispar* and *M. disstria* (Liebhold et al. 1995; Parry and Goyer 2004). Given the high levels of oxidative stress in *O. leucostigma* on *Q. alba* in this study, it is surprising that *Q. alba* is considered a favored host for the closely related larvae of *L. dispar*. There is a large amount of geographical variation in the tannin compositions of oak species, including *Q. alba* (Salminen, unpublished data), suggesting that the suitability of species such as *Q. alba* as host plants could vary geographically.

We conclude that the tannin compositions of tree leaves largely determine the effectiveness of foliar phenolics as oxidative defenses against *O. leucostigma*. The basis for this conclusion includes the following main findings: (1) Oxidatively active tree leaves contained high proportions of ellagitannins or galloyl rhamnoses among their foliar phenolics; (2) low MW phenolics contributed relatively little to the overall oxidative activities of tree leaves compared with oxidatively active tannins; (3) ascorbic acid levels were lowest in the species that also contained the highest levels of oxidatively active tannins, potentially exacerbating phenolic oxidation in the gut lumen; and (4) even a "tannin-tolerant" caterpillar such as *O. leucostigma* was unable to control the oxidation of ingested phenolics when trees such as *A. rubrum*, *A. saccharum*, or *Q. alba* were eaten.

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References

ABOU-ZAID, M. M., and NOZZOLILLO, C. 1999. 1-*O*-galloyl-α-L-rhamnose from *Acer rubrum*. *Phytochemistry* 52:1629–1631.

ABOU-ZAID, M. M., HELSON, B. V., NOZZOLILLO, C., and ARNASON, J. T. 2001. Ethyl *m*-digallate from red maple, *Acer rubrum* L., as the major resistance factor to forest tent caterpillar, Malacosoma disstria Hbn. *J. Chem. Ecol.* 27:2517–2527.

BAKER, W. L. 1972. Eastern forest insects. USDA miscellaneous publication no. 1175. Washington, DC.

BARBEHENN, R. V. 2001. Roles of peritrophic membranes in protecting herbivorous insects from ingested plant allelochemicals. *Arch. Insect Biochem. Physiol.* 47:86–99.

BARBEHENN, R. V. 2003. Antioxidants in grasshoppers: higher levels defend the midgut tissues of a polyphagous species than a graminivorous species. *J. Chem. Ecol.* 29:683–702.

BARBEHENN, R. V., BUMGARNER, S. L., ROOSEN, E., and MARTIN, M. M. 2001. Antioxidant defenses in caterpillars: role of the ascorbate recycling system in the midgut lumen. *J. Insect Physiol.* 47:349–357.

BARBEHENN, R. V., WALKER, A. C., and UDDIN, F. 2003a. Antioxidants in the midgut fluids of a tannin-tolerant and a tannin-sensitive caterpillar: effects of seasonal changes in tree leaves. *J. Chem. Ecol.* 29:1099–1116.

BARBEHENN, R. V., POOPAT, U., and SPENCER, B. 2003b. Semiquinone and ascorbyl radicals in the gut fluids of caterpillars measured with EPR spectrometry. *Insect Biochem. Mol. Biol.* 33:125–130.

BARBEHENN, R. V., CHEEK, S., GASPERUT, A., LISTER, E., and MABEN, R. 2005. Phenolic compounds in red oak and sugar maple leaves have prooxidant activities in the midguts of *Malacosoma disstria* and *Orgyia leucostigma* caterpillars. *J. Chem. Ecol.* 31:969–988.

BARBEHENN, R. V., JONES, C. P., KARONEN, M., and SALMINEN, J.-P. 2006a. Tannin composition affects the oxidative activities of tree leaves. *J. Chem. Ecol.* 32:2235–2251.

BARBEHENN, R. V., JONES, C. P., HAGERMAN, A. E., KARONEN, M., and SALMINEN, J.-P. 2006b. Ellagitannins have greater oxidative activities than gallotannins and condensed tannins at high pH: potential impact on caterpillars. *J. Chem. Ecol.* 32:2253–2267.

BARBOSA, P., and KRISCHIK, V. A. 1987. Influence of alkaloids on feeding preference of eastern deciduous forest trees by the gypsy moth *Lymantria dispar*. *Amer. Nat.* 130:53–69.

BI, J. L., and FELTON, G. W. 1995. Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21:1511–1530.

BI, J. L., MURPHY, J. B., and FELTON, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in cotton. *J. Chem. Ecol.* 23:97–117.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., WERNER, R. A., POST, K., and FRISBY, K. 1989. Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. *J. Chem. Ecol.* 15:2335–2346.

FELTON, G. W., and DUFFEY, S. S. 1992. Ascorbate oxidation reduction in *Helicoverpa zea* as a scavenging system against dietary oxidants. *Arch. Insect Biochem. Physiol.* 19:27–37.

GANT, T. W., RAMAKRISHNA, R., MASON, R. P., and COHEN, G. M. 1988. Redox cycling and sulphydryl arylation; their relative importance in the mechanism of quinone cytotoxicity to isolated hepatocytes. *Chem.-Biol. Interactions* 65:157–173.

HARUTA, M., PEDERSEN, J. A., and CONSTABEL, P. 2001. Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. *Physiol. Plant* 112:552–558.

HEMMING, J. D. C., and LINDROTH, R. L. 1995. Intraspecific variation in aspen phytochemistry: Effects on performance of gypsy moths and forest tent caterpillars. *Oecologia* 103:79–88.

HOFMANN, A. S., and GROSS, G. G. 1990. Biosynthesis of gallotannins: Formation of polygalloylglucoses by enzymatic acylation of 1,2,3,4,6-penta-*O*-galloylglucose. *Arch. Biochem. Biophys.* 283:530–532.

JOHNSON, K. S., and FELTON, G. W. 2001. Plant phenolics as dietary antioxidants for herbivorous insects: A test with genetically modified tobacco. *J. Chem. Ecol.* 27:2579–2597.

LIEBHOLD, A. M., GOTTSCHALK, K. W., MUZIKA, R.-M., MONTGOMERY, M. E., YOUNG, R., O'DAY, K., and KELLEY, B. 1995. Suitability of North American tree species to the gypsy moth: a summary of field and laboratory tests. USDA Forest Service, General Technical Report NE-211.

LINDROTH, R. L., and PETERSON, S. S. 1988. Effects of plant phenols on performance of southern armyworm larvae. *Oecologia* 75:185–189.

LINDROTH, R. L., HSIA, M. T. S., and SCRIBER, J. M. 1987. Seasonal patterns in the phytochemistry of three *Populus* species. *Biochem. Syst. Ecol.* 15:681–686.

MARTIN, J. S., MARTIN, M. M., and BERNAYS, E. A. 1987. Failure of tannic acid to inhibit digestion or reduce digestibility of plant protein in gut fluids of insect herbivores: implications for theories of plant defense. *J. Chem. Ecol.* 13:605–621.

MOILANEN, J., and SALMINEN, J.-P. 2008. Ecologically neglected tannins and their biologically relevant activity: chemical structures of plant ellagitannins reveal their *in vitro* oxidative activity at high pH. *Chemoecology*, (in press).

OSSIPOVA, S., OSSIPOV, V., HAUKIOJA, E., LOPONEN, J., and PIHLAJA, K. 2001. Proanthocyanidins of mountain birch leaves: quantification and properties. *Phytochem. Anal.* 12:128–133.

PARRY, D., and GOYER, R. A. 2004. Variation in the suitability of host tree species for geographically discrete populations of forest tent caterpillar. *Environ. Entomol.* 33:1477–1487.

ROSEN, G. M., BRITIGAN, B. E., HALPERN, H. J., and POU, S. 1999. Free Radicals: Biology and Detection by Spin Trapping. Oxford University Press, NY.

RUUHOLA, T., TIKKANEN, O.-P., and TAHVANAINEN, J. 2001. Differences in host use efficiency of larvae of a generalist moth, *Operophtera brumata* on three chemically divergent *Salix* species. *J. Chem. Ecol.* 27:1595–1615.

SALMINEN, J.-P., OSSIPOV, V., LOPONEN, J., HAUKIOJA, E., and PIHLAJA, K. 1999. Characterisation of hydrolyzable tannins from leaves of *Betula pubescens* by high-performance liquid chromatography-mass spectrometry. *J. Chrom. A.* 864:283–291.

SAS INSTITUTE 2003. The SAS system for Windows. Version 9.1. SAS Institute, Cary, NC, USA.

SUMMERS, C. B., and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Mol. Biol.* 24:943–953.

THIBOLDEAUX, R. L., LINDROTH, R. L., and TRACY, J. W. 1998. Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in Saturniid moth larvae. *Comp. Biochem. Physiol.* 120:481–487.

WAGNER, D. M. 2005. Caterpillars of Eastern North America. Princeton University Press.

WILKINSON, L. 2000. SYSTAT: The system for statistics. SYSTAT, Inc., Evanston, IL.

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Colored and White Sectors From Star-Patterned Petunia Flowers Display Differential Resistance to Corn Earworm and Cabbage Looper Larvae

Eric T. Johnson · Mark A. Berhow · Patrick F. Dowd

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Abstract Anthocyanins are likely a visual aid that attract pollinators. However, there is also the possibility that anthocyanins are present in some flowers as defensive molecules that protect them from excess light, pathogens, or herbivores. In this study, resistance due to anthocyanins from commercial petunia flowers (*Petunia hybrida*) was examined for insecticide/antifeedant activity against corn earworm (CEW, *Helicoverpa zea*) and cabbage looper (CL, *Trichoplusia ni*). The petunia flowers studied contained a star pattern, with colored and white sectors. CEW larvae ate significantly less colored sectors than white sectors in no-choice bioassays in most cases. All CEW larvae feeding on blue sectors weighed significantly less after 2 days than larvae feeding on white sectors, which was negatively correlated with total anthocyanin levels. CL larvae ate less of blue sectors than white sectors, and blue sectors from one petunia cultivar caused significantly higher CL mortality than white sectors. Partially purified anthocyanin mixtures isolated from petunia flowers, when added to insect diet discs at approximately natural concentrations, reduced both CEW and CL larva weights compared to the controls. These studies demonstrate that the colored sectors of these petunia cultivars slow the development of these lepidopteran larvae and indicate that anthocyanins play some part in flower defense in petunia.

Keywords Anthocyanins · Petunia · Insect · Resistance

E. T. Johnson (✉) · P. F. Dowd
Crop BioProtection Research,
National Center for Agricultural Utilization Research,
Agricultural Research Service,
US Department of Agriculture,
1815 North University Street,
Peoria, IL 61604, USA
e-mail: eric.t.johnson@ars.usda.gov

M. A. Berhow
New Crops and Processing Technology Research,
National Center for Agricultural Utilization Research,
Agricultural Research Service, U.S. Department of Agriculture,
1815 North University Street,
Peoria, IL 61604, USA

Introduction

Insect herbivores cause crop losses by physically damaging tissue but can also contribute to the colonization of crops by fungi, some of which synthesize toxins (e.g., mycotoxins) that can harm livestock and humans (Dowd 1998). Strengthening plant resistance to herbivore damage is an important strategy of crop management (Dowd et al. 2005). Plants are capable of synthesizing a large variety of biochemicals that serve as defensive molecules (Sadasivam and Thayumanavan 2003), mediate biotic and abiotic stresses (Arasimowicz and Floryszak-Wieczorek 2007; Dixon and Paiva 1995; Horváth et al. 2007; Korkina 2007; Wasternack 2007), and potentially contribute to human health (Sampson et al. 2002). One class of plant secondary biochemicals, the flavonoids, exhibit a wide range of functions that include protection against UV-light and pathogens, signaling during nodulation, male fertility, and auxin transport (Koes et al. 2005). Humans consume substantial quantities of flavonoids (estimated to be several hundred milligrams daily) in diets rich in fruits and vegetables (Hollman and Katan 1999). Blue and red flavonoids in flowers, fruits, and vegetables, called anthocyanins, are free-radical scavengers and antioxidants (Wang et al. 1997; Tsuda et al. 2000) that may have a role in preventing carcinogenesis and heart disease (Omenn 1995). Anthocyanins are thought to function primarily in nature as pollinator attractors

(Koes et al. 2005). Anthocyanins possibly protect leaves from excess light, water stress, or herbivores (Manetas 2006; Schaefer and Rolshausen 2006). Studies on two different types of tree species suggest that leaf anthocyanins play a role in herbivore protection (Karageorgou and Manetas 2006; Schlindwein et al. 2006). Some anthocyanins from cotton flowers can inhibit the development of insect larvae (Hedin et al. 1983; Jenkins et al. 1983).

Petunia can synthesize more than three dozen steroidal compounds that are involved in resistance to lepidopteran larvae; however, these compounds appear to be made in the leaves but not in flowers (Elliger and Waiss 1991). Petunia has served as a model species for identifying many of the regulator genes and enzymes of anthocyanin biosynthesis (Koes et al. 2005). Many anthocyanin structures from garden and wild petunias have been determined (Wiering and De Vlaming 1984; Ando et al. 1999). Blue or violet petunia flowers generally contain delphinidin-type anthocyanins, which have three hydroxyl groups on the B ring of the anthocyanin, due to the presence of flavonoid 3′,5′-hydroxylase (Holton et al. 1993). Methylation of the 3′ hydroxyl group of delphinidin results in petunidin derivatives, while an additional methylation on the 5′ hydroxyl group of petunidin results in malvidin derivatives. A large percentage of commercial petunias available contain petunidin or malvidin derivatives (Ando et al. 2004).

Preliminary experiments indicated that a variety of lepidopteran larvae can eat most but not all commercial petunia flowers (Johnson and Dowd 2003). In addition, some purified anthocyanins reduce the growth of lepidopteran larvae (Johnson and Dowd 2003, 2004). Thus, a search for petunia material that displayed differential anthocyanin production in the flowers was initiated. Extensive breeding of *Petunia hybrida* has generated a number of unique flower phenotypes that include star patterns, which exhibit alternating colored and white sectors. Since the colored and white sectors of star-patterned flowers should be genetically identical, studies that compare the feeding activities of corn earworm (CEW) larvae (*Helicoverpa zea*) and cabbage looper larvae (*Trichoplusia ni*) on these floral sectors were investigated. These insect species can feed on members of the Solanaceae family, such as potato (CL), tomato (CL and CEW), and tobacco (CEW; Metcalf et al. 1951). While these insect larvae are not natural pests of *P. hybrida*, the potential effectiveness of *P. hybrida* anthocyanins as resistance molecules against these larvae that damage important crops may ultimately improve crop protection. For example, genetic regulation of anthocyanins in maize has been well studied (Koes et al. 2005), and it may be possible to induce production of petunia-like anthocyanins in certain maize tissues for insect resistance. On the other hand, the potential insect resistance of *P. hybrida* anthocyanins may be evidence of a molecular mechanism whereby petunia flowers both attract pollinators and defend the tissue from a wide range of potential herbivores.

Methods and Materials

Plant Material Seeds were purchased from the following commercial vendors: W. Atlee Burpee & Co., Warminster, PA, USA (Razzle Dazzle Mixed); Harris® Seeds, Rochester, NY, USA (F1 Ultra Blue Star, UBS); Ball Seed Co., West Chicago, IL, USA (Carpet Blue Star). Seeds were sown in moistened Metro Mix 350 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) and germinated under a 14:10 L/D photoperiod at 25°C. Plantlets were transferred to larger pots and placed in a walk-in plant growth facility that contained 1,000 W sodium and halide lighting. Plants were kept at 24°C±2°C day and 18°C±2°C night temperatures and 50%±10% relative humidity. Plants were fertilized weekly with Peters Professional 20–20–20 general purpose fertilizer (Scotts Company, Marysville, OH, USA) at a concentration of 1 g/l or by the placement of indoor plant food spikes (Scotts Miracle-Gro Products, Marysville, OH, USA) into the soil every 30 days.

Insects Corn earworms (*H. zea*) were reared at 27°C±1°C, 40%±10% relative humidity, 14:10 L/D photoperiod as described previously (Dowd et al. 2003). CL larvae obtained from Dr. Robert Behle (USDA-Peoria) were reared under similar conditions (Behle et al. 2003). First instars were used in bioassays. The CEW and CL colonies have been propagated for approximately 180 and 120 generations, respectively.

No-Choice Bioassays Using Flower Sectors Bioassays with CEW larvae were begun soon after initial flowering of each petunia cultivar. Bioassays with CL larvae were begun 6–31 months after initial flowering. Petunia corollas of the same developmental stage (limb as flat as possible) were sectioned by color and placed separately into 5-cm diameter Petri plates with tight fitting lids (Falcon 351006, Becton Dickinson Labware, Franklin Lakes, NJ, USA). Each plate contained two (for large flowers) or four (for small flowers) sectors. Punches (7 mm) were removed from the remaining colored and white sectors and were frozen for subsequent chemical analysis. Several flowers of each cultivar were tested. Ten first instar *H. zea* or *T. ni* were added to each dish and placed in the dark at 27°C±1°C and 40%±10% relative humidity for 48 h. Corolla sectors were rated for feeding on day 2 by counting 1-mm^2 holes (or equivalent areas) with a dissecting scope as described previously (Dowd et al. 2003). Surviving larvae were frozen to weigh

later with an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus, OH, USA). Multiple bioassays were performed for each petunia cultivar and are indicated on the tables.

Quantification of Anthocyanins Anthocyanins were extracted from one or two punches overnight at 4°C in the dark with 500 μl of methanol + 0.1 M HCl (hereafter, solvent). The next day, the liquid extract was removed and the volume reduced by vacuum with an Eppendorf® Vacufuge™ Concentrator 5301 (Brinkmann Instruments, Inc. Westbury, NY, USA) at room temperature. The dried extract was suspended in 100 μl of solvent. Five hundred microliters of solvent were added to the flower punch(s), and they were ground with small plastic pestles. After another overnight extraction at 4°C in the dark, the first- and second-day extracts were combined, and volume was reduced in the Eppendorf® Vacufuge™. The residue was suspended in 200 μl of solvent and passed through a 0.45-μm syringe filter (4-mm diameter, Alltech Associates Inc. Deerfield, IL, USA). The final volume was measured with a gastight 250-μl syringe. Fifteen microliters of each sample were injected onto an Inertsil 5-μm ODS-3 column (250× 4.6 mm, Metachem Technologies Inc., Torrance, CA, USA) housed in an Agilent (Santa Clara, CA, USA) 1100 high-performance liquid chromatography (HPLC) with a diode array detector set to detect compounds at 520 nm. The mobile phases were A, 1% formic acid and B, acetonitrile and programmed to go from 0% to 39% B after 35 min, with a flow rate of 1 ml/min. The calculation for total anthocyanins consisted of all the peaks (excluding peaks that eluted within the first 5 min of the run) that had an integrated area that exceeded the lowest integrated peak area of the standard regression with malvin chloride (Fluka, Germany). The anthocyanin calculation was normalized to account for the final punch extract volume and was expressed as equivalents of malvin chloride per two flower disks.

Identification of Anthocyanins Flowers were collected approximately 3 months (UBS), 6 months (Razzle Dazzle blue), 7 months (Carpet Blue), and 23 months (Razzle Dazzle red and pink) after initial flowering and freeze dried at least overnight. Fractions were ground into a fine powder with a mortar and pestle. Typically, 100–200 g of dried flower material were needed to produce 5–25 mg of purified anthocyanins. For anthocyanin analysis, the samples were extracted with 0.12 M HCl in methanol. Fractions were sonicated for 15 min then allowed to stand overnight at room temperature. This was repeated at least twice more for each sample. Extracts from a sample were pooled, and an aliquot was removed from the vial and filtered through a 0.45-μM nylon 66 filter for liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) analysis. For preparative work, the extracts were allowed to dry in a hood, then resuspended in a lesser volume of 0.12 M HCl in water. Samples were run on a ThermoFinnigan LCQ DECA XP Plus LC–MS system with a surveyor HPLC system (autoinjector, pump, degasser, and PDA detector) and a nitrogen generator, all running under the Xcaliber 1.3 software system. The MS was run with the ESI probe in the positive mode. The source inlet temperature was set at 220°C, the sheath gas rate was set at 88 arbitrary units, and the sweep (auxiliary) gas rate was set at 12 arbitrary units. The MS was optimized for the detection of the anthocyanins by using the autotune feature of the software, while infusing a solution of malvin with the effluent of the column and tuning on an atomic mass unit of 655 $[M]^+$ for malvin. The column used was an Inertsil ODS-3 reverse phase C-18 column (3 μm, 150×3 mm, with a Metaguard column, from Varian). For anthocyanin LC–MS analysis, the initial HPLC conditions were 2% acetonitrile and 0.25% acetic acid in water, at a flow rate of 0.3 ml per min. The effluent was monitored at 520 nm on the PDA.

Isolation of Anthocyanin Composites From UBS Flowers Flowers were collected over several weeks from two UBS plants that had been flowering for approximately 5 months and placed in a −20°C freezer. Once enough material was collected, the flowers were freeze dried and extracted as above. A Buchi (Newcastle, DE, USA) Sepacore flash chromatography system with dual C-605 pump modules, C-615 pump manager, C-660 fraction collector, C-635 UV photometer, with SepacoreRecord chromatography software was used. A Buchi C-670 Cartridger system was used to load 40×150 mm flash columns with approximately 90 g of preparative C18 reverse phase bulk packing material (125 Å, 55–105 μm, Waters Corp., Milford, MA, USA). The columns were installed in the flash chromatography system and equilibrated with 5% methanol and 0.5% acetic acid in water for 5 min at a flow rate of 30 ml per min. After samples (10–15 ml) were injected, the column was developed with a binary gradient to 100% methanol over 30 min. The effluent was monitored at 520 nm, and all fractions were collected in the fraction collector by the software program. Fractions were concentrated by evaporation in the hood at room temperature.

A Shimadzu (Columbia, MD, USA) preparative HPLC system was used with dual 8A pumps, SIL 10vp autoinjector, SPD M10Avp photodiode array detector, SCL 10Avp system controller, all operating under the Shimadzu Class VP operating system. Ten milliliters of sample aliquots in methanol were injected on a Phenominex (Torrance, CA, USA) Luna C18 (2) semi-preparative reverse-phase column (10 μm, 100 Å, 250×50 cm). The column was pre-equilibrated with 1% acetic acid, 2% acetonitrile, and 97% water at a flow rate of 50 ml per min, and the effluent was

monitored at 520 nm. The column was developed to 50% acetonitrile over 45 min. UV absorbing peaks were collected by hand. The procedure was repeated to obtain sufficient purified material. Pooled material was allowed to evaporate to remove organic solvent and then freeze-dried to recover the purified anthocyanins.

Bioassays with UBS Anthocyanin Composites Dried anthocyanin composites were dissolved in distilled water and absorbed onto small discs of pinto bean diet as previously described (Dowd et al. 2007) and kept at 4°C for several hours to overnight for full absorption. Ten first instar CEW or CL were added to the dishes and, after resealing, were kept in the rearing incubator in the dark. Mortality was recorded on each day, and the larvae were frozen on the second or third day (depending on the quantity of diet remaining in controls) and subsequently weighed with an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus OH, USA).

Statistical Analyses Statistical differences in feeding and mortality rates were determined by the paired means test (proc means), while larvae survivor weights were compared by analysis of variance (ANOVA; proc glm) using SAS version 9.1 (Cary, NC, USA). Correlation analyses were completed by using "proc reg corr" with the same SAS software.

Results

Flower Sector Studies Colored and white sectors from three different petunia star cultivars were tested for corn earworm resistance. CEW mortality was low (<10%) for all of the sectors tested, and none of the cultivars displayed significantly different CEW mortality rates between the two sector types (data not shown). The Razzle Dazzle cultivar contained star-patterned flowers with different types of anthocyanins. For Razzle Dazzle red/white flowers, the feeding ratings were significantly lower on the red compared to the white sectors, but there was no significant difference in the weights of larvae feeding on the two types (Table 1). One of the Razzle Dazzle pink/white flowered plants displayed significantly lower feeding on the colored sectors, and the weights of surviving larvae feeding on the colored sectors were lower than the weights of larvae feeding on the white sectors, but the other two Razzle Dazzle pink/white flowered plants did not display the same pattern of insect resistance (Table 1). One Razzle Dazzle blue/white flowered plant showed differential feeding on the colored and white sectors, and the weights of the larvae feeding on the colored sectors were lower than the weights of larvae feeding on the white sectors, but no additional blue/white flowering plants germinated from the Razzle Dazzle seed packets obtained. Thus, more blue/white star petunia flower cultivars from other vendors were purchased, including Carpet Blue Star and Ultra Blue Star. Both cultivars showed lower feeding rates on the colored sectors and lower larvae weights on the colored sectors, which indicated that the constituents of the blue sectors consistently inhibited CEW larvae growth.

Sectors of the blue/white flowers were also fed to CL larvae. No significant differences in feeding ratings or survivor weights (data not shown) were found in Razzle Dazzle blue/white flowers (Table 2). The mortality rate of larvae feeding on the Razzle Dazzle blue sectors was higher than that of larvae feeding on the white sectors but not significantly (P=0.11). No significant differences in the

Table 1 CEW mean feeding rates (number of 1 mm^2 holes) and survivor weights (mg ± standard error) on star patterned petunia flowers after the second day of the bioassay

Cul	Col FR (*N*)[a]	Whi FR (*N*)	Col Wt ± SE (*N*)	Whi Wt ± SE (*N*)
Red1	46 (20)[b]	62 (20)[c]	0.12±0.0090 (74)[d]	0.12±0.0084 (84)[d]
Red2	46 (19)[b]	63 (19)[c]	0.11±0.0065 (146)[d]	0.12±0.0068 (171)[d]
Pink1	40 (10)[b]	78 (10)[c]	0.14±0.0073 (170)[d]	0.18±0.010 (169)[e]
Pink2	72 (30)[b]	97 (30)[c]	0.31±0.012 (170)[d]	0.28±0.012 (168)[d]
Pink3	92 (16)[b]	56 (16)[c]	ND	ND
Blu1	58 (24)[b]	89 (24)[c]	0.18±0.0086 (128)[d]	0.23±0.0089 (143)[e]
CB1	50 (9)[b]	87 (9)[c]	0.17±0.011 (60)[d]	0.22±0.014 (61)[e]
CB2	42 (9)[b]	71 (9)[c]	0.16±0.012 (69)[d]	0.20±0.011 (72)[e]
UBS1	34 (12)[b]	62 (12)[c]	0.18±0.012 (65)[d]	0.23±0.011 (76)[e]
UBS2	46 (12)[b]	73 (12)[c]	0.19±0.010 (72)[d]	0.24±0.013 (65)[e]

Cul cultivar, *Col* colored sector, *Whi* white sector, *FR* feeding rating, *wt* weight, *Red* Razzle Dazzle red/white, *Pink* Razzle Dazzle pink/white, *Blu* Razzle Dazzle blue/white, *CB* Carpet Blue Star, *UBS* Ultra Blue Star, *ND* not determined

[a] For FR, *N* refers to the number of bioassay dishes assayed; for wt, *N* refers to the number of survivor larvae weighed.

[b,c] Means that are significantly different (P<0.05) by paired means test

[d,e] Means that are significantly different (P<0.05) by ANOVA

Table 2 CL mean feeding rates (number of 1 mm^2 holes) and mortality rates on star-patterned petunia flowers after the second day of the bioassay

Cul[a]	Col FR (*N*)[b]	Whi FR (*N*)	Col MR (*N*)	Whi MR (*N*)	Col Corr (*P*)[c]	Whi Corr (*P*)[c]
Blu1	39 (8)[d]	42 (8)[d]	23% (8)[d]	8% (8)[d]	0.21 (0.61)	−0.53 (0.18)
CB1	28 (9)[d]	36 (9)[d]	23% (9)[d]	3% (9)[d]	−0.71 (0.03)	−0.40 (0.28)
CB2	27 (7)[d]	35 (7)[d]	11% (7)[d]	9.0% (7)[d]	−0.65 (0.11)	−0.52 (0.24)
UBS[f]	34 (74)[d]	44 (74)[e]	29% (74)[d]	15% (74)[e]	−0.68 (<0.001)	−0.60 (<0.001)

[a] See Table 1 for abbreviations; *MR* mortality rate
[b] For FR and MR, *N* refers to the number of bioassay dishes assayed.
[c] Correlation between FR and MR
[d,e] Means that are significantly different ($P<0.05$) by paired means test
[f] UBS mean FR and MR based on 11 different plants tested at least once.

feeding ratings, mortality rates, and survivor weights (data not shown) were found on Carpet Blue flowers (Table 2). Colored sectors from Ultra Blue Star (UBS) flowers (11 different plants were tested) were effective against CL larvae both in feeding ratings and mortality rates (Table 2). Correlations between feeding ratings and mortality ratings were significant for CB1 colored sectors and UBS colored and white sectors (Table 2).

Qualification of Anthocyanins All petunia white flower sectors contained low to no detectable anthocyanins (Table 3). The blue sectored cultivars, which displayed good resistance to CEW larvae feeding (Table 1), generally contained more malvin equivalents than the other colored cultivars. This was not true, however, for the Razzle Dazzle plant with blue-white sectored flowers, which had less malvin equivalents than Razzle Dazzle red sectors but was more resistant to CEW larvae feeding. The level of malvin equivalents in all the blue-sectored cultivars were negatively correlated to CEW larva weight. Malvin equivalent levels of both of the Razzle Dazzle pink sectored plants were seemingly negatively correlated to CEW larva weights, but in only one plant (Pink1) was this correlation significant. Neither of the Razzle Dazzle red-white sectored plants displayed a significant correlation between malvin equivalent levels and larva weights.

Identification of Anthocyanins The pigments from the blue sectored flowers were analyzed by LC–ESI–MS and contained primarily malvidin and petunidin derivatives (Table 4 and Fig. 1). The major pigments in red sectors were derivatives of cyanidin and delphinidin, while the pink sectors contained a peonidin derivative (Table 4). Structural identities were assigned only for those anthocyanins in which the experimental molecular weight matched with previously identified anthocyanins in petunia (Ando et al. 1999). The instruments used in the current study were not able to discriminate between *cis*- and *trans*-isomers, and thus, some presumed molecules were listed as either the *cis*- or *trans*- form (e.g., anthocyanin **5**). Most of the wild petunia anthocyanins with only a single sugar molecule are attached at the 3 position (Ando et al. 1999), and thus, anthocyanins **14** and **15**, which were not detected in wild petunias (Ando et al. 1999), were assumed to have their sugar attached at the 3 position. Peonidin-type anthocyanins are not present in wild petunias (Ando et al. 1999) but are present in a number of commercial *P. hybrida* lines (Ando et al. 2004). For the compounds detected in Razzle Dazzle pink sectors, it was assumed that the sugars would be attached to the peonidin base as it would be to malvidin or petunidin base for anthocyanins **10** and **13**. Because the Razzle Dazzle pink sectors are capable of producing petunidin- or peonidin-type anthocyanins, it was not possible to tell if the 933 mass from these flowers is anthocyanin **5** or **12**.

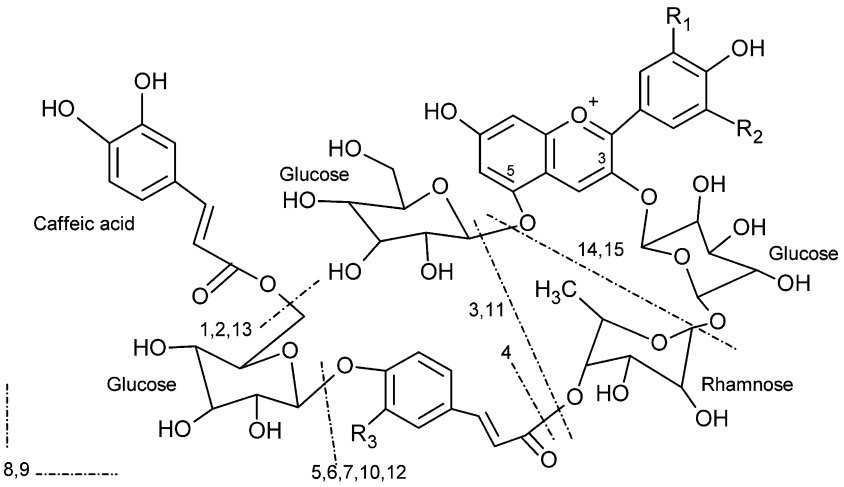
Bioassays with UBS Anthocyanin Composites Anthocyanins were partially purified from UBS flowers by using preparative chromatography. Seven different anthocyanin

Table 3 Correlation of anthocyanin levels to CEW larvae survivor weights

Cul[a]	Col Mal Eq (*N*)[b]	Whi Mal Eq (*N*)[c]	Correlation[d]	*P*[d]
Red1	183±13 (5)	0 (5)	0.06	0.46
Red2	189±14 (5)	0 (4)	−0.039	0.64
Pink1	103±8 (4)	0.7±0.7 (4)	−0.23	0.0072
Pink2	95±12 (4)	1.8±1.8 (3)	−0.17	0.072
Blu1	163±22 (8)	0 (8)	−0.24	<0.0001
CB1	343±36 (7)	0 (7)	−0.27	0.0027
CB2	377±42 (9)	0 (9)	−0.17	0.047
UBS1	266±45 (4)	0 (4)	−0.18	0.036
UBS2	311±17 (4)	0 (4)	−0.28	0.0011

See Table 1 for CEW larvae survivor weights.
[a] See Table 1 for abbreviations.
[b] Mean malvidin equivalents (μg) in two colored flower disks ± standard error
[c] Mean malvidin equivalents (μg) in two white flower disks ± standard error
[d] Correlation between mean malvidin equivalents to weights of larva survivors feeding on colored or white sectors and the resulting *P* value

Fig. 1 Structures of anthocyanins identified in this study



	R1	R2	R3
1	OH	OCH_3	H
2	OCH_3	OCH_3	H
3	OH	OH	
4	OH	OH	
5	OH	OCH_3	H
6	OCH_3	OCH_3	H
7	OCH_3	OCH_3	OH
8	OCH_3	OCH_3	H

	R1	R2	R3
9	OH	OCH_3	OH
10	OCH_3		H
11	OH	OCH_3	
12	OCH_3		OH
13	OCH_3		H
14	OH		
15	OCH_3		

composites added separately or in combination to insect diet significantly inhibited CL larvae growth (Table 5). While the combination of anthocyanin composites inhibited CEW larvae growth, only composites 1, 4A, 5A, and 5B were significantly inhibitory (Table 5). ANOVA between the following CEW means (Table 5) were not significant at $P<0.05$: 1 vs. 2, 1 vs. 3, and 5A vs. 3. No CEW or CL larvae died while feeding on the anthocyanin combinations (data not shown).

Discussion

CEW larvae generally fed less on colored than white sectors of all the petunia cultivars, which resulted in significantly lower weights in many cases (Table 1). The blue-sectored cultivars displayed superior CEW resistance compared to the red- and pink-sectored cultivars, with one exception (Razzle Dazzle Pink1). Statistically significant inverse correlations of mean CEW larva weight to mean anthocyanin levels (i.e., malvin equivalents) were found in all of the blue-sectored flower cultivars (Table 3). It may be argued that the effective CEW resistance of the blue-sectored flowers is due to a higher concentration of pigment. However, Razzle Dazzle Blu1 sectors and Razzle Dazzle Red1 sectors had statistically similar levels of malvin equivalents ($P=0.50$ by ANOVA), but the Razzle Dazzle blue sectors were more resistant to CEW feeding and caused lower CEW larva weights. Razzle Dazzle Pink1 sectors displayed good CEW resistance, while Pink2 and Pink3 colored sectors did not, perhaps due to the presence of unique allelochemicals specific to each plant. CL larvae were more sensitive to the constituents of the blue sectors because they displayed higher mortality rates than CEW larvae (compare Tables 1 and 2). While most correlations between CL mortality rates and feeding rates on Blu1 and the Carpet Blue flowers were seemingly negative (Table 2), only one was significant (CB1 on colored sectors), perhaps indicating that significant correlations would result only from more bioassays (as for UBS flowers). Correlations of CL feeding rates to mortality rates of Table 2 were −0.68 and −0.60 in UBS colored and white sectors, respectively. This suggests that anthocyanins in UBS colored sectors improves CL resistance present in the UBS white sectors but shows that other unknown defensive molecules in UBS colored and white sectors contribute to CL mortality.

Based on LC–ESI–MS analysis (not shown), the predominant anthocyanins of Razzle Dazzle blue/white and UBS flowers were anthocyanins **1** and **2**. While anthocyanin **2** was present in Carpet Blue Star flowers, the major anthocyanin was anthocyanin **1**. *P. hybrida* or garden petunias are likely the result of crosses of *P. axillaris* subsp. *axillaris* or subsp. *parodii* and *P. integrifolia* subsp. *integrifolia* or *P. inflata* (Chen et al. 2007). Anthocyanins **1** and **2** predominant in the blue star *P. hybrida* lines of this study do not occur in *P. integrifolia*, *P. inflata*, or the white flowers of *P. axillaris* subsp. *axillaris*

Table 4 Anthocyanins in petunia flower cultivars identified by LC–ESI–MS

Anthocyanins
Razzle Dazzle blue/white
Predominant anthocyanins
1095, petunidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **1**
1109, malvidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **2**
Other anthocyanins
611, delphinidin 3-rutinoside **3**
773, delphinidin 3-rutinoside-5-glucoside **4**
933, petunidin 3-*cis/trans*-*p*-coumaroylrutinoside-5-glucoside **5**
947, malvidin 3-*cis/trans*-*p*-coumaroylrutinoside-5-glucoside **6**
963, malvidin 3-trans-caffeoylrutinoside-5-glucoside **7**
1271, malvidin 3-caffeoylglucosyl *p*-coumaroylrutinoside-5-glucoside **8**
1273, petunidin 3-caffeoylglucosylcaffeoylrutinoside-5-glucoside **9**
Razzle Dazzle pink/white
Predominant anthocyanins
917, peonidin 3-*cis/trans*-*p*-coumaroylrutinoside 5-glucoside **10**
Other anthocyanins
625, petunidin 3-rutinoside **11**
933, peonidin 3-*cis/trans*-caffeoylrutinoside-5-glucoside **12** or **5**
1079, peonidin 3-glucosyl *p*-coumaroylrutinoside-5-glucoside **13**
Predominant anthocyanins
Razzle Dazzle red/white
449, Cyanidin 3-glucoside **14**, **3**
Ultra Blue Star
1, **2**
Carpet Blue Star
1
Other anthocyanins
Razzle Dazzle red/white
463, peonidin 3-glucoside **15**
Ultra Blue Star
3, **5**, **6**, **7**, **8**
Carpet Blue Star
2, **5**

Number(s) in bold refer to the anthocyanin structure described in Fig. 1.

and subsp. *parodii* (Ando et al. 1999). The major anthocyanin in *P. inflata* and *P. integrifolia* subsp. *integrifolia* is malvidin 3-*trans*-*p*-coumaroylrutinoside-5-glucoside (Ando et al. 1999). At some point in the breeding history of the *P. hybrida* blue star lines used in this study, an enzyme that places glucose on the coumaric acid group of the anthocyanins became more active.

Interestingly, the flowers with the most CEW resistance in their colored sectors (blue stars) are more similar in their anthocyanin profiles to their presumed parents (*P. axillaris* and *P. integrifolia*) than those with lower CEW resistance in their colored sectors (red and pink stars). Anthocyanin **3**, detected in Razzle Dazzle red sectors, is the predominate anthocyanin in only one wild petunia species, *P. exserta* (Ando et al. 1999). However, anthocyanin **14**, also detected abundantly in Razzle Dazzle red sectors, is not present in any wild petunias, but cyanindin 3-rutinoside is present in *P. exserta* (Ando et al. 1999). The major anthocyanin found in Razzle Dazzle pink sectors was anthocyanin **10**, which is not found in any wild petunia lines (Ando et al. 1999). The limited distribution of anthocyanin **3** and absence of anthocyanins **10** and **14** in wild petunia taxa (Ando et al. 1999) suggest that these anthocyanins are not involved in pollinator attraction and/or floral defense.

When all seven of the anthocyanin composites from UBS flowers were added to insect diet at approximately natural concentrations, they significantly reduced both CL and CEW weights compared to the controls (bottom of Table 5). This may be due to antibiosis or feeding deterrence. The reduction of CEW weights observed in the UBS colored sectors (Table 1) can be attributed partially to anthocyanins from UBS flowers (bottom of Table 5). However, CL larvae feeding on UBS colored sectors exhibited significant mortality (Table 2), while no CL mortality was observed with the combination of anthocyanin composites in insect diet (data not shown). As mentioned above, this indicates that unknown compounds beside anthocyanins contribute to CL mortality. Each of the seven individual UBS anthocyanin composites resulted in significantly reduced CL weights compared to the control (Table 5). This suggests that each of the UBS anthocyanin composites causes CL antiobiosis or feeding deterrence. However, not all of the anthocyanin composites reduced CEW weights compared to the control, which suggest that the mixture of compounds in composites 2, 3, and 4B were not as inhibitory or deterrent as the others.

Optimal defense theory predicts that flower tissue should be protected from damage to ensure fitness and that secondary

Table 5 Survivor weights (mg ± standard error) of bioassays using anthocyanin composites (3%) from UBS flowers

Composite	Anthocyanins[a]	CL Weight ± SE (*N*)	CEW Weight ± SE (*N*)
Control	–	1.7±0.14 (18)[b]	1.6±0.31 (11)[b]
1	**1**, 1113	1.1±0.092 (17)[c]	0.8±0.14 (15)[c]
2	**2**, 1126	1.0±0.083 (19)[c]	1.2±0.28 (9)[b]
3	**2**, 1127, 1149	1.1±0.080 (18)[c]	1.3±0.25 (11)[b]
4A	**5**, 951, 973	1.0±0.097 (18)[c]	0.8±0.12 (14)[c]
4B	**5**, 1271, 1288	1.1±0.12 (19)[c]	1.0±0.26 (10)[b]
5A	722, 730, 872, **6**	1.0±0.11 (18)[c]	0.8±0.12 (13)[c]
5B	**6**	1.2±0.076 (19)[c]	0.8±0.13 (10)[c]
Control	–	1.6±0.13 (19)[b]	1.1±0.17 (15)[b]
All 7[d]	All above	1.2±0.097 (18)[c]	0.6±0.064 (13)[c]

[a] Known anthocyanins and unknown anthocyanin masses

[b,c] Means of the control and experiment within a column were significantly different ($P<0.05$) by ANOVA.

[d] Each composite was added at 0.3% to make a total of 2.1% anthocyanins.

chemicals should be present constitutively rather than induced upon attack (McKey 1979). Petunia petals synthesize high levels of anthocyanins: based on Table 3, anthocyanin levels in some flower tissues (e.g., Carpet Blue) are ~6% fresh weight (punches weigh ~2.9 mg). If anthocyanin production is metabolically costly, it would be advantageous for petunia to produce petal compounds that both attract pollinators and defend the flower against herbivores. The data of this study indicate that petunia anthocyanins do inhibit growth or deter feeding of both CEW and CL larvae. This suggests that the petunia anthocyanins inhibit or deter a broad range of herbivores, but CEW and CL are likely not natural enemies of petunia. More studies that use pure wild petunia anthocyanins, wild petunia lines, and a number of their indigenous pests are needed to test fully the possible dual function of anthocyanins.

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References

ANDO, T., SAITO, N., TATSUZAWA, F., KAKEFUDA, T., YAMAKAGE, K., OHTANI, E., KOSHI-ISHI, M., MATSUSAKE, Y., KOKUBUN, H., WATANABE, H., TSUKAMOTO, T., UEDA, Y., HASHIMOTO, G., MARCHESI, E., ASAKURA, K., HARA, R., and SEKI, H. 1999. Floral anthocyanins in wild taxa of *Petunia* (Solanaceae). *Biochem. Syst. Ecol.* 27:623–650.

ANDO, T., TAKAHASHI, M., NAKAJIMA, T., TOYA, Y., WATANABE, H., KOKUBUN, H., and TATSUZAWA, F. 2004. Delphinidin accumulation is associated with abnormal flower development in petunias. *Phytochemistry* 65:2219–2227.

ARASIMOWICZ, M., and FLORYSZAK-WIECZOREK, J. 2007. Nitric oxide as a bioactive signalling molecule in plant stress responses. *Plant Sci.* 172:876–887.

BEHLE, R. W., TAMEZ-GUERRA, P., and MCGUIRE, M. R. 2003. Field activity and storage stability of *Anagrapha falcifera* nucleopolyhedrovirus (*Af*MNPV) in spray-dried lignin-based formulations. *J. Econ. Entomol.* 96:1066–1075.

CHEN, S., MATSUBARA, K., KOKUBUN, H., KODAMA, H., WATANABE, H., MARCHESI, E., and ANDO, T. 2007. Reconstructing historical events that occurred in the petunia *Hf1* gene, which governs anthocyanin biosynthesis, and effects of artificial selection by breeding. *Breed. Sci.* 57:203–211.

DIXON, R. A., and PAIVA, N. L. 1995. Stress-induced phenylpropanoid metabolism. *Plant Cell* 7:1085–1097.

DOWD, P. F. 1998. Involvement of arthropods in the establishment of mycotoxicenic fungi under field conditions, pp. 307–350, in K. K. Sinha, and D. Bhatnagar (eds.). Mycotoxins in Agriculture and Food Safety. Marcel Dekker, New York.

DOWD, P. F., ZUO, W. N., GILLIKIN, J. W., JOHNSON, E. T., and BOSTON, R. S. 2003. Enhanced resistance to *Helicoverpa zea* in tobacco expressing an activated form of maize ribosome-inactivating protein. *J. Agric.Food Chem.* 51:3568–3574.

DOWD, P. F., JOHNSON, E. T., and WILLIAMS, W. P. 2005. Strategies for insect management targeted toward mycotoxin management, pp. 517–541, in H. K. Abbas (ed.). Aflatoxin and Food Safety. CRC, Boca Raton.

DOWD, P. F., JOHNSON, E. T., and PINKERTON, T. S. 2007. Oral toxicity of beta-*N*-Acetyl hexosaminidase to insects. *J. Agric. Food Chem.* 55:3421–3428.

ELLIGER, C. A., and WAISS, A. C. Jr. 1991. Insect resistance factors in petunia, pp. 210–223, in P. A. Hedin (ed.). Naturally Occurring Pest Bioregulators. American Chemical Society, Washington, DC.

HEDIN, P. A., JENKINS, J. N., COLLUM, D. H., WHITE, W. H., PARROTT, W. L., and MACGOWN, M. W. 1983. Cyanidin-3-b-glucoside, a newly recognized basis for resistance in cotton to the tobacco budworm *Heliothis virescens* (Fab.)(Lepidoptera: Noctuidae). *Experientia* 39:799–801.

HOLLMAN, P. C. H., and KATAN, M. B. 1999. Dietary flavonoids: intake, health effects and bioavailability. *Food Chem. Toxicol.* 37:937–942.

HOLTON, T. A., BRUGLIERA, F., LESTER, D. R., TANAKA, Y., HYLAND, C. D., MENTING, J. G., LU, C. Y., FARCY, E., STEVENSON, T. W., and CORNISH, E. C. 1993. Cloning and expression of cytochrome P450 genes controlling flower colour. *Nature* 366:276–279.

HORVÁTH, E., SZALAI, G., and JANDA, T. 2007. Induction of abiotic stress tolerance by salicylic acid signaling. *J. Plant Growth Regul.* 26:290–300.

JENKINS, J. N., HEDIN, P. A., PARROTT, W. L., MCCARTY, J. C., and WHITE, W. H. 1983. Cotton allelochemics and growth of tobacco budworm larvae. *Crop Sci.* 23:1195–1198.

JOHNSON, E. T., and DOWD, P. F. 2003. Plant anthocyanin insect feeding deterrents. *Phytochemical Society of North America Annual Meeting Proceedings*, p. 8.

JOHNSON, E. T., and DOWD, P. F. 2004. Methylation of an anthocyanin increases its ability to inhibit growth of corn earworm larvae. *American Society of Plant Biologists Annual Meeting Program*, p. 125.

KARAGEORGOU, P., and MANETAS, Y. 2006. The importance of being red when young: anthocyanins and the protection of young leaves of *Quercus coccifera* from insect herbivory and excess light. *Tree Physiol.* 26:613–621.

KOES, R., VERWEIJ, W., and QUATTROCCHIO, F. 2005. Flavonoids: a colorful model for the regulation and evolution of biochemical pathways. *Trends Plant Sci.* 10:236–242.

KORKINA, L. G. 2007. Phenylpropanoids as naturally occurring antioxidants: from plant defense to human health. *Cell. Mol. Biol.* 53:15–25.

MANETAS, Y. 2006. Why some leaves are anthocyanic and why most anthocyanic leaves are red? *Flora.* 201:163–177.

MCKEY, D. 1979. The distribution of secondary compounds within plants, pp. 56–134, in G. A. Rosenthal, and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York.

METCALF, C. L., FLINT, W. P., and METCALF, R. L. 1951. Destructive and Useful Insects. McGraw-Hill, New York.

OMENN, G. S. 1995. What accounts for the association of vegetables and fruits with lower incidence of cancers and coronary heart diseases. *Ann. Epidemiol.* 5:333–335.

SADASIVAM, S., and THAYUMANAVAN, B. 2003. Molecular Host Plant Resistance to Pests. Marcel Dekker, New York.

SAMPSON, L., RIMM, E., HOLLMAN, P. C., DEVRIES, J. H., and KATAN, M. B. 2002. Flavonol and flavone intakes in US health professionals. *J. Am. Diet Assoc.* 102:1414–1420.

SCHAEFER, H. M., and ROLSHAUSEN, G. 2006. Plants on red alert: do insects pay attention? *BioEssays* 28:65–71.

SCHLINDWEIN, C. C. D., FEET-NETO, A. G., and DILLENBURG, L. R. 2006. Chemical and mechanical changes during leaf expansion of four woody species of a dry Restinga woodland. *Plant Biol.* 8:430–438.

TSUDA, T., HORIO, F., and OSAWA, T. 2000. The role of anthocyanins as an antioxidant under oxidative stress in rats. *Biofactors* 13:133–139.

WANG, H., CAO, G., and PRIOR, R. L. 1997. Oxygen radical absorbing capacity of anthocyanins. *J. Agric. Food Chem.* 45:304–309.

WASTERNACK, C. 2007. Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100:681–697.

WIERING, H., and DE VLAMING, P. 1984. Inheritance and biochemistry of pigments, pp. 49–76, in K. C. Sink (ed.). Petunia. Springer, New York.

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Antifeedant and Phytotoxic Activity of the Sesquiterpene *p*-Benzoquinone Perezone and Some of its Derivatives

Eleuterio Burgueño-Tapia · Lucia Castillo · Azucena González-Coloma · Pedro Joseph-Nathan

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Abstract The sesquiterpene *p*-benzoquinone perezone (**1**), isolated from *Perezia adnata* var. *alamani* (Asteraceae), and its non-natural derivatives isoperezone (**2**), dihydroperezone (**3**), dihydroisoperezone (**4**), and anilidoperezone (**5**) were tested as antifeedants against the herbivorous insects *Spodoptera littoralis*, *Leptinotarsa decemlineata*, and *Myzus persicae*. Compounds **1**–**5** exhibited strong antifeedant activity against *L. decemlineata* and *M. persicae*, and elicited a low response by *S. littoralis*. Antifeedant activity on *L. decemlineata* and *M. persicae* increased when the hydroxyl group at C-3 in perezone (**1**) was changed to C-6 to give isoperezone (**2**). The same effect was found with hydrogenation of the double bond of the alkyl chain of **(1)** to yield dihydroperezone (**3**). In contrast, hydrogenation of this double bond in isoperezone (**2**) to give dihydroisoperezone (**4**) led to a reduction in antifeedant activity. Determination of the phytotoxic activity of **1**–**5** revealed that **3** had a significant inhibition effect on *Lactuca sativa* radicle length growth.

E. Burgueño-Tapia
Departamento de Química Orgánica, Escuela Nacional de Ciencias Biológicas, Instituto Politécnico Nacional, Prolongación de Carpio y Plan de Ayala, Col. Santo Tomás, México, D.F. 11340, México

L. Castillo · A. González-Coloma
Instituto de Ciencias Agrarias, CCMA, CSIC, Serrano 115-bis, 28006 Madrid, España

P. Joseph-Nathan (✉)
Departamento de Química, Centro de Investigación y de Estudios Avanzados del Instituto Politécnico Nacional, Apartado 14-740, México, D.F. 07000, México
e-mail: pjoseph@nathan.cinvestav.mx

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Introduction

Natural quinones and some derivatives have attracted attention as insecticidal (Xu et al. 2003), antifeedant (Marimoto et al. 2002; Wellsow et al. 2006), and phytotoxic compounds (Hejl et al. 1993; Barbosa et al. 2001; Lima et al. 2003;, Hejl and Koster 2004). To date, however, only a small group of sesquiterpenoid quinones has been tested with respect to these biological activities (Motti et al. 2007; Burgueño-Tapia et al. 2008). Perezone (**1**), a sesquiterpenoid *p*-benzoquinone described by Río de la Loza (1852), was the earliest natural product isolated in crystalline form discovered in the New World (Fig. 1). It originally was isolated from roots of *Perezia adnata* var. *alamani* (Asteraceae), popularly known as 'raiz del pipitzahuac'. Its structure was characterized (Archer and Thomson 1965; Bates et al. 1965; Wagner et al. 1965; Walls et al. 1965b) 113 years after its discovery, as a direct consequence of the structural elucidation of α- and β-pipitzol (Walls et al. 1965a), obtained by heating **1**. Ever since its original isolation more than 155 years ago, perezone has been the subject of extensive chemical studies (Joseph-Nathan and Santillan 1989; Roura-Pérez et al. 2007), including its rearrangement to isoperezone (**2**) (Rodríguez-Hernández et al. 1994; Burgueño-Tapia and Joseph-Nathan 1997).

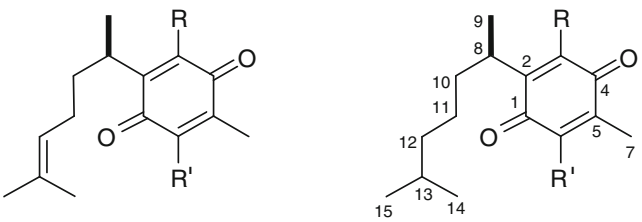
Some pharmacological effects have been reported for perezone: hypoglycemic action (Alarcón-Aguilar et al. 1997), induction of a biphasic dose-dependent contractile response in calcium-free intestinal smooth muscle preparation (García et al. 1995), an increase in intestinal motility (Enríquez et al. 1980), lack of sensitizing capacity (Hausen

and Soriano-García 1987), electron donor-acceptor characteristics (Carabez and Sandoval 1988), impairment of Ca^{2+} homeostasis in adrenal cortex mitochondria (Cuéllar et al. 1987), protection from the deleterious effects of experimental ischemia and reperfusion (Téllez et al. 1999), and inhibition of ADP-, epinephrine-, and collagen-induced platelet aggregation (de la Peña et al. 2001). Furthermore, plant defense-related properties (insect antifeedant effects and phytotoxicity) of structurally similar quinones have been recently reported (Burgueño-Tapia et al. 2008).

In continuation of our studies on the chemical and plant defense-related properties of natural products and their derivatives (González-Coloma et al. 2005; Reina et al. 2006 Burgueño-Tapia, et al. 2007, 2008), we investigated the antifeedant activity and toxic effects of perezone isolated from *Perezia adnata* var. *alamani* (Asteraceae), and its derivatives **2**–**5** (Fig. 1) on the polyphagous lepidopteran species *Spodoptera littoralis*, the Colorado potato beetle *Leptinotarsa decemlineata*, and the aphid *Myzus persicae*. Moreover, we studied the phytotoxic activity of these compounds on *Lactuca sativa*.

Methods and Materials

General Silica gel 60 Merck (230–400 mesh ASTM) was used for column chromatography. Optical rotations were measured in $CHCl_3$ on a Perkin-Elmer 341 polarimeter. High-resolution mass spectrometry (HRMS) measurements were conducted on an Agilent LCTOF high-resolution mass spectrometer at the UCR Mass Spectrometry Facility, University of California, Riverside, CA. Nuclear magnetic resonance (NMR) measurements were carried out by using 5-mm probes at 22°C from $CDCl_3$ solutions with tetramethylsilane (TMS) as the internal standard. 1D ^1H and ^{13}C spectra were acquired under standard conditions on Varian Mercury spectrometers operated at 300 and 75 MHz, respectively. The 2D hydrogen-detected heteronuclear shift correlation spectra were obtained by using the gHMQC and gHMBC pulse sequences with 512 time increments, 64 transients having been collected for each time increment with a relaxation delay of 1.0 sec



	R	R'
1	OH	H
2	H	OH
5	OH	NH-C_6H_5

	R	R'
3	OH	H
4	H	OH

Fig. 1 Compounds studied; side chain unsaturated (**1**, **2,** and **5**); side chain saturated (**3** and **4**). **1**=Perezone; **2**=Isoperezone; **3**=Dihydroperezone; **4**=Dihydroisoperezone; **5**=Anilidoperezone

Compounds Perezone (**1**) and its derivatives **2**, **3**, and **5** were available from previous studies (Burgueño-Tapia et al. 1997; Burgueño-Tapia and Joseph-Nathan 2000).

Dihydroisoperezone (**4**): A sample of 10 mg of 5% Pd/C was added to a solution of isoperezone (50 mg, 0.2 mmol) in AcOEt (5 ml). The air in the flask was replaced by H_2, and the solution was vigorously stirred at room temperature for 12 hr. The mixture was filtered through filter paper, and the filtrate was stirred at room temperature for an additional 12 hr to yield 47.9 mg (95% yield) of **4** as orange plates. mp: 121–122°C (mp 122–123°C, Rodríguez-Hernández et al. 1994). $[\alpha]_{589}$ +20.8, $[\alpha]_{578}$ 23.6, $[\alpha]_{546}$ 45.3 (c 1.06, $CHCl_3$). HRESIMS *m/z* $[M + Na]^+$ 273.1474 (calcd 273.1466). ^1H NMR: δ 6.98 (1H, brs, interchangeable with D_2O, OH), 6.44 (1H, d, *J*=1.2 Hz, H-3), 2.89 (1H, m, H-8), 1.94 (3H, s, H-7), 1.50 (1H, m, H-13), 1.45 (2H, m, H-10), 1.24 (2H, m, H-11), 1.14 (2H, m, H-12), 1.12 (3H, d, *J*=7.0 Hz, H-9), 0.85 (6H, d, *J*=6.6 Hz, H-14 and H-15). ^{13}C NMR: δ 188.2 (C-4), 183.3 (C-1), 151.3 (C-6), 149.7 (C-2), 133.0 (C-3), 116.7 (C-5), 38.8 (C-12), 35.8 (C-10), 31.3 (C-8), 27.8 (C-13), 25.0 (C-11), 22.6 (C-14), 22.5 (C-15), 19.3 (C-9), 7.8 (C-7).

Insect Bioassays Colonies of *Spodoptera littoralis* and *Leptinotarsa decemlineata* were reared on artificial diet and potato foliage (*Solanum tuberosum*), respectively (Poitout and Bues 1974). *Myzus persicae* was reared on bell pepper (*Capsicum annuum*) plants. All insects were maintained at 24±1°C, 60–70% r.h. with a 16:8 hr (L:D) photoperiod in a growth chamber. The environmental conditions for the assays described below were the same as those used for rearing. In both the feeding and settling bioassays, compounds **1** – **5** were tested. In addition, the model antifeedants ryanodine, which is a diterpene alkaloid (González-Coloma et al. 1999), silphinene, which is a tricyclic sesquiterpene (González-Coloma et al. 2002b), and polygodial, which is a sesquiterpene dialdehyde (Moreno-Osorio et al. 2008), were included as positive controls for *S. littoralis* and the aphids, respectively.

Feeding Assays These were conducted with newly molted *S. littoralis* 6th-instar larvae and adults of *L. decemlineata*. Leaf disks of *C. anuum* or *S. tuberosum* (1.0 cm^2) were treated on the upper surface with 10 µl of the test substance (5 µg/µl acetone). Three *S. littoralis* larvae or three adults of *L. decemlineata* were placed in a Petri dish with four 1-cm^2 leaf disks (two treated with test substance and two controls

with solvent only) (five to 10 Petri dishes in total per compound tested). Each compound was tested three times. Each assay was terminated after consumption of about 50–75% of the control disks. Feeding inhibition was calculated as % FI = [1 − (T/C) × 100], where T and C are the consumed leaf areas of treated and control leaf disks, respectively. These feeding bioassays were statistically evaluated by the Wilcoxon signed rank test.

Settling Assays Ten *M. persicae* adults were placed in an agar-coated box with one leaf disk cut in two pieces (each 1 cm^2), one for control and one treated with the test substance on the upper surface with 10 µl of the test substance (5 µg/µl acetone). In total, 20 boxes were used per compound tested. Each compound was tested three times. The bioassays were terminated after 24 hr (Gutiérrez et al. 1997; Reina et al. 2001). Settling bioassay data based on the number of aphids settled on the treated and control disks were statistically evaluated by the Wilcoxon signed rank test.

Forced Feeding To study the toxic effects of ingestion of compounds **1**–**5**, we forced pre-weighed newly molted *S. littoralis* 6th-instars to feed. A 6th instar was orally injected with 40 µg of the test compound in 4 µl of DMSO (treatment) or solvent alone (control) with a Rheodyne Hamilton syringe (50 µl) attached to a Hamilton micro-dispenser, as described by Reina et al. (2001). The syringe tip was inserted into the mouth of the larvae (maximum of 5 mm), and then larvae were forced to feed until no regurgitation was observed. In total, 20 larvae were "fed" this way per compound tested. At the end of the experiments (72 hr), food consumption and growth were calculated on a dry weight basis. A covariance analysis (ANCOVA1) of food consumption (ΔI) and biomass gains (ΔB) with initial larval weight (BI) as covariate (covariate $P>0.05$) was performed to test for significant effects of the compounds on these variables. An additional ANOVA analysis and covariate adjustment on ΔB with ΔI as covariate (ANCOVA2) were performed on those compounds that significantly reduced ΔB to understand their postingestive mode of action (antifeedant and/or toxic) (Reina et al. 2001). Rotenone, an insecticidal isoflavone, was included as a positive control (González-Coloma et al. 2002a).

Phytotoxic Evaluation These experiments were conducted with lettuce, *Latuca sativa* var. Carrascoy (Asteraceae) seeds as described by Moiteiro et al. (2006). Germination was monitored daily. Radicle length was measured at the end of the experiment after 7 d (20 digitalized radicles randomly selected for each experiment) with the application Image J Version 1.37r, 2006 (http://rsb.info.nih.gov./ij/). An analysis of variance (ANOVA) was performed on germination and radicle length data. Juglone was included as a positive control (Burgueño-Tapia et al. 2007).

Results and Discussion

The preparation of dihydroisoperezone (**4**) from isoperezone (**2**) has been previously described (Rodríguez-Hernández et al. 1994), although ^1H and ^{13}C NMR data were not reported. Compound **4** was obtained as orange plates, mp 121–122°C, after 5% Pd/C catalyzed hydrogenation of isoperezone (**2**) to give the respective hydroquinone, which was air-oxidized to quinone **4** in an EtOAc solution. Complete oxidation of the hydroquinone was monitored by TLC and ^1H NMR. The complete ^1H and ^{13}C NMR data assignment was made by comparison with data of isoperezone (**2**) (Burgueño-Tapia and Joseph-Nathan 1997) and by using one- and two-dimensional NMR experiments, including COSY, DEPT, gHSQC, and gHMBC.

Table 1 shows the antifeedant effects of (**1**–**5**). These *p*-benzoquinones were effective antifeedants to adult *L. decemlineata*, but not to larvae of *S. littoralis* as previously shown for hydroxyperezone and its derivatives (Burgueño-Tapia et al. 2008). The most active compounds against *L. decemlineata* were isoperezone (**2**) and anilidoperezone (**5**),

Table 1 Antifeedant effects of **1**–**5*** against *Spodoptera littoralis* larvae; *Leptinotarsa decemlineata*, and *Myzus persicae* adults. percent settling of *M persicae* adults on control (%C) and treated (%T) leaf disks

Compound*	*S. littoralis*[a,b,c]	*L. decemlineata*[a,b,c]	*M. persicae* [c,d]	
	%FI	%FI	%C	%T
1	40±8	59±4[d]	78±10	22±10[d]
2	29±8	89±6[d]	90±8	10±8[d]
3	38±10	78±4[d]	84±10	16±10[d]
4	10±4	Nt	71±12	29±12[d]
5	41±9	86±5[d]	40±12	60±12
Ryanodine[e]	100±0	–	–	–
Silphinene[f]	–	99±2	–	–
Polygodial[g]	–	–	98±6	2±2[d]

[a] Means ±SE values are given
[b] % FI = 1 − (*T*/*C*) × 100, where *T* and *C* are the consumed leaf area of treated and control leaf disks, respectively, dose of 50 µg/cm^2. *N*=15
[c] $P<0.05$, Wilcoxon Signed Rank test
[d] %*C* and %*T* are percent aphids settled on control and treated leaf disks, respectively, dose of 50 µg/cm^2. *N*=60
[e] From González-Coloma et al. 1999
[f] From González-Coloma et al. 2002b
[g] From Moreno-Osorio et al. 2008
1=Perezone; **2**=Isoperezone; **3**=Dihydroperezone; **4**=Dihydroisoperezone; **5**=Anilidoperezone

followed by dihydroperezone (**3**), with activity levels within the range of the positive control silphinene, the major component of *Senecio palmensis* (Asteraceae) (González-Coloma et al. 2002b), for **2** and **5**. Hydrogenation of the alkyl double bond in perezone (**1**) to give dihydroperezone (**3**) increased this activity. The same effect was observed when the hydroxyl group at C-3 in **1** was changed to C-6 in **2**. The introduction of an anilido group at C-6 in **1** to afford **5** also increased this antifeedant effect.

Compounds **1**–**4** showed strong inhibition of settling by *M. persicae* (% settling on treated disks <30%), **2** being the most active (% setting on treated disks 10%) with activity levels similar to the positive control polygodial (Moreno-Osorio et al. 2008), followed by **3**, **1**, and **4** (Table 1). Similar to the structure-activity pattern observed for the antifeedant effects on *L. decemlineata*, the hydrogenation of the alkyl double bond in **1** to give **3** increased activity, while reduction of this alkyl double bond in isoperezone (**2**) to give dihydroisoperezone (**4**) reduced this effect.

Table 2 shows the nutritional effects of **1**–**5** on *S. littoralis* larvae. A covariance analysis (ANCOVA1) of food consumption (ΔI) and biomass gains (ΔB) with initial larval weight as covariate (covariate $p>0.05$) was performed to test for significant effects of the test compounds on these variables. An additional ANOVA analysis and covariate adjustment on ΔB with ΔI as covariate (ANCOVA2) were performed for those compounds that significantly reduced ΔB in order to gain insight into their post-ingestive mode of action (antifeedant and/or toxic) (Raubenheimer and Simpson 1992; Horton and Redak 1993; Reina et al. 2001). Compounds **1**–**3** and **5** had a similar negative effect on biomass gain (ΔB) and consumption (ΔI) within the range of activity of the positive control rotenone (González-Coloma et al. 2002a). Treatment effects on ΔB disappeared with covariance adjustment, indicating that these compounds are post-ingestive growth inhibitors. Perezone (**1**) inhibits mitochondrial electron transport through a process that differs from that of rotenone (Carabez and Sandoval 1988), and this could explain the post-ingestive effects of **1** and its derivatives on *S. littoralis*.

Table 2 Biomass gain (ΔB) and consumption (ΔI) effects (% control) **1**–**5*** (40μg/larvae) on *Spodoptera littoralis* larvae [†]

Compound	ΔB[a,b]	ΔI[b,c]	pANCOVA2[a,d]
1	52±6[e]	58±9[e]	0.706
2	66±14[e]	56±9[e]	0.257
3	56±2[e]	59±11[e]	0.599
4	80±8	84±6	n.s.
5	54±12[e]	56±10[e]	0.814
Rotenone[f]	56±2[e]	62±9[e]	<0.05

n.s. not significant
[a] Means ±SE values are given (N=20)
[b] ΔB = change in insect body weight (mg dry weight)
[c] ΔI = mg food consumed (mg dry weight)
[d] Treatment p level, ANCOVA2 (ΔI as covariate)
[e] $P<0.05$, ANCOVA1 (initial larval weight as covariate),
[f] From González-Coloma et al. 2002a
*Compounds as in Table 1

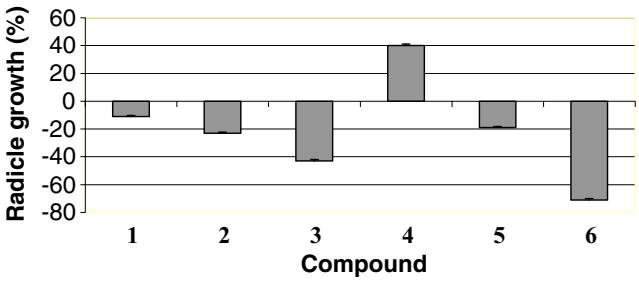


Fig. 2 Activity of the test compounds (50 μg/cm^2) on *Lactuca sativa* radicle length growth (mean ± SE, N=20). $P<0.05$, LSD test for all compounds except **1**. Compounds **1**–**5** as in Fig.1. Compound **6** is the positive control juglone (Burgueño-Tapia et al. 2008)

The test compounds did not affect germination of *L. sativa* seeds (% germination after 24 hr >99), but showed variable effects on radicle length growth (Fig. 2). Compounds **3, 2**, and **5** significantly reduced growth of radicle length (43%, 23%, and 19% growth inhibition, respectively, $P<0.05$, LSD test). Similar to the structure–activity pattern observed for the antifeedant effects, the alkyl double bond hydrogenation of **1** to yield dihydroperezone (**3**) and the change of the C-3 hydroxyl group of **1** to C-6 (**2**) increased this activity. In addition, hydrogenation of the side chain double bond in isoperezone (**2**) to give dihydroisoperezone (**4**) resulted in stimulation of radicle growth (40% growth increase, $P<0.05$, LSD test). On the other hand, the related compound hydroxyperezone with a second hydroxyl group at the 6 position reduced *L. sativa* germination after 24 hr (21–76% germination) and inhibited radicle growth (67–50% inhibition) more effectively (Burgueño-Tapia et al. 2008). Furthermore, sterification of its hydroxyl groups reduced post-ingestive effects on *S. littoralis*, while reduction of the alkyl double bond in leucohydroxyperezone tetraacetate increased it (Burgueño-Tapia et al. 2008). In this study, we have demonstrated that the position of the hydroxyl substituent along with the unsaturation/saturation of the alkyl chain can modulate strongly the antifeedant, post-ingestive, and phytotoxic effects of the perezone molecule.

Perezone is an electron-donor and electron-acceptor compound that behaves similarly to naphthoquinones (Carabez and Sandoval 1988). A recent study showed the importance of weak interactions of the olefin quinone type in the reduction process of quinones (Roura-Pérez et al. 2007). Chain size and flexibility is important for perezone's redox properties since it allows the approach of the

donating isopropenyl group to the quinone ring. These interactions can protect the quinone in biological media and even modify electrochemical potentials. In addition, it is possible that quinones with different side chains interact with different redox enzyme systems altogether (Roura-Pérez et al. 2007). These intra- and intermolecular interactions may likely explain the structure-dependent variation in the biological effects described here for perezone (**1**) and its derivatives.

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References

ALARCÓN-AGUILAR, F. J., ROMÁN-RAMOS, R., JIMÉNEZ-ESTRADA, M., REYES-CHILPA, R., GONZÁLEZ-PAREDES, B., and FLORES-SÁENZ, J. L. 1997. Effects of three Mexican medicinal plants (*Asteraceae*) on blood glucose levels in healthy mice and rabbits. *J. Ethnopharmacol* 55:171–177.

ARCHER, D. A., and THOMSON, R. H. 1965. The structure of perezone. *Chem. Comm.* 14: 354–355.

BARBOSA, L. C. A., FERREIRA, M. L., and DEMUNER, A. J. 2001. Preparation and phytotoxicity of sorgoleone analogues. *Quím. Nova.* 24:751–755.

BATES, R. B., PAKNIKAR, S. K., and THALACKER, V. P. 1965. A 1,3-addition by a hydroxyquinone: The structure of perezone. *Chem. & Ind.*1793.

BURGUEÑO-TAPIA, E., and JOSEPH-NATHAN, P. 1997. Detailed studies of perezone rearrangements. *Monatsh. Chem.* 128:651–658.

BURGUEÑO-TAPIA, E., and JOSEPH-NATHAN, P. 2000. ^{13}C NMR substituent chemical shifts in hydroxy-*p*-benzoquinones. *Magn. Reson. Chem.* 38:390–393.

BURGUEÑO-TAPIA, E., GONZÁLEZ-COLOMA, A., MARTÍN-BENITO, D., and JOSEPH-NATHAN, P. 2007. Antifeedant and phytotoxic activity of cacalolides and eremophilanolides. *Z. Naturforsch.* 6c2:362–366.

BURGUEÑO-TAPIA, E., GONZÁLEZ-COLOMA, A., CASTILLO, L., and JOSEPH-NATHAN, P 2008. Antifeedant and phytotoxic activity of hydroxyperezone and related molecules. *Z. Naturforsch* 63c:221–225.

CARABEZ, A., and SANDOVAL, F. 1988. The action of the sesquiterpenic benzoquinone perezone on electron-transport in biological membranes. *Arch. Biochem. Biophys.* 260:293–300.

CUÉLLAR, A., CARABEZ, A., and CHÁVEZ, E. 1987. Ca^{2+} releasing effects of perezone on adrenal cortex mitochondria. *Life Sci.* 41:2047–2054.

DE LA PEÑA, A., IZAGUIRRE, R., BAÑOS, G., VIVEROS, M., ENRÍQUEZ, R. G., and FERNÁNDEZ-G, J. M. 2001. Effect of perezone, aminoperezone and their corresponding isomers isoperezone and isoaminoperezone upon *in vitro* platelet aggregation. *Phytomedicine.* 8:465–468.

ENRÍQUEZ, R., ORTEGA, J., and LOZOYA, X. 1980. Active components in *Perezia* roots. *J. Ethnopharmacol.* 2:389–393.

GARCÍA, X., ALCÁNTARA-SARABIA, G., CARTAS-HEREDIA, L., and GIJÓN, E. 1995. Actions of perezone on rat smooth muscle. *Gen. Pharmacol.* 26:1741–1745.

GONZÁLEZ-COLOMA, A., GUTIÉRREZ, C., HÜBNER, H., ACHENBACH, H., TERRERO, D., and FRAGA, B. M. 1999. Selective insect antifeedant and toxic action of ryanoid diterpenes. *J. Agric. Food Chem.* 47:4419–4424.

GONZÁLEZ-COLOMA, A., GUADAÑO, A., DE INÉS, C., MARTÍNEZ-DIAZ, R., and CORTES, D. 2002a. Selective action of acetogenin mitochondrial Complex I inhibitors. *Z. Naturforsch.* 57c:1028–1034.

GONZÁLEZ-COLOMA, A., VALENCIA, F., MARTÍN, N., HOFFMANN, J. J., HUTTER, L., MARCO, J. a., and REINA, m. 2002b. Silphinene sesquiterpenes as model insect antifeedants. *J. Chem. Ecol.* 28:117–129.

GONZÁLEZ-COLOMA, A., GUADANO, A., TONN, C. E., and SOSA, M. E. 2005. Antifeedant/insecticidal terpenes from Asteraceae and Labiatae species native to Argentinean semi-arid lands. *Z. Naturforsch.* 60c:855–861.

GUTIÉRREZ, C., FERERES, A., REINA, M., CABRERA, R., and GONZÁLEZ-COLOMA, A. 1997. Behavioral and sub-lethal effects of structurally-related lower terpenes on *Myzus persicae*. *J. Chem. Ecol.* 23:1641–1650.

HAUSEN, B. M., and SORIANO-GARCÍA, M. 1987. The sensitizing capacity of perezone. *Contact Dermatitis.* 17:259–260.

HEJL, A. M., EINHELLIG, F. A., and RASMUSSEN, J. A. 1993. Effects of juglone on growth, photosynthesis and respiration. *J. Chem. Ecol.* 19:559–568.

HEJL, A. M., and KOSTER, K. L. 2004. The allelochemicals sorgoleone inhibits root H+-ATPase and water uptake. *J. Chem. Ecol.* 30:2181–2191.

HORTON, R. D., and REDAK, R. A. 1993. Further comments on analysis of covariance in insect dietary studies. *Entomol. Exp. Appl.* 69:263–275.

JOSEPH-NATHAN, P., and SANTILLAN, R. L. 1989. The chemistry of perezone and its consequences, pp. 763–813, in Atta-ur-Rahman (ed.). Studies in Natural Products Chemistry, Vol. 5 Structural Elucidation (Part B)Elsevier, Amsterdam.

LIMA, L. S., BARBOSA, L. C. A., DE ALVERENGA, E. S., DEMUNER, A. J., and DA SILVA, A. A. 2003. Synthesis and phytotoxicity evaluation of substituted *para*-benzoquinones. *Aust. J. Chem.* 56:625–630.

MARIMOTO, M., TANIMOTO, K., SAKATANI, A., and KOMAI, K. 2002. Antifeedant activity of an anthraquinone aldehyde in *Galium aparine* L. against *Spodoptera litura* F. *Phytochemistry.* 60:163–166.

MOITEIRO, C., MARCELO-CURTO, M. J., MOHAMED, N., BAILÉN, M., MARTÍNEZ-DÍAZ, R., and GONZÁLEZ-COLOMA, A. 2006. Bio-valorization of friedelane triterpenes derived from the cork processing industry byproducts. *J. Agric. Food. Chem.* 54:3566–3571.

MORENO-OSORIO, L., CORTES, M., ARMSTRONG, V., BAILÉN, M., and GONZÁLEZ-COLOMA, A. 2008. Antifeedant activity of some polygodial derivatives. *Z. Naturforsch*. 63c: in press.

MOTTI, C. A., BOURGUET-KONDRCKI, M.-L., LONGEON, L., DOYLE, J. R., LLEWELING, L. E., TAPIOLES, D. M., and YING, P. 2007. Comparison of the biological properties of several marine sponge-derived sesquiterpenoid quinones. *Molecules.* 12:1376–1388.

POITOUT, S., and BUES, R. 1974. Elevage de plusiereurs espèces de lépidoptères Noctuidae sur milieu artificiel riche et sur milieu artificiel simplifié. *Ann. Zool. Ecol. Anim.* 2:79–91.

RAUBENHEIMER, D., and SIMPSON, S. J. 1992. Analysis of covariance: an alternative to nutritional indices. *Entomol. Exp. Appl.* 62:221–231.

REINA, M., GONZÁLEZ-COLOMA, A., GUTIÉRREZ, C., CABRERA, R., RODRÍGUEZ, M. L., FAJARDO, V., and VILLARROEL, L. 2001. Defensive chemistry of *Senecio miser*. *J. Nat. Prod.* 64:6–11.

REINA, M., GONZÁLEZ-COLOMA, A., DOMÍNGUEZ-DÍAZ, D., CABRERA, R., JIMÉNEZ, M. C., RODRÍGUEZ, M. L., and VILLARROEL, L. 2006. Bioactive eremophilanolides from *Senecio poepigii*. *Nat. Prod. Res.* 20:13–19.

RÍO DE LA LOZA, L. Discurso pronunciado por el catedrático de Química Médica de la Escuela de Medicina (November 23th, 1852), pp. 94–100, in Escritos de Leopoldo Río de la Loza, compiled by J. M. Noriega,. Imprenta de Ignacio Escalante, México (1911).

RODRÍGUEZ-HERNÁNDEZ, A., BARRIOS, H., COLLERA, O., ENRÍQUEZ, R. G., ORTIZ, B., SÁNCHEZ-OBREGÓN, R., WALLS, F., YUSTE, F., REYNOLDS, W. F., and YU, M. 1994. Isomerization of perezone into isoperezone and preparation of dihydroisoperezinone. *Nat. Prod. Lett.* 4:133–139.

ROURA-PÉREZ, G., QUIROZ, B., AGUILAR-MARTÍNEZ, M., FRONTANA, C., SOLANO, A., GONZÁLEZ, I., BAUTISTA-MARTÍNEZ, J. A., JIMÉNEZ-BARBERO, J., and CUEVAS, G. 2007. Remote position substituents as modulators of conformational and reactive properties of quinones. Relevance of the π/π intramolecular interaction. *J. Org. Chem.* 72:1883–1894.

TÉLLEZ, J. F., CARVAJAL, K., CRUZ, D., CARABEZ, A., and CHÁVEZ, E. 1999. Effect of perezone on arrythmias and markers of cell injury during reperfusion in the anesthetized rat. *Life Sci.* 16:1615–1623.

WAGNER, E. R., MOSS, R. D., BROOKER, R. M., HEESCHEN, J. P., POTTS, W. J., and DILLING, M. 1965. A correction of the structure of perezone. *Tetrahedron Lett* 4233–4239.

WALLS, F., PADILLA, J., JOSEPH-NATHAN, P., GIRAL, F., and ROMO, J. 1965a. The structures of - and -pipitzols. *Tetrahedron Lett*. 1577–1582.

WALLS, F., SALMÓN, M., PADILLA, J., JOSEPH-NATHAN, P., and ROMO, J. 1965b. La estructura de la perezona. *Bol. Inst. Quím. Univ. Nal. Autón. Méx.* 17:3–15.

WELLSOW, J., GRAYER, R. J., VEITCH, N. C., KOKUBUN, T., LELLI, R., KITE, G. C., and SIMMONDS, M. S. J. 2006. Insect-antifeedant and antibacterial activity of diterpenoids from species of *Plectranthus*. *Phytochemistry.* 67:1818–1825.

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Variation of Insect Attracting Odor in Endophytic *Epichloë* Fungi: Phylogenetic Constrains *Versus* Host Influence

Fabrizio Steinebrunner · Florian P. Schiestl · Adrian Leuchtmann

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Abstract Odor is a key trait for pollinator attraction in flowering plants, and many studies have investigated odor evolution in the light of pollinator selection by emphasizing the importance of the plant phylogenetic history. By contrast, little is known on the evolution of odors in fungus–insect interactions. In this study, profiles of three volatile compounds that are emitted by grass-inhabiting *Epichloë* fungi (Clavicipitaceae, Ascomycota) and that have a confirmed or likely role in the attraction of gamete-transferring *Botanophila* flies were investigated. We collected headspace samples from stromata of six European *Epichloë* species (including various host races) that originated from different locations in Switzerland, France, Poland, and UK for conducting gas chromatography analyses. Odor profiles exhibited considerable variation, but profiles of most species overlapped and did not discriminate at the species level. The exception was *Epichloë festucae*, which had a profile dominated by methyl (*Z*)-3-methyldodec-2-enoate. Based on an *Epichloë* phylogeny, there was some hierarchical structuring regarding levels of chokol K, another confirmed *Botanophila* attractant. However, patterns of odor profiles appeared to be largely dependant on particular *Epichloë*–host associations. The observed variation may be the result of complex selective pressures imposed by *Botanophila* gametic vectors, local environment, and mycoparasites.

F. Steinebrunner · A. Leuchtmann (✉)
Plant Ecological Genetics, Institute of Integrative Biology (IBZ),
ETH Zürich,
8092 Zürich, Switzerland
e-mail: adrian.leuchtmann@env.ethz.ch

F. P. Schiestl
Institute of Systematic Botany, University of Zürich,
Zollikerstrasse 107,
8008 Zürich, Switzerland

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Introduction

Odor is a key component of many sessile organisms in communicating with other organisms in an ecosystem. It may be important, alone or in concert with visual stimuli, for the attraction of insects that are involved in pollination or dispersal by eliciting searching, alighting, and feeding (Knudsen 2002; Plepys et al. 2002). Even though pollinator attraction is viewed as the primary function of odor, other functions that include plant defense or protection against abiotic stresses have been documented (Pellmyr and Thien 1986; Dudareva et al. 2006). While odor has received much attention in angiosperms, only a few studies have focused on odors of fungi and even fewer on the role of odor in fungal–insect interactions (Hedlund et al. 1995; Raguso and Roy 1998; Guevara et al. 2000). Recently, the biological function of volatiles for sexual reproduction of *Epichloë* fungi has been unraveled (Schiestl et al. 2006; Steinebrunner et al. 2008a).

Epichloë fungi (Clavicipitaceae, Ascomycota) are endophytes of pooid grasses with which they form constitutive, life-long symbioses. Infections are symptomless during vegetative growth of the host. Only at the flowering stage does the fungus produce external fruiting structures (stromata) on grass inflorescences. *Epichloë* is self-incompatible and has to be fertilized by gametes of the opposite mating type for sexual reproduction. Gametes (spermatia) are transferred by specialized flies of the genus *Botanophila* that feed and develop on fungal stromata (Bultman et al. 1995). Immediately after oviposition, the flies actively

fertilize the stroma, which involves spreading of viable spermatia containing feces over the stroma surface (Bultman et al. 1998). Thus, fly activity is indispensable for the formation of sexual ascospores that are wind dispersed and may infect new host grasses. Conversely, *Botanophila* larvae develop on the stroma and rely on the growing fungal tissue as a food source. In general, the *Epichloë–Botanophila* association is a balanced mutualism for which overexploitation by *Botanophila* larvae does not occur (Bultman et al. 2000). However, if the conditions are conducive, the mutualism may be disrupted to form an interaction of simple foraging by fly larvae (Rao and Baumann 2004).

The phylogeny of genus *Epichloë* based on multiple gene sequences is well established (Schardl et al. 1997; Craven et al. 2001). *Epichloë* comprises ten species, which have been circumscribed as distinct mating populations with one exception (Schardl et al. 1997). The species differ in host range and are restricted to either Eurasia or North America, following the native distribution of the host grasses (Leuchtmann 2003). A recent study of *Epichloë*-associated *Botanophila* revealed that at least six distinct fly taxa are involved in gamete transfer in Europe and North America (Leuchtmann 2007). While phylogenetic analyses indicated that seven *Epichloë* species coevolved by common descent with grass hosts (Schardl et al. 1997), comparison of molecular phylogenetic relationships of *Botanophila* with the associated *Epichloë* hosts did not suggest co-evolution of fungus and fly (Leuchtmann 2007).

Odor communication between *Epichloë* and the *Botanophila* flies is based on a relatively simple mechanism that involves two system specific volatiles, namely, chokol K and methyl (*Z*)-3-methyldodec-2-enoate (MME; Schiestl et al. 2006; Steinebrunner et al. 2008a). These compounds (and perhaps others) are assumed to be key traits responsible for *Botanophila* attraction and are likely adaptive for *Epichloë* and possibly *Botanophila* flies. In addition, chokol K is fungitoxic (Koshino et al. 1989) and has been shown to reduce spore germination of *Epichloë* mycoparasitic fungi (Steinebrunner et al. 2008b). Thus, the production of chokol K and MME in *Epichloë* may be the result of complex selection pressures.

In plant–insect interactions, odor profiles are often species specific (Levin et al. 2001; Grison-Pige et al. 2002; Knudsen et al. 2006), which potentially enables pollinators to recognize a particular plant species. Likewise, in the *Epichloë* system, species-specific odor profiles may allow for specific *Botanophila* attraction and, thus, could maximize successful cross-fertilization. Among three *Epichloë* species, differences in the production of chokol K and MME have been reported (Steinebrunner et al. 2008a). However, regarding *Botanophila* flies, there is little empirical evidence for host fidelity. With one exception, larval catches from *Epichloë* stromata indicate that *Botanophila* species are typically associated with several *Epichloë* species (Leuchtmann 2007). Similarly, a common *Botanophila* species did not differentiate between synthetic odor blends that imitate different *Epichloë* species offered in traps but visited all traps at equal rates (Steinebrunner et al. 2008a). On the other hand, an earlier study that examined gamete mixtures in the fly feces suggested that flies predominantly visited one host while visiting others less frequently (Bultman and Leuchtmann 2003). Therefore, *Botanophila* flies may display "flower" constancy, a tendency to visit stromata of one species repeatedly while ignoring suitable stromata of another *Epichloë* species.

A given odor profile may not only be viewed as result of selective biotic forces acting on its producer but may simply be a by-product of metabolic processes, or have remained, although the adaptive context has changed (Levin et al. 2003; Raguso et al. 2003). Therefore, interpretation of odor profiles should be made in a phylogenetic context. A further complication in studying odor in endophytic *Epichloë* is that its production may be modulated by the host grass, the *Epichloë* genotype, and/or the interaction of the two.

In the present study, we examined *Epichloë* odor production at species and at host race levels. We relied on the analysis of stromata emitted, previously described compounds (or its variants) by using headspace sampling. The following questions were asked: (1) How variable are odor profiles among *Epichloë* species? Are the profiles species specific? Is there evidence for fluctuations among years? (2) Are odor data phylogenetically informative so that they could be used to reconstruct phylogenetic relationships?

Our study is the first describing variation of insect attracting odor in *Epichloë* fungi and offering insight into phylogenetic patterns of odor production on a genus-wide scale.

Methods and Materials

Study Taxa and Sampling Plants infected by *Epichloë* fungi originated from various locations in Switzerland, France, Poland, UK, and USA and had been maintained for up to 14 yr in pots at an experimental garden in Zürich, Switzerland. In total, 14 different host plant–endophyte associations represented by 1–24 genetically distinct accessions were included in the study (Table 1). Before and during the experiment, plants were watered regularly and received liquid fertilizer once a week. To induce stromata formation for odor collection, potted plants were vernalized outside during winter. The following spring when stromata emerged (which usually coincides with host flowering), plants were moved into the laboratory, and volatiles emitted

Table 1 List of *Epichloë* associations used in the present study with number of accessions (genotypes) and geographic origin

Fungal species	Host species	Abbreviation	No. of accessions	Geographic origin[a]	Country
E. baconii	*Agrostis tenuis*	Eba–At	4	ZH	Switzerland
E. baconii	*Calamagrostis villosa*	Eba–Cv	3	GR	Switzerland
E. bromicola	*Bromus erectus*	Eb–Be	17	SH, AG, LU, Vesancy	Switzerland, France
E. clarkii	*Holcus lanatus*	Ec–Hl	8	VD	Switzerland
E. festucae	*Festuca rubra*	Ef–Fr	6	VD, VS, Vesancy, Lexington	Switzerland, France, USA
E. sylvatica	*Brachypodium sylvaticum*	Es–Bs	8	ZH	Switzerland
E. typhina	*Anthoxanthum odoratum*	Et–Ao	3	VD, TI	Switzerland
E. typhina	*Dactylis glomerata*	Et–Dg	24	VD, ZH, SH, Vesancy	Switzerland, France
E. typhina	*Brachypodium pinnatum*	Et–Bp	7	VD, ZH	Switzerland
E. typhina	*Phleum pratense*	Et–Php	1	Cardiff	UK
E. typhina	*Poa nemoralis*	Et–Pn	5	VD, ZH	Switzerland
E. typhina	*Poa pratensis*	Et–Pp	3	ZH, VS	Switzerland
E. typhina	*Poa trivialis*	Et–Pt	2	ZH	Switzerland
E. typhina	*Puccinellia distans*	Et–Pd	2	Gniebnia, Janikowo	Poland

[a] Swiss canton (*AG* Aargau, *GR* Grison, *LU* Luzern, *SH* Schaffhausen, *TI* Ticino, *VD* Vaud, *VS* Valais, *ZH* Zürich) or town if outside Switzerland

from the stromata were sampled with headspace sorption within the first 3 d after emergence. Sequential measurements on stromata from *Epichloë typhina* infecting *Phleum pratense* at days 1, 3, 6, 10, 13, 15, 20, and 23 after stroma emergence demonstrated that volatile production did not decrease considerably until day 3 (Fig. 1) suggesting that our sampling provided an accurate measure for maximum odor production. To estimate consistency of odor emission among years, odor was sampled from a representative subset of plants that included 15 accessions and 7 host grasses in 2 consecutive years (2005 and 2006).

Collection of Fungal Volatiles To collect volatiles, stromata-bearing tillers were covered individually with polyethylene terephtalate bags (Nalo®, Kalle GmbH, Germany) and the air pulled out by a vacuum pump (SCK Inc.) at a rate of approximately 100 ml/min. Volatiles were trapped on custom-made micropipette filters containing 5 mg of Porapak Q (Mesh size 80/100, Alltech Associates Inc., USA; Salzmann et al. 2006). If available, two to four stromata per plant were sampled individually, except for some plants where only one stroma-bearing tiller emerged. Before sampling, the Porapak Q adsorbent was cleaned with 100 µl of dichlormethane and 100 µl of hexane (Merck, Uvasol). Blank air samples were collected to check for background contaminants. After a sampling period of 24 hr (including day and night at room temperature), adsorbed volatiles were eluted from Porapak Q with 50 µl of a mixture of hexane/acetone (9:1; Merck), sealed in glass vials, and stored at −20°C. Headspace sorption is the method of choice for collecting compounds of medium to high volatility that are produced *de novo* and emitted immediately, as those from *Epichloë* stromata examined here, and relatively long sampling periods were necessary to collect sufficiently concentrated samples. Interval sampling of the key volatiles chokol K and MME did not show differences in emission between day and night (Steinebrunner, unpublished data), thus suggesting continuous odor production. Nevertheless, flies are active only during day, preferably at warm and sunny conditions between 10 A.M. and 4 P.M.

In vitro production of volatiles was examined in isolates of *Epichloë* grown on potato dextrose agar (Difco) in Petri dishes (Steinebrunner et al. 2008b). For collecting volatiles, Petri dishes with 2-wk-old colonies were placed in polyethylene terephtalate bags (Nalo®, Kalle GmbH), then

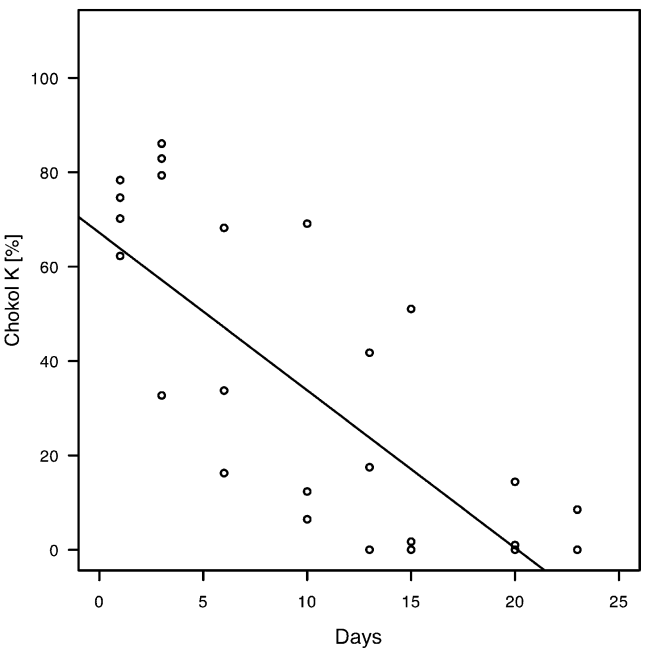

Fig. 1 Scatterplot of repeated headspace measurements from *Epichloë typhina* stromata on *Phleum pratense* (*Et–Php*) at days 1, 3, 6, 10, 13, 15, 20, and 23 after stromata emergence

the cover was lifted, and the air above the growing *Epichloë* cultures inside the bag was sampled as described above.

Quantitative GC Analyses and GC-Mass Spectrometry For quantitative analysis, 100 ng of *n*-octadecane were added to samples as an internal standard. One microliter of each sample was injected splitless into a gas chromatograph (GC; Agilent 6890N) equipped with an HP5 column (5%-phenyl-metylpolysiloxane, 30 m×0.32 mm diameter×0.25 μm film thickness) and a flame ionization detector. The column was temperature-programmed at 50°C for 1 min, followed by a temperature increase to 300°C at a rate of 10°C min^{-1}, with a final hold of 10 min at 300°C. The carrier gas was hydrogen (Carbagas, local distributor), and the GC was operated at constant flow (2 ml min^{-1}). Data acquisition and data analysis were done by using the Agilent Chemstation software package (Agilent Technologies, Palo Alto, CA, USA).

Previously known compounds (chokol K and MME) were identified by retention time comparison with authentic standards (Schiestl et al. 2006; Steinebrunner et al. 2008a). Identification of unknown compounds was done by using GC/mass spectrometry (MS) (HP G 1800 A, Hewlett Packard Inc., Palo Alto, CA, USA) with the operating conditions as described above, following tentative identification of the mass spectra on the basis of the NIST database information. To calculate absolute amounts, the internal standard method proposed by Schomburg (1990) was used.

Phylogenetic Analysis To establish a possible link between phylogeny of *Epichloë* taxa and the observed odor profiles, a maximum likelihood (ML) tree based on variations of intron sequences of β-tubulin (*tub2*) of representative strains of European *Epichloë* species plus the American outgroup taxon *E. brachyelytri* was constructed. The sequences used have been published in Craven et al. (2001) and were retrieved from GenBank (National Center for Biotechnology Information, Bethesda, MD, USA; http://www.ncbi.nlm.nih.gov/) with one exception. *E. typhina* from *Puccinellia distans* (*Et–Pd*) was newly sequenced in the course of this study, following the methods described in Craven et al. (2001), and submitted to GenBank (accession number EU375739). Aligned sequences were analyzed with PAUP *4.0b10 (Swofford 2003) employing ML and maximum parsimony analyses. ModelTest v.3.7 (Posada and Crandall 1998) was used to select the best fitting model (K80+G) for general time reversible ML analysis by using the tree bisection and reconnection branch swapping algorithm. Odor profiles from *in vitro* and *in vivo* results, including three compounds [chokol K, MME, and methyl 2,4,8-trimethylundecanoate (MTE)], plus a group of unknown diterpenes were then mapped into the ML phylogenetic tree. Phylogenetic reconstruction was done separately with odor data, but as expected, the resulting tree was poorly resolved due to the relatively small data set.

Statistical Analyses All comparisons of odor compounds from different *Epichloë* species and host races are based on relative amounts. We use relative values for technical reasons to provide a more reliable measure for comparison. Absolute values from samples that are not simultaneously processed are difficult to compare due to variations in absorption and elution of filters. Relative values of single compounds were calculated by dividing the amount of each compound by the sum of all other compounds in the headspace (excluding the solvent peak). Homogeneity of variances of data was tested with Levene's test. If no adequate transformation were found, data were analyzed by using the nonparametric Kruskal–Wallis test. Principal component analysis based on the three investigated compounds (chokol K, MME, and MTE) was used to illustrate the relationship among species. Sequential odor emission measurements in *E. typhina* from *Phleum pratense* (*Et–Php*) were analyzed with one-way analysis of variance based on log-transformed values. For comparisons of odor emission among years of selected genotypes, the 95% confidence interval (95% CI), based on the differences of the means of the 2 yr, was calculated. If zero was included in the CI, the emission was not regarded as significantly different. The CI calculations were done by using the bootstrapping method "boot" from the R library (boot) and are based on 10,000 replicates. All statistical analyses were done with the software R (R Development Core Team 2005).

Results

Epichloë Volatiles Analyses of headspace samples focused on three volatile compounds, chokol K, MME, and a trimethylester tentatively identified as MTE. The identification of MTE was done with MS-spectrum comparison from the NIST database and resulted in an 85.3% match of spectra (Fig. 2). Production of these volatiles are confined to the stroma tissue and could not be detected in samples taken from plant leaves or stems (data not shown). Chokol K and MME have been shown previously to attract *Botanophila* flies (Steinebrunner et al. 2008a). The third compound, MTE, is reported from *Epichloë* for the first time. MTE has not yet been shown to play a role in odor communication; however, due to its similar structure to MME and high abundance in some *Epichloë* associations, such a function seems likely. The rest of the headspace was made up of various other compounds for which no activity was indicated, and no attempts were made to identify them.

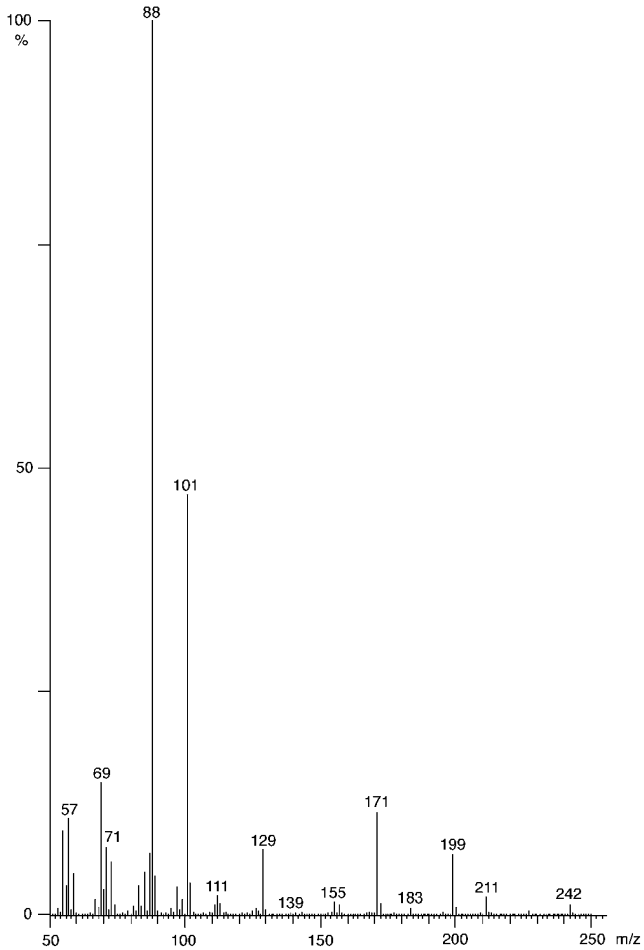

Fig. 2 Mass spectrum of the volatile compound from *Epichloë typhina* on *Puccinellia distans* identified as methyl 2,4,8-trimethylundecanoate (*MTE*) using the NIST database

Relative amounts of chokol K, as determined in headspace samples from fungal stromata, differed among the *Epichloë* species tested (Kruskal–Wallis test, χ=40.86, *df*=5, *P*< 0.001). Proportion of this compound was highest in *Epichloë sylvatica* (*Es*) comprising more than 56% on average of the total amount in headspace samples (Table 2). In other species, average amounts of chokol K were lower with 23% in *Epichloë clarkii* (*Ec*) and 10% in *E. typhina* (*Et*), while only trace amounts (<2%) were found in *Epichloë baconii* (*Eba*) and *Epichloë bromicola*. No chokol K at all was detected in samples from *Epichloë festucae* (*Ef*).

Chokol K production differed among the eight host races of *E. typhina* (Kruskal–Wallis test, χ=15.29, *df*=7, *P*= 0.018). Average emissions were relatively high from *E. typhina* stromata on *Anthoxanthum odoratum* (*Et–Ao*), *Brachypodium pinnatum* (*Et–Bp*), *Phleum pratense* (*Et–Php*), and *Poa trivialis* (*Et–Pt*) with proportions between 29% and 52% (Table 2). Lower emissions (<2%) were measured from stromata of *E. typhina* on *Dactylis glomerata* (*Et–Dg*), *Puccinellia distans* (*Et–Pd*), *Poa nemoralis* (*Et–Pn*), and *Poa pratensis* (*Et–Pp*). Exceptionally large genotypic differences were observed among accessions of the *Et–Bp* association with regard to the chokol K production: Five accessions were strong chokol producers (49.74%±9.27 SEM, *N*=5), while the other two produced only trace amounts (0.041%±0.01, *N*=2).

Proportions of the second volatile MME in headspace samples were also different among *Epichloë* species (Kruskal–Wallis test, χ=24.68, *df*=5, *P*<0.001). Only in samples from *E. festucae*, however, was the average amount high (39%), whereas in all other associations, no or little MME was found (Table 2). Interestingly, the other two volatiles were not detected in the *E. festucae* association.

The third volatile MTE showed a similar pattern of uneven distribution among *Epichloë* species (Kruskal–Wallis test, χ=11.35, *df*=5, *P*=0.045). With more than 57% on average, MTE was the main compound found in samples from *E. typhina* on *Puccinellia distans* (*Et–Pd*), where it appears to have replaced the other methyl ester MME. In all other associations, MTE did not exceed 3% of the total headspace (Table 2).

Principal component analysis (PCA) based on patterns of the three volatile compounds did not discriminate among *Epichloë* species, except for *E. festucae* (Fig. 3). *E. festucae* (*4*) was separated clearly from the other species owing to its unique pattern with the single dominating volatile MME. The correlation vectors show that a stronger correlation existed between chokol K and each of the methyl esters than between MME and MTE themselves. The greater length of the chokol K vector compared to the other vectors indicates that this compound is the best represented variable in the PCA.

Odor profiles of 14 *Epichloë* accessions representing seven host grasses were evaluated in 2 consecutive years. Mean year differences of volatile emissions were not significantly different from zero at 95% CI for all three compounds (chokol K, −4.8% to 13.53%; MME, 4.5% to 5.40%; MTE, −0.1% to 0.22%) suggesting that volatile production by a genotype is consistent and not affected by seasonal variations.

Headspace samples collected from axenic *Epichloë* cultures *in vitro* were screened for the three volatile compounds. Of these compounds, only chokol K was detected, but in small amounts and not in all isolates (Table 2). Chokol K was found in cultures of *E. sylvatica* (two isolates), *E. clarkii* (two isolates), and in most *E. typhina* associations (one to two isolates), but not in cultures of *E. bromicola* (two isolates), *E. baconii* (two isolates), and *E. festucae* (two isolates). Generally, genotypes that produced high levels of chokol K *in vivo* also produced chokol K *in vitro*. In addition, many isolates that

Table 2 Mean relative amounts (%) with standard errors (±SE) of the volatile compounds chokol K, methyl (*Z*)-3-methyldodec-2-enoate (*MME*), methyl 2,4,8-trimethylundecanoate (*MTE*), and unidentified diterpenes found in the headspace collected from stromata of different *Epichloë* associations (*in vivo*) and from isolates in pure culture (*in vitro*)

Association[a]	*In vivo*			*In vitro*	
	Chokol K Mean ± SE	MME Mean ± SE	MTE Mean ± SE	Chokol K Mean ± SE	Diterpenes Mean ± SE
E. bromicola–Be	0.17±0.05	0.29±0.07	0.03±0.02	0.00	0.00
E. baconii (all)	0.52±0.25	2.03±0.88	1.33±0.93	0.00	19.24±10.06
Eba–At	0.45±0.29	3.52±1.01	2.33±1.51	0.00	0.00
Eba–Cv	0.63±0.52	0.06±0.06	0.00	0.00	38.49±15.00
E. clarkii–Hl	23.28±6.08	0.59±0.21	0.26±0.11	0.18±0.13	10.85±8.86
E. festucae–Fr	0.00	38.07±3.62	0.00	0.00	0.00
E. sylvatica–Bs	56.21±6.79	0.81±0.45	0.20±0.19	0.03±0.02	1.39±0.39
E. typhina (all)	10.20±2.94	1.47±0.30	2.67±1.84	0.02±0.11	13.05±3.98
Et–Ao	29.35±6.89	1.87±1.69	0.50±0.02	1.04±0.90	0.00
Et–Bp	35.54±11.18	1.59±0.57	0.02±0.02	0.06±0.06	12.66±9.68
Et–Dg	1.06±0.67	1.97±0.48	0.13±0.09	0.03±0.03	54.73±28.41
Et–Pd	0.23±0.23	0.00	57.29±19.88	0.12±0.08	19.16±6.68
Et–Php	45.78±1.70	0.00	4.19±0.91	0.00	7.31±6.21
Et–Pn	0.53±0.27	0.48±0.22	0.02±0.01	0.00	0.00
Et–Pp	0.15±0.02	0.31±0.09	0.30±0.14	0.32±0.20	0.00
Et–Pt	51.72±7.44	0.14±0.14	1.26±0.39	0.19±0.19	8.20±5.01

[a] For host abbreviations, see Table 1.

were found to produce no or only little chokol K under *in vitro* conditions produced a range of diterpenes that were not detected in headspace samples from stromata *in vivo*, notably, *E. baconii* from *Calamagrostis villosa* and *E. typhina* from *D. glomerata* (Table 2). Others produced neither chokol K nor diterpenes. These diterpenes are yet unidentified and are referred to in this study as unknown diterpenes (W. Boland, Jena, personal communication).

Phylogenetic Analysis The ML tree attempts to link the phylogeny of *Epichloë* species with observed odor profiles (Fig. 4). Following Craven et al. (2001), *Epichloë* species are grouped into two major clades: the "main group of *Epichloë* species" including *E. bromicola*, *E. baconii*, and *E. festucae* (at the base of the phylogram) and the "*E. typhina* complex" including *E. typhina* (with its host races), *E. clarkii*, and *E. sylvatica*. Here, we designate the well-supported, less basal subclade (88% bootstrap support) within the *E. typhina* complex as "*E. typhina* top clade" excluding the more basal *Et–Pn* and *Et–Pp* associations.

High levels of chokol K with a proportion of more than 10% in the headspace (levels 3 and 4) were found only in *Epichloë* accessions that were placed in the *E. typhina* top clade (Fig. 4). An exception was the accessions of *E. typhina* infecting *Puccinellia distans* from Poland (*Et–Pd*), whose profiles were dominated by MTE. All other accessions included in the more basal clades produced low amounts or no chokol K (levels 0 and 1). Regarding

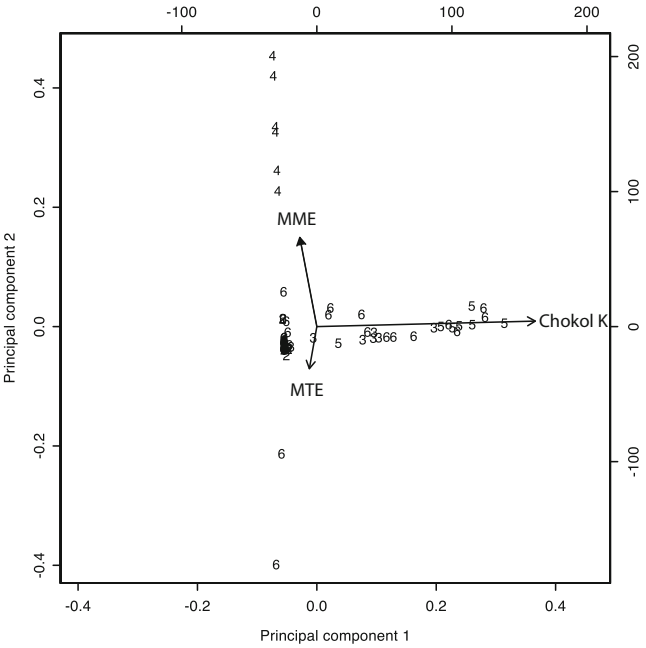

Fig. 3 Principal component analysis of odor compounds chokol K, methyl (*Z*)-3-methyldodec-2-enoate (*MME*), and methyl 2,4,8-trimethylundecanoate (*MTE*) found in headspace samples from stromata of different *Epichloë* species (1 *E. baconii*; 2 *E. bromicola*; 3 *E. clarkii*; *4 E. festucae*; *5 E. sylvatica*; *6 E. typhina*). The first two principal component analysis (*PCA*) factor loadings of the 93 analyzed accessions (genotypes) explain 73% of the total variance. *Correlation arrows* give the direction in which the relative amounts of odor compounds from particular genotypes increase. The *angles between the arrows* correspond to the correlation of the odor variables among each other, while the *arrow lengths* indicate how well a particular compound is represented in the dataset

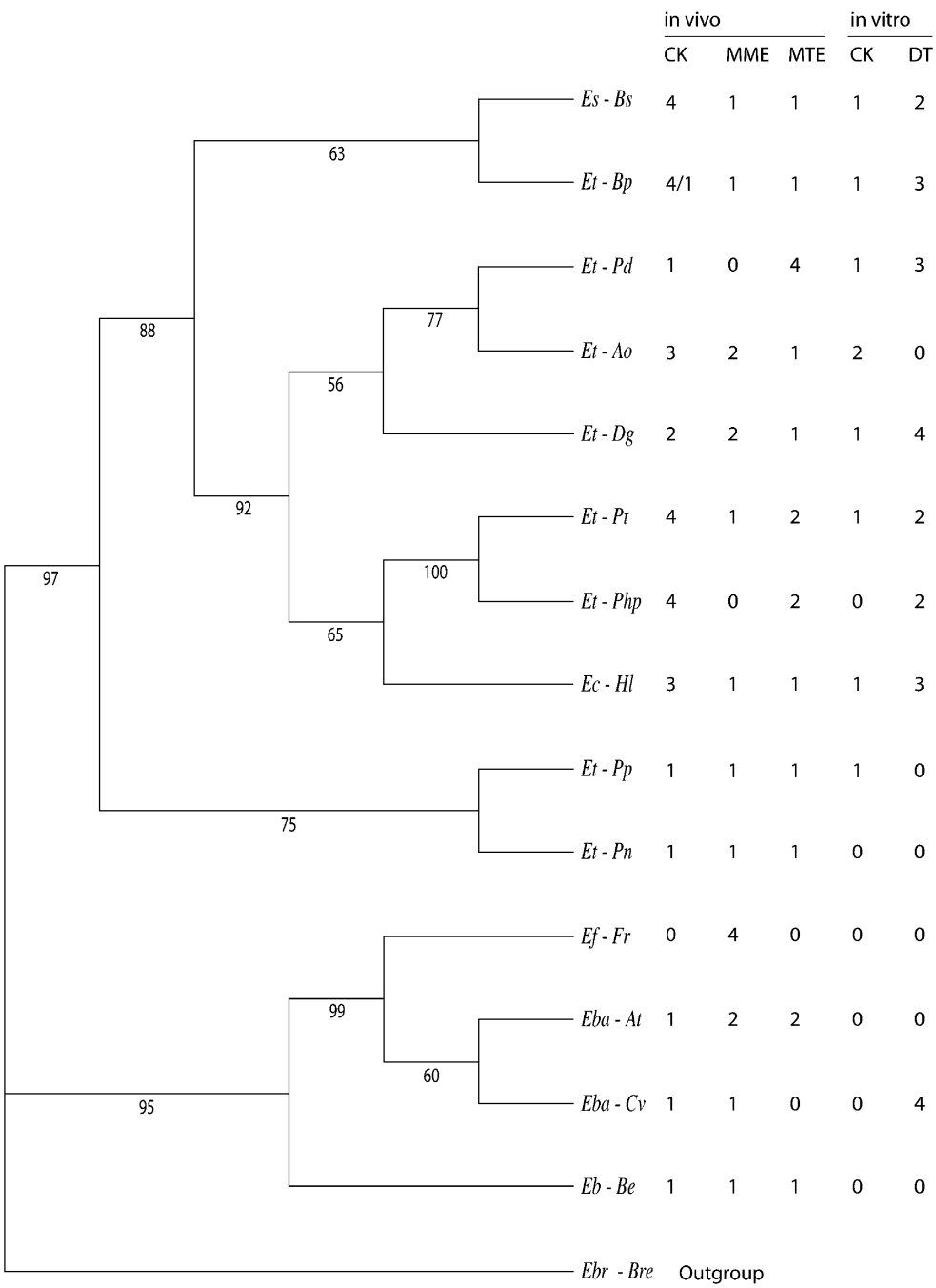

Fig. 4 Maximum likelihood tree (-ln 1600.19) based on fungal *tub2* sequences generated by PAUP*4.0b10 and using the K80+G model of evolution. *Numbers below the branches* are posterior probabilities (based on 1,000 bootstrap replications). Tabular figures at terminal positions indicate the relative abundance (*0* absent; *1* <1%; *2* 1–10%; *3* 10–30%; *4* 30–70%; *5* >70%) of odor compounds [*CK* chokol K; *MME* methyl (*Z*)-3-methyldodec-2-enoate; *MTE* methyl 2,4,8-trimethylundecanoate; *DT* unknown diterpenes] from *in vivo* and *in vitro* measurements. The tree is rooted with the outgroup taxon *E. brachyelytri* from *Brachyelytrum erectum* (*Ebr–Bre*). Other species and grass host abbreviations are as in Table 1

MME, low and moderate levels (0–2) were found in species of all major clades with one exception. The odor profile of *E. festucae* of the *Epichloë* main group was characterized by a high level of MME (level 4) as the dominant volatile. Similarly, the third volatile MTE was present in all major clades at low to moderate levels (0–2) with the exception of association *Et–Pd* mentioned above. The unknown diterpenes (DT) produced *in vitro* were present only in species of the top clade, again with one exception (*Eba–Cv* of the main group) and thus mostly occurred in parallel with high levels of chokol K *in vivo*.

Discussion

Evolution of odor profiles in *Epichloë* fungi may be complex, since host grass and fungal genotypes, the gamete transferring *Botanophila* flies, and even *Epichloë* mycoparasites may influence odor evolution. *Epichloë* species and host races examined in this study exhibited considerable variation in profiles of three volatile compounds, chokol K, MME, and MTE, that have a confirmed or likely role in odor communication. However, profiles overlapped among species, and only few associations exhibited distinct

profiles. Moreover, there was substantial variation within species, particularly in *E. typhina*, that naturally infects a range of different host species.

What is the Basis of Odor Variation? The plant material used in our study was from vegetatively propagated accessions, each representing an individual genotypic combination of a host and its systemic endophyte. Among naturally occurring associations, there is a high degree of host specificity, with many *Epichloë* species apparently forming host races or cryptic species on different hosts (Schardl et al. 2007; Zabalgogeazcoa et al. 2008). Observed variations in odor profiles among and within species may, therefore, be attributed to these more or less stable associations. However, with the sampling scheme used in this study, it is not possible to differentiate between host and fungus contributions to odor variation in *Epichloë* species that infect multiple hosts, such as *E. typhina*. To answer this question, a particular endophyte genotype would have to be introduced into different host species experimentally. In closely related *Neotyphodium* endophytes, investigations on the genetic control of toxic alkaloid secondary metabolites have been made by using experimental infections or reciprocal crosses between infected host plants. These investigations suggested that the ability to produce alkaloids is a property of the fungal genotype, but that the levels of fungal alkaloids may be modified to some extent by plant genotype (Hill et al. 1991; Roylance et al. 1994; Easton et al. 2002), while the influence of environmental factors is usually small (Agee and Hill 1994). In another study that compared endophyte haplotypes in different host genotypes, the host grass significantly influenced alkaloid production (Faeth et al. 2002). In our study, levels of chokol K and MME collected from the same host–fungus associations in different years remained almost constant between years, suggesting that there is a genetic basis for the observed odor variation and that environmental factors may play a minor role.

In vitro studies may be useful to dissect fungus and plant influences on production of odor compounds. In the present study, only chokol K, but not MME and MTE, were produced under *in vitro* conditions (Table 2). These findings confirm results from a previous study, which suggested that chokol K production was genetically encoded in *Epichloë*, but that for the methylester MME precursory substances of plant origin may be needed (Steinebrunner et al. 2008b). Alternatively, plant-specific signaling may be required for expression of genes involved in the biosynthetic pathway of MME and MTE, as has been shown for the endophyte produced secondary metabolite peramine that protects host plants from insect herbivory (Tanaka et al. 2005) and for an indole-diterpene mycotoxin of *Neotyphodium lolii* (Young et al. 2006). Moreover, in pure culture when host influences are eliminated, chokol K was detected only in species of the *E. typhina* complex but was not produced by other *Epichloë* species of the main clade, suggesting that species depend differently upon plant signaling.

Phylogenetic Analysis Mapping of odor compounds into the corresponding phylogenetic tree of *Epichloë* taxa did not show a strong phylogenetic signal of odor profiles. However, there was a tendency for *Epichloë*–grass associations in the *E. typhina* top clade to produce high levels of chokol K (Fig. 4), suggesting that chokol K production was favored early in the speciation of *E. typhina*. However, weak and strong chokol K producing genotypes of *E. typhina* were sometimes found within the same association, notably *E. typhina* on *B. pinnatum*, while for association *Et–Pd*, only low levels of chokol K were observed. A phylogeny based on odor alone showed a different tree topology, although it was poorly resolved (data not shown). Incongruence between odor phenotypes and the genetic relationship of the producer have been reported for various plants (Williams and Whitten 1999; Barkman 2001; Levin et al. 2003). It has been argued that environmental variation, adaptation to pollinators, and biosynthetic interrelation of compounds would make it difficult to use odor as a character for phylogenetic inference (Levin et al. 2003). Our study did not put the emphasis on validating odor characters for phylogenetic tree reconstruction but on combining DNA sequence and odor information to locate possible evolutionary key events in the phylogeny of *Epichloë*. Such an event may have been the ability to produce high amounts of chokol K, which occurred at the base of the *E. typhina* top clade after branching from the more basal *E. typhina* host races (*Et–Pp*, *Et–Pn*). Chokol K appears to play a role in the attraction of gamete transferring *Botanophila* flies (Schiestl et al. 2006; Steinebrunner et al. 2008a), and its increased production by *E. typhina* may have favored host range expansion and evolution of new host races.

Variability of Odor Profiles within and among Species Although there was some structuring with regard to compound levels, odor profiles based on chokol K, MME, and MTE mostly overlapped among species (Fig. 3). Exceptions were the profile of *E. festucae* that was characterized by the dominance of methyl ester MME and the profile of the *Et–Pd* association that showed a high level of MTE instead of MME. Thus, *Epichloë* taxa appear to be little differentiated at the species level with respect to odor profiles, but high variability was observed among genotypes, particularly in associations with *E. typhina*. Several studies have assessed variability of flower odors among plant species and investigated the use of odor profiles for

drawing species boundaries (Azuma et al. 1997; Williams and Whitten 1999; Levin et al. 2003; Raguso et al. 2003; Huber et al. 2005). While some of these studies found that each species had its own unique profile (Levin et al. 2003; Huber et al. 2005), others reported a high degree of homoplasy (Williams and Whitten 1999).

Odor emission by *Epichloë* stromata is thought to be important for the attraction of gamete-transferring *Botanophila* flies, and thus, patterns or levels of compounds may be selectively adaptive. In a trapping study that used synthetic versions of chokol K and MME, attraction of *Botanophila* flies was increased with increasing amounts of these compounds (Steinebrunner et al. 2008a), suggesting that high-odor emission would be advantageous. However, different, taxonomically closely related *Botanophila* species showed no preference for specific blends that represented different *Epichloë* taxa, notably *E. clarkii* with high levels of chokol K and *E. festucae* with high levels of MME.

The variability in odor profiles observed among genotypes of *E. typhina* from different accessions may be viewed in the light of Thompson's geographical mosaic theory. This theory predicts that geographic differences in interactions among local selection pressures, genetic drift, and migration create a range of possible outcomes (Thompson 1999). For example, the distinct MTE dominated odor profile in the *Et–Pd* association from Poland may be the result of different selection pressures imposed by the local environment, the *Botanophila* gametic vectors, or the host plant. *Puccinellia distans* is a halophyte naturally occurring on marine and inland salines, but infected plants examined in this study originated from highly disturbed anthropogenic habitats in the vicinity of an industrial plant (Lembicz 1998; Olejniczak and Lembicz 2007). Thus, migration of an *Et–Pd* association into this unusual environment and the interaction with the locally occurring *Botanophila* flies may have resulted in the distinct profile observed for these genotypes. Unfortunately, this hypothesis could not be confirmed because stroma-forming plants of *Puccinellia* are rare outside the industrial sites and were not available. However, local adaptation of the asexual *Neotyphodium* stage in terms of alkaloid production or herbivore defense has been documented in *Achnatherum robustum* (Faeth et al. 2006) and in a subarctic fescue (Koh and Hik 2007), suggesting that local adaptation may be a common occurrence in endophyte/grass symbiota.

Chokol K is not only involved in odor communication with *Botanophila* flies but, based on its antifungal properties, may also play a role in protecting *Epichloë* stromata from mycoparasites (Schiestl et al. 2006; Steinebrunner et al. 2008b). High levels of chokol K typically found in associations of the *E. typhina* top clade may have also been selectively favored under circumstances of intensive mycoparasite pressure. However, it is not known whether *E. typhina* associations are exposed to such pressure more often compared to the other species and whether mycoparasite resistance is adaptive.

Finally, differences at the *Epichloë* population level may leave a signature on odor profiles. Depending on whether populations are large or small, selection pressures on volatile production may be enhanced or relaxed. Thus, in small disjunctive populations, *Botanophila* flies will have to be attracted over a wider distance to guarantee outcrossing, which may select for increased odor production. Moreover, population size differences among species and locations could facilitate genetic drift.

In summary, profiles of three odor compounds produced by *Epichloë* stromata were often distinctive for particular grass–*Epichloë* associations but did not discriminate at the species level, except for *E. festucae*. Although there was some hierarchical structuring regarding levels of chokol K emission based on the *Epichloë* phylogeny, odor profiles appeared to be largely dependent on particular *Epichloë*–host associations. The observed profiles may be the result of complex selective pressures imposed by *Botanophila* gametic vectors, local environment, and mycoparasites. Future studies should focus on dissecting genetic and environmental factors responsible for the production of odor compounds and on testing host and *Epichloë* genotype contributions with experimentally manipulated associations by using cross-infections.

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References

AGEE, C. S., and HILL, N. S. 1994. Ergovaline variability in *Acremonium*-infected tall fescue due to environment and plant genotype. *Crop Sci.* 34:221–226.

AZUMA, H., TOYOTA, M., ASAKAWA, Y., YAMAOKA, R., GARCIA-FRANCO, J. G., DIERINGER, G., THIEN, L. B., and KAWANO, S. 1997. Chemical divergence in floral scents of *Magnolia* and allied genera (Magnoliaceae). *Plant Spec. Biol.* 12:69–83.

BARKMAN, T. J. 2001. Character coding of secondary chemical variation for use in phylogenetic analyses. *Biochem. Syst. Ecol.* 29:1–20.

BULTMAN, T. L., and LEUCHTMANN, A. 2003. A test of host specialization by insect vector as a mechanism for reproductive isolation among entomophilous fungal species. *Oikos.* 103:681–687.

BULTMAN, T. L., WHITE, J. F., BOWDISH, T. I., WELCH, A. M., and JOHNSTON, J. 1995. Mutualistic transfer of *Epichloë*-spermatia by *Phorbia* flies. *Mycologia.* 87:182–189.

BULTMAN, T. L., WHITE, J. F., BOWDISH, T. I., and WELCH, A. M. 1998. A new kind of mutualism between fungi and insects. *Mycol. Res.* 102:235–238.

BULTMAN, T. L., WELCH, A. M., BONING, R. A., and BOWDISH, T. I. 2000. The cost of mutualism in a fly–fungus interaction. *Oecologia.* 124:85–90.

CRAVEN, K. D., HSIAU, P. T. W., LEUCHTMANN, A., HOLLIN, W., and SCHARDL, C. L. 2001. Multigene phylogeny of *Epichloë* species, fungal symbionts of grasses. *Ann. Missouri Bot. Gard.* 88:14–34.

DUDAREVA, N., NEGRE, F., NAGEGOWDA, D. A., and ORLOVA, I. 2006. Plant volatiles: Recent advances and future perspectives. *Crit. Rev. Plant Sci.* 25:417–440.

EASTON, H. S., LATCH, G. C. M., TAPPER, B. A., and BALL, O. J. P. 2002. Ryegrass host genetic control of concentrations of endophyte-derived alkaloids. *Crop Sci.* 42:51–57.

FAETH, S. H., BUSH, L. P., and SULLIVAN, T. J. 2002. Peramine alkaloid variation in Neotyphodium-infected Arizona fescue: Effects of endophyte and host genotype and environment. *J. Chem. Ecol.* 28:1511–1526.

FAETH, S. H., GARDNER, D. R., HAYES, C. J., JANI, A., WITTLINGER, S. K., and JONES, T. A. 2006. Temporal and spatial variation in alkaloid levels in *Achnatherum robustum*, a native grass infected with the endophyte *Neotyphodium*. *J. Chem. Ecol.* 32:307–324.

GRISON-PIGE, L., HOSSAERT-MCKEY, M., GREEFF, J. M., and BESSIERE, J. M. 2002. Fig volatile compounds—a first comparative study. *Phytochemistry.* 61:61–71.

GUEVARA, R., RAYNER, A. D. M., and REYNOLDS, S. E. 2000. Orientation of specialist and generalist fungivorous ciid beetles to host and non-host odors. *Physiol. Entomol.* 25:288–295.

HEDLUND, K., BENGTSSON, G., and RUNDGREN, S. 1995. Fungal odor discrimination in two sympatric species of fungivorous collembolans. *Funct. Ecol.* 9:869–875.

HILL, N. S., PARROTT, W. A., and POPE, D. D. 1991. Ergopeptine alkaloid production by endophytes in a common tall fescue genotype. *Crop Sci.* 31:1545–1547.

HUBER, F. K., KAISER, R., SAUTER, W., and SCHIESTL, F. P. 2005. Floral scent emission and pollinator attraction in two species of *Gymnadenia* (Orchidaceae). *Oecologia.* 142:564–575.

KNUDSEN, J. T. 2002. Variation in floral scent composition within and between populations of *Geonoma macrostachys* (Arecaceae) in the western Amazon. *Am. J. Bot.* 89:1772–1778.

KNUDSEN, J. T., ERIKSSON, R., GERSHENZON, J., and STAHL, B. 2006. Diversity and distribution of floral scent. *Bot. Rev.* 72:1–120.

KOH, S., and HIK, D. S. 2007. Herbivory mediates grass–endophyte relationships. *Ecology.* 88:2752–2757.

KOSHINO, H., YOSHIHARA, T., TOGIYA, S., TERADA, S., TSUKADA, S., OKUNO, M., NOGUCHI, A., SAKAMURA, S., ICHIHARA, A., SHIMANUKI, T., TAJIMI, A., and SATO, T. 1989. Antifungal compounds from stromata of *Epichloë typhina* on *Phleum pratense*, pp. 244–251. Proceedings 31. Symposium on the Chemistry of Natural Products. Sapporo, Japan.

LEMBICZ, M. 1998. Life history of *Puccinellia distans* (L.) Parl. (Poaceae) in the colonisation of anthropogenic habitats. *Phytocoenosis (N.S.).* 10:1–32.

LEUCHTMANN, A. 2003. Taxonomy and diversity of Epichloë endophytes, pp. 169–194, in J. F. White Jr, C. W. Bacon, N. L. Hywel-Jones, and J. W. Spatafora (eds.). Clavicipitalean FungiMarcel Dekker, New York.

LEUCHTMANN, A. 2007. *Botanophila* flies on *Epichloë* host species in Europe and North America: no evidence for co-evolution. *Entomol. Exp. Appl.* 123:13–23.

LEVIN, R. A., RAGUSO, R. A., and MCDADE, L. A. 2001. Fragrance chemistry and pollinator affinities in Nyctaginaceae. *Phytochemistry.* 58:429–440.

LEVIN, R. A., MCDADE, L. A., and RAGUSO, R. A. 2003. The systematic utility of floral and vegetative fragrance in two genera of Nyctaginaceae. *Syst. Biol.* 52:334–351.

OLEJNICZAK, P., and LEMBICZ, M. 2007. Age-specific response of the grass *Puccinellia distans* to the presence of a fungal endophyte. *Oecologia.* 152:485–494.

PELLMYR, O., and THIEN, L. B. 1986. Insect reproduction and floral fragrances—keys to the evolution of the angiosperms. *Taxon.* 35:76–85.

PLEPYS, D., IBARRA, F., FRANCKE, W., and LOFSTEDT, C. 2002. Odor-mediated nectar foraging in the silver Y moth, *Autographa gamma* (Lepidoptera: Noctuidae): Behavioural and electrophysiological responses to floral volatiles. *Oikos.* 99:75–82.

POSADA, D., and CRANDALL, K. A. 1998. MODELTEST: Testing the model of DNA substitution. *Bioinformatics.* 14:817–818.

R Development Core Team. 2005. R: A Language and Environment for Statistical Computing. Vienna.

RAGUSO, R. A., and ROY, B. A. 1998. 'Floral' scent production by *Puccinia* rust fungi that mimic flowers. *Mol. Ecol.* 7:1127–1136.

RAGUSO, R. A., LEVIN, R. A., FOOSE, S. E., HOLMBERG, M. W., and MCDADE, L. A. 2003. Fragrance chemistry, nocturnal rhythms and pollination "syndromes" in *Nicotiana*. *Phytochemistry.* 63: 265–284.

RAO, S., and BAUMANN, D. 2004. The interaction of a *Botanophila* fly species with an exotic *Epichloë* fungus in a cultivated grass: fungivore or mutualist? *Entomol. Exp. Appl.* 112:99–105.

ROYLANCE, J. T., HILL, N. S., and AGEE, C. S. 1994. Ergovaline and peramine production in endophyte-infected tall fescue: Independent regulation and effects of plant and endophyte genotype. *J. Chem. Ecol.* 20:2171–2183.

SALZMANN, C. C., BROWN, A., and SCHIESTL, F. P. 2006. Floral scent emission and pollination syndromes: Evolutionary changes from food to sexual deception. *Int. J. Plant Sci.* 167:1197–1204.

SCHARDL, C. L., LEUCHTMANN, A., CHUNG, K. R., PENNY, D., and SIEGEL, M. R. 1997. Coevolution by common descent of fungal symbionts (*Epichloë* spp.) and grass hosts. *Mol. Biol. Evol.* 14: 133–143.

SCHARDL, C. L., LEUCHTMANN, A., and MCDONALD, B. A. 2007. Relationships of *Epichloë* typhina isolates from different host grasses, pp. 451–455, in A. J. Popay, and E. R. Thom (eds.). Proceedings of the 6th International Symposium on Fungal Endophytes of GrassesNew Zealand Grassland Association (Inc.), Dunedin, New Zealand.

SCHIESTL, F. P., STEINEBRUNNER, F., SCHULZ, C., VON REUSS, S., FRANCKE, W., WEYMUTH, C., and LEUCHTMANN, A. 2006. Evolution of 'pollinator'-attracting signals in fungi. *Biol. Lett.* 2:401–404.

SCHOMBURG, G. 1990. Gas Chromatography: A Practical Course. Wiley-VCH, Weinheim, New York.

STEINEBRUNNER, F., TWELE, R., FRANCKE, W., LEUCHTMANN, A., and SCHIESTL, F. P. 2008a. Role of odor compounds in the attraction of gamete vectors in endophytic *Epichloë* fungi. New Phytol. 178:401.

STEINEBRUNNER, F., SCHIESTL, F. P., and LEUCHTMANN, A. 2008b. Ecological role of volatiles produced by *Epichloë*: differences in antifungal toxicity. FEMS Microbiol. Ecol. 64:307–316.

SWOFFORD, D. L. 2003. PAUP: Phylogenetic Analysis Using Parsimony and Other Methods. Sinauer, Sunderland, Massachusetts.

TANAKA, A., TAPPER, B. A., POPAY, A., PARKER, E. J., and SCOTT, B. 2005. A symbiosis expressed non-ribosomal peptide synthetase from a mutualistic fungal endophyte of perennial ryegrass confers protection to the symbiotum from insect herbivory. *Mol. Microbiol.* 57:1036–1050.

THOMPSON, J. N. 1999. Specific hypotheses on the geographic mosaic of coevolution. *Am. Nat.* 153:S1–S14.

WILLIAMS, N. H., and WHITTEN, W. M. 1999. Molecular phylogeny and floral fragrances of male euglossine bee-pollinated orchids: A study of *Stanhopea* (Orchidaceae). *Plant Spec. Biol.* 14:129–136.

YOUNG, C. A., FELITTI, S., SHIELDS, K., SPANGENBERG, G., JOHNSON, R. D., BRYAN, G. T., SAIKIA, S., and SCOTT, B. 2006. A complex gene cluster for indole-diterpene biosynthesis in the grass endophyte *Neotyphodium lolii*. *Fungal Genet. Biol.* 43:679–693.

ZABALGOGEAZCOA, I., GARCÍA CIUDAD, A., LEUCHTMANN, A., VÁZQUEZ DE ALDANA, B. R., and GARCÍA CRIADO, B. 2008. Effects of choke disease in the grass *Brachypodium phoenicoides*. Plant Pathol. published online DOI 10.1111/j.1365–3059.2007. 01784.x.

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Tritrophic Effects of Xanthotoxin on the Polyembryonic Parasitoid *Copidosoma sosares* (Hymenoptera: Encyrtidae)

Evan C. Lampert · Arthur R. Zangerl · May R. Berenbaum · Paul J. Ode

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Abstract Plant chemistry can have deleterious effects on insect parasitoids, which include the reduction in body size, increased development time, and increased mortality. We examined the effects of xanthotoxin, a linear furanocoumarin, on the polyembryonic encyrtid wasp *Copidosoma sosares*, a specialist parasitoid that attacks the parsnip webworm, *Depressaria pastinacella*, itself a specialist on furanocoumarin-producing plants. Furanocoumarins, allelochemicals abundant in the Apiaceae and Rutaceae, are toxic to a wide range of herbivores. In this study, we reared parasitized webworms on artificial diets containing no xanthotoxin (control) or low or high concentrations of xanthotoxin. Clutch sizes of both male and female *C. sosares* broods were more than 20% smaller when they developed in hosts fed the diet containing high concentrations of xanthotoxin. Xanthotoxin concentration in the artificial diet had no effect on the development time of *C. sosares*, nor did it have an effect on the body size (length of hind tibia) of individual adult male and female *C. sosares* in single-sex broods. Webworms fed artificial diets containing low or high concentrations of xanthotoxin were not significantly smaller, and their development time was similar to that of webworms fed a xanthotoxin-free diet. Mortality of webworms was not affected by xanthotoxin in their artificial diet. Therefore, dietary xanthotoxin did not appear to affect *C. sosares* via impairment of host health. However, unmetabolized xanthotoxin was found in *D. pastinacella* hemolymph where *C. sosares* embryos develop. Hemolymph concentrations were fourfold greater in webworms fed the high-xanthotoxin-containing diet than in webworms fed the low-xanthotoxin-containing diet. We failed to detect any xanthotoxin metabolism by either *C. sosares* embryos or precocious larvae. Therefore, the observed tritrophic effects of xanthotoxin are likely to be due to the effects of xanthotoxin after direct contact in the hemolymph rather than to the effects of compromised host quality.

Keywords Plant chemistry · Xanthotoxin · Tritrophic interactions · Fitness · Cytochrome P450 detoxification · *Depressaria pastinacella* · Furanocoumarin

E. C. Lampert · P. J. Ode (✉)
Department of Entomology, North Dakota State University,
Fargo, ND 58105, USA
e-mail: paul.ode@ndsu.edu

A. R. Zangerl · M. R. Berenbaum
Department of Entomology,
University of Illinois at Urbana–Champaign,
Urbana, IL 61801, USA

Present address:
E. C. Lampert
Department of Ecology and Evolutionary Biology,
University of Colorado at Boulder,
Boulder, CO 80309, USA

Introduction

The development of insect parasitoids is often influenced by the diet of their hosts. When hosts are herbivorous insects, plant defensive chemistry can adversely affect the fitness of third and sometimes higher (e.g., Harvey et al. 2003) trophic levels (Ode 2006). The diet of the host insect may affect developing parasitoids indirectly by altering host suitability or directly by exposing the parasitoid to unmetabolized defensive chemicals.

Numerous studies suggest negative associations between plant chemistry and/or plant species identity and parasitoid fitness measures in the field (reviewed in Ode 2006).

Whereas correlative studies are relatively numerous, few studies have explicitly demonstrated that plant chemistry is responsible for the oft-observed negative correlations between levels of plant defensive chemistry and parasitoid fitness proxies (Ode 2006). A handful of investigators have used artificial diets, to which known quantities of specific plant allelochemicals were added, to establish a cause-and-effect relationship between plant chemistry and parasitoid fitness. For instance, the ichneumonid *Hyposoter exigua* Viereck suffered morphological deformities, increased mortality and development time, and decreased body size and longevity when reared from the noctuid *Helicoverpa zea* (Boddie) larvae feeding on diets containing high concentrations of the alkaloid α-tomatine (Campbell and Duffey 1979, 1981). Another series of studies documented the negative effects of dietary nicotine supplements on the generalist parasitoid *Hyposoter annulipes* (Cresson) and the specialist braconid parasitoid *Cotesia congregata* (Say) (Thurston and Fox 1972; Barbosa et al. 1986, 1991; El-Heneidy et al. 1988).

In this study, we used artificial diets to explore the tritrophic effects of the furanocoumarin xanthotoxin on several fitness correlates of the polyembryonic wasp, *Copidosoma sosares* (Walker) (Hymenoptera: Encyrtidae). Like all copidosomatine encyrtids (Strand et al. 1991), *C. sosares* is a polyembryonic egg-larval parasitoid (Hardy 1996; Ode et al. 2004; Guerrieri and Noyes 2005). *C. sosares* produces three types of broods: all male, all female, and mixed sex. All-male and all-female broods result from a single unfertilized or fertilized egg, respectively; mixed-sex broods develop from one egg of each sex (Hardy 1996; Ode et al. 2004). As the host continues its larval development, *C. sosares* eggs proliferate clonally, resulting in 100 to 400 genetically identical offspring (Ode et al. 2004). The vast majority of embryos undergo morphogenesis during the host's last instar, completely consuming the host except for the exoskeleton, thus forming a "mummy" (Hardy 1996). As the mummy is formed, the *C. sosares* larvae begin to pupate. Typically, adult wasps emerge 10–12 d later. One or two embryos develop early (during the earlier host instars) into "precocious" or "soldier" larvae (Hardy 1996). In the well-studied congener *C. floridanum* (Ashmead), soldier larvae are implicated in defending reproductive individuals against multiparasitoids (Harvey et al. 2000) and superparasitoids (Giron et al. 2004) as well as in mediating conflicts over sex ratio in mixed-sex broods (Grbić et al. 1992).

C. sosares is a specialist parasitoid of the parsnip webworm, *Depressaria pastinacella* (Duponchel) (Lepidoptera: Elachistidae). The parsnip webworm is a specialist herbivore that feeds on reproductive structures of plants in the genera *Pastinaca* and *Heracleum* (Apiaceae), both of which are rich in furanocoumarins (Berenbaum and Zangerl 2006). Field studies conducted in western Europe, where this system is native, showed that two furanocoumarins are negatively associated with fitness correlates of *C. sosares* (Ode et al. 2004). Webworms feeding on host plants containing higher levels of isopimpinellin were less likely to be successfully parasitized by *C. sosares*; survivorship as well as clutch size of *C. sosares* broods were reduced when developing in webworms that fed on plants containing higher xanthotoxin levels.

Furanocoumarins are toxic to a wide range of herbivores (Berenbaum 1990; Berenbaum and Zangerl 1996). In addition to tritrophic effects observed in the field (Ode et al. 2004), laboratory studies that use artificial diets demonstrate tritrophic effects on two other insect parasitoids. Survivorship of *C. floridanum* decreased when their generalist hosts *Trichoplusia ni* (Hübner) (Lepidoptera: Noctuidae) fed on an artificial diet with high concentrations of a mixture of three furanocoumarins: bergapten, psoralen, and xanthotoxin (Reitz and Trumble 1996). Similarly, *Archytas marmoratus* (Townsend) (Diptera: Tachinidae) experienced reduced survivorship when its host, *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae), was reared on an artificial diet with the same high-concentration mixture (Reitz and Trumble 1997).

The observed tritrophic effects of furanocoumarins on *C. sosares* may be the result of compromised webworm quality if the costs of detoxification or excretion are sufficient to reduce the nutritional quality or size of the webworm. Alternatively or in addition, some furanocoumarins may pass, unmetabolized, into the hemolymph of the webworm where developing *C. sosares* embryos would directly encounter these potentially toxic compounds. Webworms fed an artificial diet containing 0.3% fresh weight xanthotoxin had trace amounts of xanthotoxin in their hemolymph, and webworms feeding on *P. sativa* fruit had trace amounts of six furanocoumarins in their hemolymph. These findings suggest that immature *C. sosares* do encounter these compounds (McGovern et al. 2006). Moreover, metabolism assays failed to show any ability of *C. sosares* larva to metabolize xanthotoxin (McGovern et al. 2006).

We manipulated the amount of xanthotoxin present in artificial diets fed to parasitized webworms to establish the effects of xanthotoxin on *C. sosares*. Given the negative correlations between xanthotoxin and *C. sosares* fitness proxies observed in the field, we also measured the amount of unmetabolized xanthotoxin present in the webworm hemolymph. Although *C. sosares* larvae apparently are incapable of metabolizing xanthotoxin (McGovern et al. 2006), they spend a majority of their developmental time as polygerms (embryos) or precocious larvae. Host larvae continue to feed as the *C. sosares* polygerm proliferates, potentially exposing developing *C. sosares* directly to chemicals from the host's diet. Precocious larvae could

also potentially detoxify furanocoumarins that their reproductively destined clone mates would otherwise encounter. Therefore, we examined the ability of *C. sosares* embryos and precocious larvae to metabolize xanthotoxin.

Methods and Materials

Effect of Xanthotoxin on C. sosares Approximately 200 adult female and male *C. sosares* were collected as they emerged from 100 field-collected mummies from 45 cow-parsnip (*Heracleum lanatum* Michx.) plants in July 2005 from two sites (N 44°05′, W 103°38′ and N 44°25′, W 103° 53′) 44.3 km apart in the in the Black Hills National Forest. Males and females were allowed to mate and placed at 5°C and a 8:16-hr light/dark photoperiod for 4 mo to break the reproductive diapause. After this period, *C. sosares* females were placed individually into 100-mm-diameter plastic Petri dishes for 24 hr and allowed to oviposit in freshly laid webworm eggs on parsnip leaves. Neonate parasitized webworms hatched 6–7 d later. Approximately 100 additional mated adult females were collected with a handheld aspirator from the same sites in the Black Hills National Forest on 18 June 2006. Females were transported back to the laboratory where they were allowed to oviposit in freshly laid webworm eggs as described above. Webworms collected from *H. lanatum* in the Black Hills National Forest were the source of the host eggs used in both years. Once webworm eggs hatched, a trimmed paintbrush was used to place individual neonate larvae randomly into 44-ml diet cups (Solo®). Diet cups contained ~15 ml of semidefined artificial either with no xanthotoxin (control), a low (0.313 ng/μg fresh weight diet), or a high concentration of xanthotoxin (4.71 ng/μg fresh weight). These concentrations reflect the range of xanthotoxin concentrations observed in our field collections of *H. lanatum* seed material throughout the western USA (unpublished data). Furanocoumarins were dissolved in 50–150 ml acetone. α-Cellulose was placed in the furanocoumarin–acetone mixture and left in a fume hood 1–2 d to dry completely before incorporation into the artificial diet (Nitao and Berenbaum 1988). Parasitized webworms were allowed to develop in a growth chamber set to 27°C and a 16:8-hr light/dark photoperiod.

Cups containing parasitized webworms were checked daily, and the dates at which mummies were formed were recorded. Mummies were placed individually into 18× 180-mm glass tubes stopped with cloth plugs and returned to the growth chamber. They were checked daily until adult wasps emerged, whereupon the date was recorded, and the wasps were frozen at −20°C until clutch size and body size could be measured. The number of emerged adult wasps of each sex was counted, as were the numbers of unemerged adults, pupae, and larvae inside mummies. Clutch size was calculated as the total number of individual *C. sosares* per host (i.e., the primary clutch size (sensu Ode and Strand 1995)): emerged plus unemerged adults (secondary clutch size), pupae, and larvae. Within-brood mortality was calculated as the total number of dead pupae and larvae divided by the primary clutch size. Mummies that failed to produce any emerged adults by 30 d postmummy formation were dissected, and body size and sex of any unemerged adults were recorded along with primary clutch size and within-brood mortality. Both metatibiae were removed from 10 randomly selected wasps of each sex from each mummy and measured with an ocular micrometer at ×50 magnification. Lengths of both tibiae were averaged for each wasp, and the measurements from 10 wasps were averaged for each mummy. Tibial length was chosen as a measure of body size, as tibial length correlates strongly with egg load and male mating ability in the congener *C. floridanum* (Ode and Strand 1995).

The effects of diet composition and sexual composition (all male, all female, and mixed sex) on primary clutch size and development time were analyzed separately with two-way analyses of variance (ANOVAs; PROC GLM; SAS Institute 2003). The effect of diet composition on within-brood mortality was analyzed with a logistic model that treated the response, mortality, as a binomial count (PROC LOGISTIC; SAS Institute 2003). The effect of diet composition on body size was analyzed with an analysis of covariance, with clutch size as a covariate. Tukey's honestly significant difference (HSD) multiple comparisons tests were performed when the overall test was significant (PROC GLM; SAS Institute 2003).

Xanthotoxin Effects on Host Quality Effects of xanthotoxin on *C. sosares* may be the result of compromised host quality. To examine this possibility, we randomly assigned unparasitized neonate parsnip webworms individually to one of the three artificial diets as described. Cups with unparasitized webworms were examined each morning for the formation of pupae. The sex of pupae was determined by the location of the bursal scar (Nitao and Berenbaum 1988); the scar is on the ninth abdominal tergite in males and eighth abdominal tergite of females. Pupae whose sex could not be determined were removed from the analyses. Pupal mass was recorded to the nearest 0.0001 g with an analytical balance. After measurements, pupae were placed individually into empty diet cups in the growth chamber until moths emerged, and the total number of days from the time that neonates were placed on diet until emergence of adult moths was recorded. The effects of diet and gender on *D. pastinacella* pupal mass and development time were analyzed with separate two-way ANOVAs (PROC GLM;

SAS Institute 2003). Pupae that failed to emerge after 30 d were dissected to determine whether death occurred before or after an adult developed inside. The mortality of *D. pastinacella* was calculated as the proportion of neonates that failed to develop into adult moths; mortality was compared across the three artificial diets with a likelihood ratio chi-square test (PROC FREQ; SAS Institute 2003).

Webworm Hemolymph Analysis To quantify unmetabolized xanthotoxin encountered by developing *C. sosares* embryos and precocious larvae, we removed parasitized fourth-instar webworms (*C. sosares* individuals are either embryos or precocious larvae at this point) from each diet treatment (26 total=nine from low-xanthotoxin diets, 17 from high-xanthotoxin diets). Webworms were anesthetized with CO_2, and prolegs were snipped with fine spring scissors. Hemolymph drained from these wounds was drawn with a pipette and transferred into 0.5-ml centrifuge tubes.

Webworms from which hemolymph was collected were dissected lengthwise in physiological saline, and any *C. sosares* precocious larvae and polygerms were removed and placed separately into 1.5-ml sample tubes. Precocious larvae (three samples from low-xanthotoxin diet and four from high-xanthotoxin diet) and polygerm (five samples from low-xanthotoxin diet and five from high-xanthotoxin diet) were frozen at −80°C until metabolic assays could be run (see below). Hemolymph samples plus the tubes in which they were contained were weighed to the nearest milligram and dried in a lyophilizer, and the residues were extracted with 30 μl methanol. The tubes from which the samples were drawn were washed, dried, and reweighed to quantify the fresh mass of hemolymph analyzed. Ten microliters of the extract was analyzed by high-pressure liquid chromatography (HPLC; Waters, Milford, MA, USA, gradient HPLC, with diode-array detector and autosampler). Solvent A was 1% formic acid in water, and solvent B was acetonitrile (linear gradient from 85% A to 100% B over 30 min, followed by 5 min at 100% B, flow rate 1 ml min^{-1}). Furanocoumarins were separated with a C-18 column (Shiseido, Capcell, 4.5×250 mm, 5-μm particles). Differences in hemolymph titers of xanthotoxin (ng mg^{-1} fresh weight of hemolymph) among webworms reared on the different diets were analyzed with an ANOVA (PROC GLM; SAS Institute 2003).

Metabolism of Xanthotoxin by C. sosares Embryos and Precocious Larvae Despite encountering furanocoumarins in host hemolymph, *C. sosares* larvae do not exhibit detectable metabolic detoxification of xanthotoxin (McGovern et al. 2006). To examine the possibility that embryos and/or precocious larvae are able to detoxify xanthotoxin, collected embryos and precocious larvae (see above) were analyzed according to McGovern et al. (2006). In brief, samples were homogenized and incubated for 30 min at 30°C in a reaction mixture that contained 500 ng xanthotoxin and an NADPH-regenerating system. The reactions were halted by adding 0.1 N HCl. Ten microliters of each reaction mixture was removed for protein quantification by using a dye assay (BioRad, Hercules, CA, USA) before HPLC analysis of unmetabolized furanocoumarins (extracted with 300 μl of ethyl acetate).

Results

Effect of Xanthotoxin on C. sosares Experiments that examined the effects of xanthotoxin on *C. sosares* clutch size, survivorship, and body size were repeated in 2005 and 2006. In none of the analyses was the year in which the experiment was conducted significant ($P>0.2$ in all tests). Therefore, we pooled data across 2005 and 2006 in the analyses presented below. A total of 269 *C. sosares* broods developed on the three diets: 28 all male, 27 all female, and three mixed sex on the control diet; 56 all male, 36 all female, and six mixed sex on low xanthotoxin; and 74 all male, 31 all female, and eight mixed sex on high-xanthotoxin diets.

C. sosares that developed in webworms fed with the artificial diet with a high concentration of xanthotoxin produced smaller primary clutch sizes than those that developed in webworms fed with diets with low xanthotoxin concentrations or no xanthotoxin (Fig. 1; $F_{2,\ 260}=$

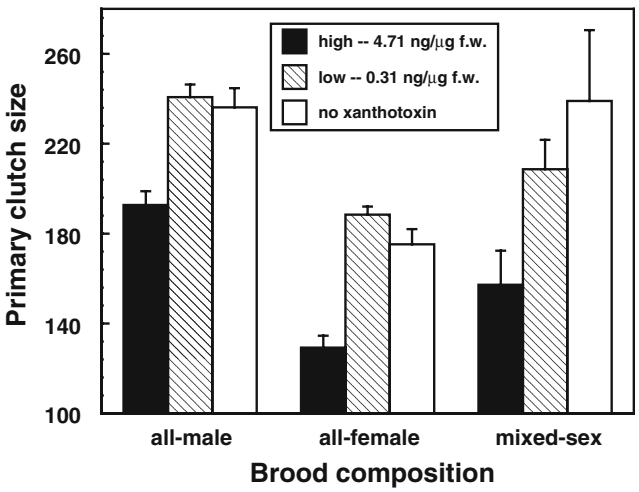

Fig. 1 Effects of dietary xanthotoxin on the mean (±SEM) primary clutch sizes (see text for explanation) of all-male, all-female, and mixed-sex broods of *Copidosoma sosares*. Clutch sizes from webworms fed a high-xanthotoxin diet were significantly smaller than clutch sizes from either the low-xanthotoxin or xanthotoxin-free diets; clutch sizes from the low-xanthotoxin and xanthotoxin-free diets did not differ (Tukey's HSD multiple comparisons test)

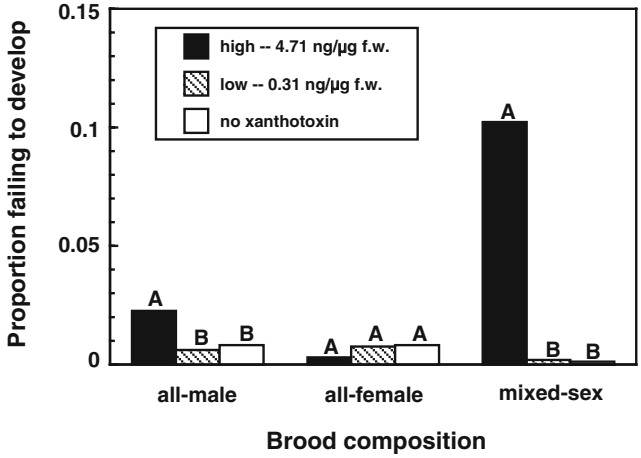

Fig. 2 Effects of dietary xanthotoxin on the proportion of the primary clutch that failed to develop into adults of all-male, all-female, and mixed-sex broods of *Copidosoma sosares*. Within each brood type (all-male, all-female, and mixed-sex), bars with different letters indicate significantly different odds of larval mortality as a function of diet type. Data were analyzed with logistic regression models (see text)

24.60, $P<0.001$). All-male and mixed-sex clutch sizes were larger than all-female clutch sizes irrespective of diet on which they were reared ($F_{2,\ 260}=54.60$, $P<0.001$). Xanthotoxin concentration in the artificial diet had a similar effect on all-male, all-female, and mixed-sex broods (diet×sex interaction $F_{4,\ 260}=0.70$, $P=0.594$).

Within-brood mortality was affected by the concentration of xanthotoxin in the diet (diet term: Wald $\chi^2=45.58$, $P<0.001$), and this effect differed among the three types of *C. sosares* broods (diet×sex interaction term: Wald $\chi^2=71.85$, $P<0.001$; Fig. 2). Whereas the odds of an individual female surviving to adulthood in an all-female brood were not influenced by diet composition (Wald $\chi^2=4.02$, $P=0.134$), this was not the case for individuals in all-male or mixed-sex broods. The odds of an individual male failing to complete development to adulthood in a host on a high-xanthotoxin diet were 3.3 times greater than the odds for developing in a host fed a xanthotoxin-free diet (Wald $\chi^2=69.37$, $P<0.001$) but did not differ between low-xanthotoxin and xanthotoxin-free diets (Wald $\chi^2=3.10$, $P=0.078$). Likewise, the odds of an individual in a mixed-sex brood failing to complete development in a webworm fed a high-xanthotoxin diet was nearly 58 times greater than the odds of failing to complete development on a xanthotoxin-free diet (Wald $\chi^2=16.25$, $P<0.001$); the odds of larval mortality did not differ between the low-xanthotoxin and xanthotoxin-free diets (Wald $\chi^2=0.0123$, $P=0.912$). Larval survivorship to adulthood was 89% or above in all cases (Fig. 2).

After accounting for the effects of clutch size and diet, individual females were larger than males (male tibial length=0.54±0.01 mm; female=0.48±0.01 mm; $F_{1,\ 241}=233.72$, $P<0.001$). Neither artificial diet type nor the interaction with sex affected body size (diet: $F_{2,\ 241}=1.37$, $P=0.256$; diet×sex: $F_{2,\ 241}=0.72$, $P=0.486$).

Although individuals in all-female and mixed-sex broods developed more quickly than individuals in all-male broods (male=40.6±3.4 d; female=37.7±3.4 d; mixed=34.0±3.2 d; $F_{2,\ 257}=13.87$, $P<0.001$), xanthotoxin concentration had no effect on the amount of time parasitized webworms fed on diet (diet: $F_{2,\ 261}=2.32$, $P=0.101$; diet×sex: $F_{4,\ 261}=2.05$, $P=0.088$).

Xanthotoxin Effects on Host Quality A total of 241 *D. pastinacella* successfully pupated on the experimental diets: 23 male and 21 female moths on the xanthotoxin-free diet, 41 male and 58 female moths on the low-xanthotoxin diet, and 46 male and 52 female moths on the high-xanthotoxin diet.

The amount of xanthotoxin added to the artificial diet had no effect on the mass of *D. pastinacella* pupae ($F_{2,\ 234}=2.15$, $P=0.520$). Likewise, after adjusting for the effects of xanthotoxin, males and females did not differ in pupal mass nor in how mass responded to dietary xanthotoxin (sex: $F_{1,\ 234}=0.11$, $P=0.743$; sex×diet: $F_{2,\ 234}=0.66$, $P=0.520$). The number of days from when neonates were placed on the diet until moth emergence was not influenced by xanthotoxin in the diet ($F_{2,\ 154}=0.25$, $P=0.779$). Development time from neonates until adulthood did not differ between males and females ($F_{1,\ 154}=1.05$, $P=0.306$). Development time of males and females did not differentially respond to diet composition ($F_{2,\ 154}=0.70$, $P=0.497$). Finally, mortality rates of *D. pastinacella* were similar across the control, low-xanthotoxin, and high-xanthotoxin diets (likelihood ratio $\chi^2=3.47$, 2 *df*, $P=0.177$).

Webworm Hemolymph Analysis Hemolymph from webworms fed with the artificial diet high in xanthotoxin (4.71 ng/µg fresh weight) had almost four times the unmetabolized xanthotoxin concentration as hemolymph from webworms fed with the artificial diet low in xanthotoxin (0.313 ng/µg fresh weight; Fig. 3; $F_{1,\ 15}=7.11$, $P=0.018$).

Metabolism of Xanthotoxin by C. sosares Embryos and Precocious Larvae Neither *C. sosares* embryos nor precocious larvae showed any detectable levels of metabolic detoxification of xanthotoxin. Of 34 reactions, only 17 showed detectable levels of protein ranging from 34 to 4,700 mg. The amount of unmetabolized xanthotoxin remaining after the metabolic assays of both *C. sosares* embryos and precocious larvae was no different from the mean of the four time-zero controls (*t* test, $t=0.013$, $P=0.99$). Comparison of the eight reactions with more than 1 mg of protein to time-zero controls gave a similar result ($t=0.252$, $P=0.807$).

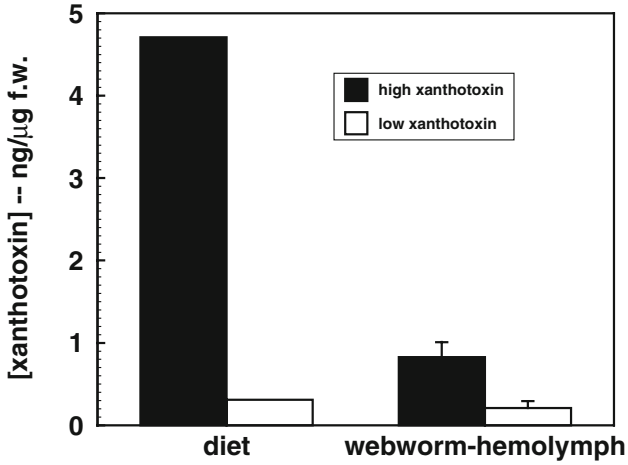

Fig. 3 Unmetabolized xanthotoxin (ng/μg fresh weight hemolymph) in *Depressaria pastinacella* hemolymph fed with a high-xanthotoxin (4.71 ng/μg fresh weight) artificial diet or a low-xanthotoxin (0.313 ng/μg fresh weight) artificial diet

Discussion

A previous field study that examined the correlation between the concentration of seven furanocoumarins in the host plants, *Pastinaca sativa* and *Heracleum sphondylium* (a common host plant in Europe), and the fitness measures of *C. sosares* showed that one furanocoumarin, xanthotoxin, was negatively correlated with clutch size as well as within-brood survivorship (Ode et al. 2004). In this study, we demonstrate that xanthotoxin added to an artificial diet consumed by webworms is responsible for both of these tritrophic correlations observed in the field. Clutch sizes from webworms reared on an artificial diet with a high level of xanthotoxin were 20–30% smaller than those reared on diets containing 15-fold less xanthotoxin or on a xanthotoxin-free diet. Further corroborating observed correlations in the field (Ode et al. 2004), our results demonstrate that within-brood survivorship of all-male and mixed-sex broods but not of all-female broods was reduced by high levels of xanthotoxin in diets compared to low levels or xanthotoxin-free diets. However, while the effects of xanthotoxin are statistically significant, within-brood larval mortality was low across all brood type and diet combinations relative to observed survivorship of field-collected broods (cf. Ode et al. 2004), suggesting that factors other than xanthotoxin have an impact on larval mortality of *C. sosares*.

Specialist parasitoids are less strongly affected by plant defensive chemistry than generalist parasitoids (Gunasena et al. 1990; Barbosa et al. 1991; Harvey et al. 2003, 2005; Sznajder and Harvey 2003). However, despite being a highly specialized parasitoid of a specialist herbivore, *C. sosares* exhibited significant variation in clutch size and survivorship in response to variation in the concentration of xanthotoxin in the host diet. Similarly, Barbosa et al. (1991) found that nicotine resulted in increased mortality in the specialist parasitoid *C. congregata*, whereas its sphingid host, *Manduca sexta* L., was not as strongly affected. Such studies suggest that specialist parasitoids are not necessarily immune to the negative effects of plant chemistry.

Whereas xanthotoxin has demonstrable effects on *C. sosares* fitness proxies, we were unable to detect any negative effects on host quality in terms of reduced webworm mass, increased development time, or increased mortality. It does not appear that the observed effects of xanthotoxin on *C. sosares* are the result of compromised host quality in response to dietary xanthotoxin. However, it is possible that other furanocoumarins or synergistic combinations, not considered in this study, could reduce host quality. For instance, the angular furanocoumarin sphondin is negatively correlated with the proportion of the linear furanocoumarin bergapten in seeds, and both are associated with resistance to webworms (Berenbaum et al. 1986). The concurrent presence of bergapten and xanthotoxin may compromise the ability of *D. pastinacella* to metabolize xanthotoxin. Diets high in such furanocoumarins conceivably could reduce host quality to the point that *C. sosares* fitness is also reduced. On the other hand, previous field correlative studies (Ode et al. 2004) indicate that xanthotoxin alone, out of seven analyzed furanocoumarins, was negatively correlated with *C. sosares* fitness.

Xanthotoxin likely affects *C. sosares* fitness by directly acting as a toxin. Although webworms are remarkable for their efficiency in metabolizing xanthotoxin (Zangerl and Berenbaum 1993), our results, in addition to those of McGovern et al. (2006), show that developing *C. sosares* do encounter unmetabolized xanthotoxin in the hemolymph of their webworm hosts. Furthermore, hemolymph titers of xanthotoxin increased fourfold in webworms fed with a diet that contained high levels of xanthotoxin compared to webworms consuming a diet low in xanthotoxin, indicating that changes in webworm diet, be they artificial or natural, correspond directly to the amount of xanthotoxin encountered by developing *C. sosares*.

Despite the presence of potentially toxic levels of xanthotoxin during development, we found no evidence that either *C. sosares* embryos or precocious larvae are able to metabolize it. Successful development of *C. sosares* embryos and larvae (see McGovern et al. 2006) likely depends in part on the ability of the webworm to detoxify furanocoumarins as well as the furanocoumarin content of the host plant tissues consumed by the webworms. Webworms from different geographical regions vary in their metabolic capacities (Berenbaum and Zangerl 1998; Zangerl and Berenbaum 2003; Berenbaum and Zangerl 2006). Similarly, plant populations vary considerably in

terms of the furanocoumarin profiles produced, both within a species (Berenbaum and Zangerl 1998; Zangerl and Berenbaum 2003) and among species (Zangerl and Berenbaum 2003; Ode et al. 2004). In western Europe, where this tritrophic interaction is native, *C. sosares* clutch size and survivorship are greater on *H. sphondylium* (a host plant that contains relatively low levels of xanthotoxin) than it is on *P. sativa* (which typically contains 10 times the levels of xanthotoxin as *H. sphondylium*). Together, variation among webworm populations in terms of detoxification abilities and among host plant populations/species in terms of furanocoumarin profiles may have a strong influence on the establishment success of *C. sosares*.

The establishment success of *C. sosares* may be influenced ultimately by the trophic complexity in which the selective interactions between wild parsnip and parsnip webworm are embedded. The interaction intensity between parsnip and webworm determines the variation in wild parsnip furanocoumarin profiles and parsnip webworm detoxification abilities (Berenbaum and Zangerl 2006). The level of trophic complexity involving parsnips and webworms in The Netherlands is, by and large, greater than in midwestern North America. Higher trophic complexity in The Netherlands populations is indicated by the presence of alternative host plants and attacks by parasitic wasps, both of which are largely absent in midwestern North American parsnip populations. As a possible consequence of decreased trophic complexity, xanthotoxin concentrations in midwestern North American populations of parsnip are generally higher than in populations in The Netherlands (Ode et al. 2004; Berenbaum and Zangerl 2006). If *C. sosares* was introduced to midwestern North America (whether intentionally, accidentally, or naturally), it may experience, to its detriment, significantly elevated levels of xanthotoxin in its webworm hosts. Alternatively, such effects of xanthotoxin may be negated by the elevated detoxification abilities of midwestern North American webworms. An understanding of the effects of reduced trophic complexity on the selective intensity between plant and herbivore is important not only for interpreting the evolution of trophic relationships but also for predicting and understanding the outcome of biological control introductions.

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References

BARBOSA, P., GROSS, P., and KEMPER, J. 1991. Influence of plant allelochemicals on the tobacco hornworm and its parasitoid, *Cotesia congregata*. *Ecology* 72:1567–1575.

BARBOSA, P., SAUNDERS, J. A., KEMPER, J., TRUMBULE, R., OLECHNO, J., and MARTINAT, P. 1986. Plant allelochemicals and insect parasitoids: effects of nicotine on *Cotesia congregata* (Say) (Hymenoptera: Braconidae) and *Hyposoter annulipes* (Cresson) (Hymenoptera: Ichneumonidae). *J. Chem. Ecol.* 12:1319–1328.

BERENBAUM, M. R. 1990. Evolution of specialization in insect-umbellifer associations. *Annu. Rev. Entomol*. 35:319–343.

BERENBAUM, M. R., and ZANGERL, A. R. 1996. Phytochemical diversity: adaptation or random variation?, pp. 1–24, in J.T. ROMEO, J.A. SAUNDERS, and P. BARBOSA (eds.). Recent Advances in Phytochemistry: Phytochemical Diversity and Redundancy in Ecological Interactions, vol. 30. Plenum, New York, NY.

BERENBAUM, M. R., and ZANGERL, A. R. 1998. Chemical phenotype matching between a plant and its insect herbivore. *Proc. Natl. Acad. Sci. USA* 95:13743–13748.

BERENBAUM, M. R., and ZANGERL, A. R. 2006. Parsnip webworms and host plants at home and abroad: trophic complexity in a geographic mosaic. *Ecology* 87:3070–3081.

BERENBAUM, M. R., ZANGERL, A. R., and NITAO, J. K. 1986. Constraints on chemical coevolution: wild parsnips and the parsnip webworm. *Evolution* 40:1215–1228.

CAMPBELL, B. C., and DUFFEY, S. S. 1979. Tomatine and parasitic wasps: potential incompatibility of plant antibiosis with biological control. *Science* 205:700–702.

CAMPBELL, B. C., and DUFFEY, S. S. 1981. Alleviation of a-tomatine-induced toxicity to the parasitoid, *Hyposoter exiguae*, by phytosterols in the diet of the host, *Heliothis zea*. *J. Chem. Ecol.* 7:927–946.

EL-HENEIDY, A. H., BARBOSA, P., and GROSS, P. 1988. Influence of dietary nicotine on fall armyworm, *Spodoptera frugiperda* and its parasitoids, the ichneumonid wasp *Hyposoter annulipes*. *Entomol. Exp. Appl.* 46:227–232.

GIRON, D., DUNN, D. W., HARDY, I. C. W., and STRAND, M. R. 2004. Aggression by polyembryonic wasp soldiers correlates with kinship but not resource competition. *Nature* 430:676–679.

GRBIĆ, M., ODE, P. J., and STRAND, M. R. 1992. Sibling rivalry and brood sex ratios in polyembryonic wasps. *Nature* 360:254–256.

GUERRIERI, E., and NOYES, J. 2005. Revision of the European species of *Copidosoma* Ratzeburg (Hymenoptera: Encyrtidae), parasitoids of caterpillars (Lepidoptera). *Syst. Entomol.* 30:97–174.

GUNASENA, G. H., VINSON, S. B., and WILLIAMS, H. J. 1990. Effects of nicotine on growth, development, and survival of the tobacco budworm (Lepidoptera, Noctuidae) and the parasitoid *Campoletis sonorensis* (Hymenoptera, Ichneumonidae). *J. Econ. Entomol.* 83:1777–1783.

HARDY, I. C. W. 1996. Precocious larvae in the polyembryonic parasitoid *Copidosoma sosares* (Hymenoptera: Encyrtidae). *Entomol. Ber.* 56:88–92.

HARVEY, J. A., CORLEY, L. S., and STRAND, M. R. 2000. Competition induces adaptive shifts in caste ratios of a polyembryonic wasp. *Nature* 406:183–186.

HARVEY, J. A., DAM, N. M., and GOLS, R. 2003. Interactions over four trophic levels: foodplant quality affects development of a hyperparasitoid as mediated through a herbivore and its primary parasitoid. *J. Anim. Ecol.* 72:520–531.

HARVEY, J. A., NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31:287–302.

MCGOVERN, J. L., ZANGERL, A. R., ODE, P. J., and BERENBAUM, M. R. 2006. Furanocoumarins and their detoxification in a tri-trophic interaction. *Chemoecology* 16:45–50.

NITAO, J. K., and BERENBAUM, M. R. 1988. Laboratory rearing of the parsnip webworm, *Depressaria pastinacella* (Lepidoptera: Oecophoridae). *Ann. Entomol. Soc. Am.* 81:485–487.

ODE, P. J. 2006. Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. *Annu. Rev. Entomol.* 51:163–185.

ODE, P. J., and STRAND, M. R. 1995. Progeny and sex allocation decisions of the polyembryonic wasp *Copidosoma floridanum*. *J. Anim. Ecol.* 64:213–224.

ODE, P. J., BERENBAUM, M. R., ZANGERL, A. R., and HARDY, I. C. W. 2004. Host plant, host plant chemistry, and the polyembryonic parasitoid *Copidosoma sosares*: indirect effects in a tritrophic interaction. *Oikos* 104:388–400.

REITZ, S. R., and TRUMBLE, J. T. 1996. Tritrophic interactions among linear furanocoumarins, the herbivore *Trichoplusia ni* (Lepidoptera: Noctuidae), and the polyembryonic parasitoid *Copidosoma floridanum* (Hymenoptera: Encyrtidae). *Environ. Entomol.* 25:1391–1397.

REITZ, S. R., and TRUMBLE, J. T. 1997. Effects of linear furanocoumarins on the herbivore *Spodoptera exigua* and the parasitoid *Archytas marmoratus*: host quality and parasitoid success. *Entomol. Exp. Appl.* 84:9–16.

Sas Institute 2003. SAS/STAT User's Guide, Version 9.1.3. Cary, NC, SAS Institute.

STRAND, M. R., BAEHRECKE, E. H., and WONG, E. A. 1991. The role of host endocrine factors in the development of polyembryonic parasitoids. *Biol. Control* 1:144–152.

SZNAJDER, B., and HARVEY, J. A. 2003. Second and third trophic level effects of differences in plant species reflect dietary specialisation of herbivores and their endoparasitoids. *Entomol. Exp. Appl.* 109:73–82.

THURSTON, R., and FOX, P. M. 1972. Inhibition by nicotine of emergence of *Apanteles congregatus* from its host, the tobacco hornworm. *Ann. Entomol. Soc. Am.* 65:547–550.

ZANGERL, A. R., and BERENBAUM, M. R. 1993. Plant chemistry, insect adaptations to plant chemistry, and host plant utilization patterns. *Ecology* 74:47–54.

ZANGERL, A. R., and BERENBAUM, M. R. 2003. Phenotype matching in wild parsnip and parsnip webworms: causes and consequences. *Evolution* 57:806–815.

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Predatory Mite Attraction to Herbivore-induced Plant Odors is not a Consequence of Attraction to Individual Herbivore-induced Plant Volatiles

Michiel van Wijk · Paulien J. A. De Bruijn · Maurice W. Sabelis

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Abstract Predatory mites locate herbivorous mites, their prey, by the aid of herbivore-induced plant volatiles (HIPV). These HIPV differ with plant and/or herbivore species, and it is not well understood how predators cope with this variation. We hypothesized that predators are attracted to specific compounds in HIPV, and that they can identify these compounds in odor mixtures not previously experienced. To test this, we assessed the olfactory response of *Phytoseiulus persimilis,* a predatory mite that preys on the highly polyphagous herbivore *Tetranychus urticae.* The responses of the predatory mite to a dilution series of each of 30 structurally different compounds were tested. They mites responded to most of these compounds, but usually in an aversive way. Individual HIPV were no more attractive (or less repellent) than out-group compounds, i.e., volatiles not induced in plants fed upon by spider-mites. Only three samples were significantly attractive to the mites: octan-1-ol, not involved in indirect defense, and *cis*-3-hexen-1-ol and methyl salicylate, which are both induced by herbivory, but not specific for the herbivore that infests the plant. Attraction to individual compounds was low compared to the full HIPV blend from Lima bean. These results indicate that individual HIPV have no *a priori* meaning to the mites. Hence, there is no reason why they could profit from an ability to identify individual compounds in odor mixtures. Subsequent experiments confirmed that naive predatory mites do not prefer tomato HIPV, which included the attractive compound methyl salicylate, over the odor of an uninfested bean. However, upon associating each of these odors with food over a period of 15 min, both are preferred. The memory to this association wanes within 24 hr. We conclude that *P. persimilis* possesses a limited ability to identify individual spider mite-induced plant volatiles in odor mixtures. We suggest that predatory mites instead learn to respond to prey-associated mixtures of volatiles and, thus, to odor blends as a whole.

Keywords Herbivore-induced plant volatiles · Tritrophic system · Learning · Memory · Synthetic odor perception

M. van Wijk (✉) · P. J. A. De Bruijn · M. W. Sabelis
Population Biology Section,
Institute for Biodiversity and Ecosystem Dynamics,
P.O. Box 94084, 1090 GB Amsterdam, The Netherlands
e-mail: wijk@science.uva.nl

Introduction

Natural plant odors are usually blends of many molecules. Upon infestation by herbivores, plants change the composition of the odors they emit (Arimura et al. 2005). This information is used by the third trophic level to locate and prey on the herbivores that feed on the plant (Sabelis et al. 2006). The induced odor thus constitutes a signal to predatory arthropods, but exactly which features of this induced odor are perceived as the signal? Predators may either respond to one or a few of the induced compounds that they recognize individually in the mixture. Alternatively, predators could respond to the new odor mixture as a whole. If herbivore species induce the same volatile compounds in the plants they feed on, and if predators possess the ability to identify specific compounds in odor mixtures, the population of predators could evolve a set genetically fixed olfactory responses to these specific compounds. Predators with such innate responses to specific prey-related odors can minimize the exploration of plants that are not infested with their prey by limiting their olfactory acuity to a subset, of specific, ecologically

relevant compounds (Bernays 2001; Egan and Funk 2006). Alternatively, predators could maximize their olfactory acuity for a wide variety of odors and perceive the plant odor mixture as a whole, i.e., distinct from its components. Consequently, individual compounds have no *a priori* meaning to such predators. Although this forces the predators to cope with much more information, it will facilitate the ability to differentiate among odors with chemically overlapping compositions. This may, for example, help them learn the difference between the odors that emanate from plants harboring suitable prey and plants harboring unsuitable prey. Here, we ask, if we can find a preference for individual herbivore-induced plant compounds in a population of the predatory mite *Phytoseiulus persimilis*.

Phytoseiulus persimilis preferably preys on the two-spotted spider mite *Tetranychus urticae* Koch. The predatory mites are blind and rely on odors to locate distant prey patches (Sabelis and Van der Baan 1983; Sabelis et al. 1984a, b). By using olfactory cues that emanate from spider mite-infested plants, the predatory mites are able to discern plants with prey from plants without (Sabelis and Van der Baan 1983; Dicke and Sabelis 1988; Dicke et al. 1990a, 1998; Dicke 1994; Sabelis et al. 1999). The prey, *T. urticae*, is highly polyphagous and has been reported to feed on more than 900 species of plants in 124 genera (Bolland et al. 1998). Infestation by spider mites induces different host plant species to emit different blends (Van Den Boom et al. 2004).

Under natural conditions, the quantitative and qualitative odor emission from plants is not only affected by herbivory of a predator's prey, but also by several other independent biotic and abiotic factors. Different herbivore species induce different blends of herbivore-induced plant volatiles (HIPV) in the same plant species (De Moraes et al. 1998; De Boer et al. 2004, 2005). The same herbivore induces different HIPV in different plant species (Van Den Boom et al. 2004). The HIPV composition changes with leaf age (Takabayashi et al. 1994) and during the onset of infestation (Kant et al. 2008). Genetic variation within herbivore and plant species also affects HIPV production (Degen et al. 2004; Kant et al. 2008). Biotic factors such as herbivore-vectored viruses (Eigenbrode et al. 2002; Jimenez-Martinez et al. 2004) and abiotic factors such as fertility of the soil, light, and temperature, all qualitatively and quantitatively, affect the odor composition of a plant (Gouinguene and Turlings 2002; Vallat et al. 2005). Under natural conditions, these factors together generate a wealth of olfactory cues that vary in time, space, and possibly also information (Sabelis et al. 2006).

Phytoseiulus persimilis is able to adjust its olfactory response based on experience. Rearing the predatory mites from egg to adulthood on different species of spide mite-infested plants induces a preference for these plant odors (Takabayashi and Dicke 1992; Krips et al. 1999). Prolonged feeding (24 hr or more) of adults in the presence of HIPV blends also induced an acquired olfactory preference for these HIPV (Krips et al. 1999; Drukker et al. 2000; de Boer and Dicke 2004a; De Boer et al. 2005). This ability to learn from experience may help dispersing predatory mites detect spider mite-infested plants that they are already familiar with. The polyphagous nature of the prey, however, makes it worthwhile to explore not previously experienced odors as well. How can the dispersing predatory mites determine which odor sources are worthwhile exploring and which are not? Predatory mites could generalize their preference from odors that have been previously experienced in association with their prey to similar odors not previously experienced. On the other hand, predatory mites could possess an innate preference for specific spider mite-induced compounds that they identify in odor mixtures not previously experienced. The literature offers evidence for and against both possibilities. Drukker et al. (2000) found that *P. persimilis* females reared in the absence of plant odors did not innately prefer the odor of spider mite-infested plants over that of uninfested plants. Results obtained by de Boer and Dicke (2004a) suggested that *P. persimilis* preferred methyl salicylate only if the mites had experience with a methyl salicylate containing odor-blend. Hence, an innate preference for methyl salicylate seems to be absent. This acquired attraction to methyl salicylate could indicate that *P. persimilis* generalizes the acquired preference from the mixture *to* the individual compound. This generalization could be facilitated by the fact that the few (about 20) olfactory receptor cells that *P. persimilis* possesses may perceive only a fraction of an odor mixture (Jackson 1974; Jagers op Akkerhuis et al. 1985; van Wijk et al. 2006). On the other hand, the acquired preference for methyl salicylate reported by de Boer and Dicke (2004a) could also indicate that *P. persimilis* possess the ability to identify the presence of this particular compound in a mixture, which would suggest that olfaction in *P. persimilis* is elemental. More evidence in support of elemental odor perception can be found in the reported attractiveness of several typical spider mite-induced plant compounds (Dicke et al. 1990b; de Boer and Dicke 2004a, b; Kappers et al. 2005). An innate preference for specific compounds in combination with the ability to identify these compounds in unfamiliar mixtures could explain how *P. persimilis* has been able to prefer, without prior experience, the odor of spider mite-infested plants over that of uninfested conspecifics for several plant species (van den Boom et al. 2002). Thus, some experiments with *P. persimilis* do not provide evidence for an innate preference for HIPV, whereas others indicate that individual compounds could represent attractants by themselves and as part of odor mixtures.

In this paper, we aim to elucidate whether individual compounds can represent a signal that is recognized as a distinct element of the odor mixture that spider mite-infested plants emit. Acceptance of this hypothesis requires two prerequisites: first, individual spider mite-induced compounds should be attractive, and second, the mites should be able to identify the attractive components in mixtures. To test the first prerequisite, we investigated if individual compounds that are typically induced by spider mites elicit a specific behavioral response that differs from the response elicited by compounds that—to the best of our knowledge—are not involved in indirect plant defense. We used the information about the olfactory preference for individual compounds gained in the first experiment to test the second prerequisite. In this experiment, we assessed whether the mites can utilize their preference for specific spider mite-induced plant compounds to select without prior experience the odor that emanates from a spider mite-infested plant, which emits attractive HIPV, over the odor of an uninfested plant. We further assessed whether the presence of HIPV affects the ability to learn about odors, and we asked how long the mites maintain this memory.

Methods and Materials

Plants and Mites Lima bean plants (*Phaseolus lunatus*) were reared in a climate room (22°C, 60% RH, 16:8 LD) from seeds until they were 2 wk old. Next, they were infested with two-spotted spider mites, *T. urticae*. The predatory mites (*P. persimilis*) were reared in a climate room (25°C, 80% RH, 16:8 LD) on detached spider mite-infested Lima bean leaves. Every day (except on the weekend), the predatory mites received fresh spider-mite-infested Lima bean leaves, and the culture was harvested. The frequent harvesting of the mites ensured that the females used were one to a few days old since their final molt. The predatory mites were originally obtained in 2001 from field samples at various sites near the coast of Sicily, Italy. The predatory mites were collected from spider mite-infested plants, and the plants were from different genera in three different plant families (*Convolvulaceae*, *Asteraceae*, *Euphorbiaceae*). Before testing the olfactory response, all mites were brought in a hungry state, which mimics the conditions before and after dispersal (Sabelis and Afman 1994). To this end, adult female predatory mites were kept in Eppendorf tubes, deprived of water and food for 16–22 hr (24°C.).

Odors The response to a panel of 30 odors was tested. Most were obtained from Fluka with the exception 2,3-dimethyl-pyrazine, which was obtained from Sigma-Aldrich. Octan-1-ol and butan-1-ol were obtained from Sigma, *cis*-3-hexenyl acetate, dodecyl-acetate, nerolidol, proionic acid were obtained from Aldrich, methyl salicylate from Sigma-Aldrich, and β-ocimene (70% E- and 30% *Z*- isomers) from R. C. Treatt & Co. (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, (TMTT) were provided by Dr. W. Boland of the Max Planck Institute for Chemical Ecology, Jena, Germany.

Olfactory Response Tests The response to each compound in the odor panel was tested by using a "choice arena". The basic arena consisted of an upside-down Petri dish (diam 9 cm) (Fig. 1a). An opening at the bottom of the arena was connected to a vacuum pump (flow 0.42 l/min). Prior to the experiment, groups of about 20 female predatory mites were placed in a cartridge that could be fitted between the vacuum pump and the choice arena. For each replicate experiment, freshly made odor sources and a new cartridge with a new group of predatory mites were provided. Insect glue barriers divided the arena in two sides while leaving an opening at the center of the bottom of the arena (Fig. 1a). One side of the arena contained a filter paper (diam 1 cm) with the 0.5-µl odor (dissolved in hexane), while the alternative side contained a control filter paper treated with the solvent only (0.5 µl hexane). The odor sources were prepared in a fume, and the solvent was allowed to evaporate for exactly 1 min before the odor source was placed in the set-up. Mites were released from the cartridge, and after 3 min, the mites at each side of the arena were counted. Each odor was tested in five concentrations, covering a concentration range that spans five orders of magnitude (pure and diluted with a factor 10, 10^2, 10^3, and 10^4). The response to each concentration of each odor was tested in six replicate experiments, and each contained 20 predatory mites. Fresh arenas were used for each odor, and the choice arena was rotated between replicate experiments to correct for any unforeseen directional bias. To avoid contamination of the air that entered the choice arena, it was placed in a flow cabinet that was continuously supplied with clean air. Assuming individual mites make independent choices, the preference of the mites for either side of the arena was evaluated with a replicated *G*-test (Sokal and Rohlf 1995). In short, significant values of G_t indicate an overall deviation from a 50:50 distribution. This statistic is broken down into two components that characterize/evaluate different aspects of the deviation; G_h and G_p. Significance of G_h indicates heterogeneity among replicate experiments, while significance of G_p indicates a deviation from an even distribution in the overall pooled result.

To aid graphical display of the data, we calculated the following preference index to each odor-sample: [(mites at odor side—mites at control side)/total amount of mites) × 100]. In this way, repellent odors were assigned a negative

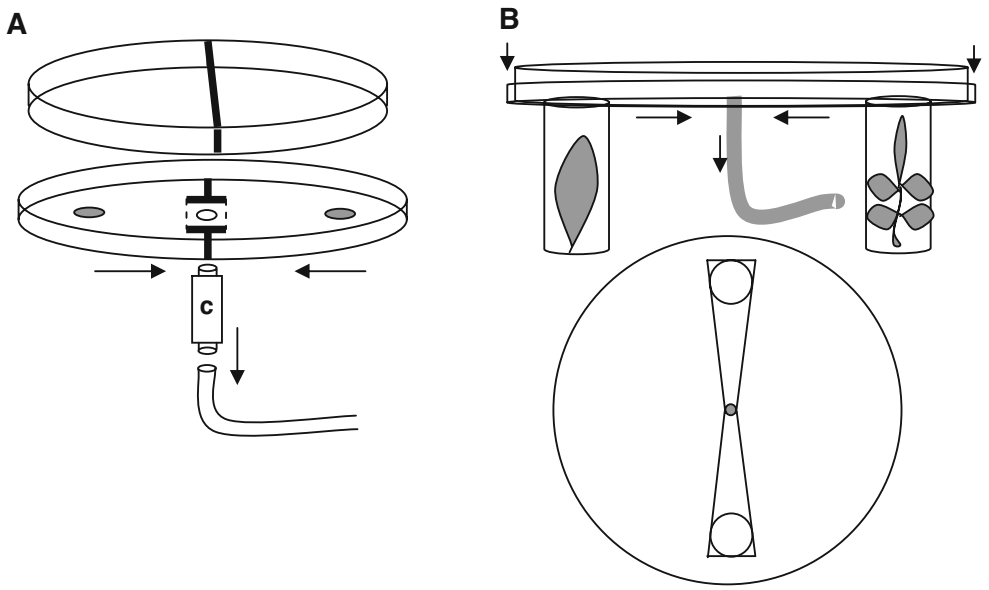

Fig. 1 1A: The "choice arena" consists of an upside down Petri dish. At the center, the arena connects to a cartridge (c) that holds the predatory mites. The cartridge is connected to the vacuum pump. Arrows indicate the direction of the radial airflow in the arena. Odors are applied on filter paper (gray circles).The choice arena is divided in the two sides by a thin layer of insect glue (thick black lines). 1B: Modified version of 1 A that can be fitted with two veils that hold a tomato or a bean leaf placed on moist cotton wool. Gauze covered holes connect the veils to the choice arena. A single mite is released into the center and from 30 min the time it spends in each of the odor fields (triangles in the lower panel) is measured

preference index (−100 to 0), and attractive odors a positive preference index (0 to 100).

Natural Odors In this set of experiments, individual mites were placed into an experimental arena. This consisted of an upside-down Petri dish (diam 14 cm) with two vials mounted just below an opening in the bottom (Fig. 1b). The vials held either an uninfested bean leaf (*Phaseolus vulgaris*) or an infested tomato leaf (*Lycopersicon esculentum*). Tomato plants were infested with *T. urticae* more than 1 wk before the onset of the experiment. Spider mite-infested tomato was selected because *P. persimilis* that originated from the same laboratory population are attracted to its odor when reared on spider mite-infested tomato leaves (Kant et al. 2004). To establish a radial airflow, a vacuum pump was connected to an opening in the center of the arena. Odor fields were defined as the triangular area that had a base just wide enough to encompass the opening above the vial that contained the odor source, while the tip encompassed the opening where the setup was mounted to the vacuum pump (Fig. 1b). The mites could freely move over the bottom of the arena, and the time spent in each odor field was continuously measured for 30 min. The mites were allowed to associate odors with the presence of food in an arena. To this end, washed spider mite eggs were placed on gauze that covered an odor source. To make sure that the odor sources were the only cues that the mites used during the behavioral observation in the *post*-experience phase, the mites were always observed in an identical, but different arena. The mites' ability to learn about the absence of food in a particular odor field was assessed in two ways: first, by restricting the mites to one of the odor sources in the absence of food, and second by offering the same unrewarded odor at both sides, while the mites were not restricted to one of the odor sources and could leave the odor field.

Results

Validation of the Choice Arena Several tests were conducted to validate the experimental setup. First, the airflow in the choice arena was visualized with smoke derived from droplets of chloric acid and ammonia. This revealed a steady radial airflow along the bottom of the choice arena. When the fumes were applied to one side of the arena, they never entered the other side, while gauze that covered the bottom of the cartridge in the vacuum entrance was progressively covered with NH_4Cl salt starting from one side only. This indicated that the separation of the odor plumes extended to the bottom of the cartridge.

Unforeseen bias in the setup was investigated with a choice test in the absence of odor sources (Fig. 2). This resulted in an even distribution among both sides of the choice arena. The average preference index was −0.25. None of the six replicate experiments had a significant bias

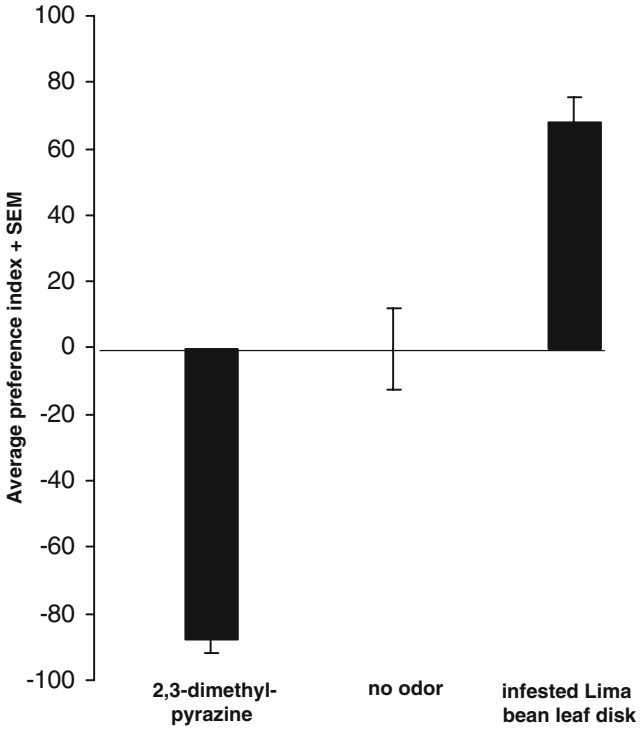

Fig. 2 Control experiments: 0.5 μl 2,3-dimethyl pyrazine is highly repellent and the mites easily avoid the side of the choice arena that contains this odor. In the absence of odors, the mites are evenly distributed along both sides of the choice arena. A small leaf disks excised from spider mite-infested lima bean leaf is attractive to the mites

to either side, and the data were not anymore heterogeneous than expected (G_p^{ns}, G_h^{ns}, G_t^{ns}). Subsequently, two positive control experiments were conducted. In the first, an excised, spider mite-infested, lima bean leaf-disc (diam 1.5 cm) was placed on moist filter paper at one side, whereas only moist filter paper was placed at the other side. This resulted for each of the six replicate experiments in a significant attraction of starved *P. persimilis*, and the overall attraction was also significant (G_p^*, G_h^{ns}, G_t^*). The average preference index was 67.8. Finally, an odor that was *a priori* known to be highly repellent to the mites, 2,3-dimethyl pyrazine (0.5 μl), was offered at one side, while a control paper was placed at the other. This resulted for each of the six replicate experiments in a significant avoidance of the odor with an overall significant aversion (G_p^*, G_h^{ns}, G_t^*). The average preference index was −87.5.

Response to the Odor-Panel The overall response to odors changed as a function of the concentration (Fig. 3). High concentrations elicited on average the most aversive response. Also, the strength of the response increased with increasing concentration. This correlation between the (log) odor concentration and the strength of the response (R^2= 0.77) was, however, present only for odor samples that were significantly repellent (G_t*, G_p*, and G_h^{ns}) (Table 1). For the nine moderately attractive samples (G_p* and G_h^{ns}), such a correlation was absent (R^2=0.00002).

Thus, the predatory mites perceived most odors in the panel. Out of 30 odors, 24 elicited a response (e.g., (P ($G_{p,\ df=1}$) < 0.05)) at one or more of the tested concentrations (Table 1). The six that did not elicit a response were acetone, α-humulene, α-pinene, linalool, propan-1-ol, and *trans*-caryophylene. Overall, 27 out of 150 choice tests were more heterogeneous than expected under the null hypothesis (P ($G_{h,\ df=5}$) < 0.05) (Table 1). These heterogeneous results were more prevalent at the higher concentration range (pure to 100× diluted odor samples) than in the lower concentration range (1,000 and 10,000× diluted samples). Based on a significance of G_p, 20 out of 30 odors at the highest concentration elicited a response, of which 19 were

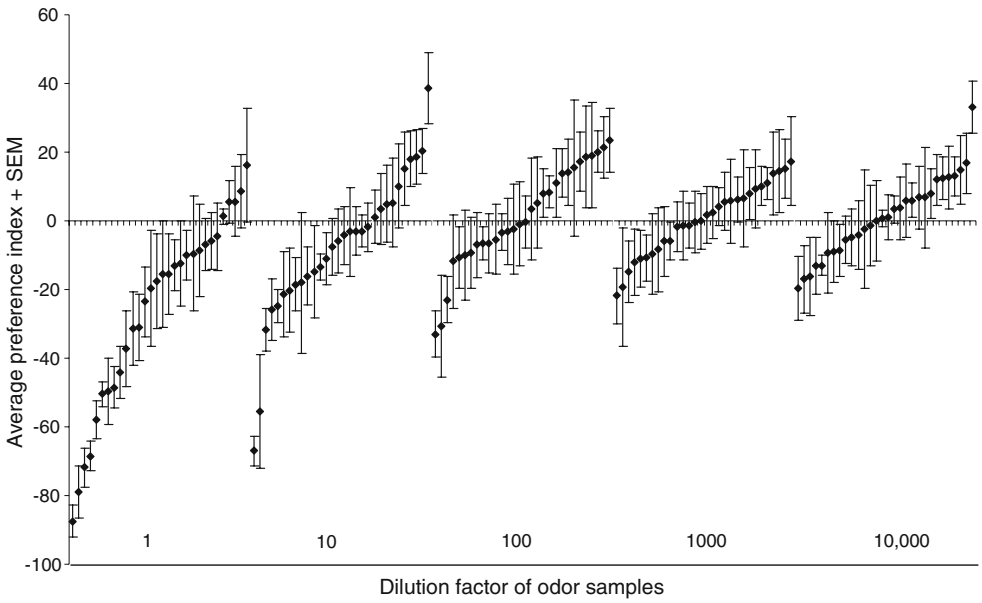

Fig. 3 The response that odors elicit from *P. persimilis* as a function of the odor concentration. Along the *x*-axis, the odors are sorted from repellent to attractive for each tested concentration in decreasing order. The strength of the response decreases with decreasing odor concentration. The number of repellent samples is correlated strongly with increasing odor concentration, whereas this correlation is absent for the occurrence of attractive odor samples. At the lowest concentration, most odors do not elicit a significant response

Table 1 The table contains the response of the mites to all 30 tested compounds at each of the five concentrations

Compound	Dilution	Significant G values	Average preference index	SEM	*P* value *G* test			Spider mite induced	Reference
					P Gt	*P* Gp	*P* Gh		
1-trans-2-hexenol	0	Gp Gt	−50.43	11.380	0.000	0.000	0.923	SIPV	van den Boom et al. 2004
1- trans-2-hexenol	1:10	Gp Gt	−31.77	17.738	0.028	0.001	0.829		
1- trans-2-hexenol	1:100		−0.91	32.439	0.213	0.922	0.138		
1- trans-2-hexenol	1:1000		−1.29	18.691	0.787	0.866	0.678		
1- trans-2-hexenol	1:10000	Gp	−19.61	25.070	0.053	0.016	0.249		
2,3-dimethyl-pyrazine	0	Gp Gt	−87.48	13.640	0.000	0.000	0.325		
2,3-dimethyl-pyrazine	1:10	Gp Gh Gt	−18.04	52.455	0.000	0.046	0.000		
2,3-dimethyl-pyrazine	1:100		−3.37	15.433	0.948	0.698	0.912		
2,3-dimethyl-pyrazine	1:1000		−1.73	19.997	0.793	0.655	0.711		
2,3-dimethyl-pyrazine	1:10000		3.34	12.014	0.961	0.601	0.944		
2-benzyl-ethanol	0	Gp Gt	−48.54	17.166	0.000	0.000	0.617		
2-benzyl-ethanol	1:10		−18.49	21.150	0.340	0.063	0.649		
2-benzyl-ethanol	1:100		8.38	13.680	0.923	0.431	0.931		
2-benzyl-ethanol	1:1000		−5.98	27.394	0.349	0.574	0.270		
2-benzyl-ethanol	1:10000		1.07	18.213	0.857	0.866	0.766		
3-octanone	0	Gp Gh Gt	−79.00	21.178	0.000	0.000	0.015		
3-octanone	1:10	Gp Gh Gt	−55.50	42.991	0.000	0.000	0.000		
3-octanone	1:100	Gp	−22.98	19.095	0.090	0.007	0.599		
3-octanone	1:1000		5.53	23.156	0.502	0.508	0.429		
3-octanone	1:10000		−9.37	30.917	0.163	0.541	0.117		
acetic acid	0	Gp Gt	−44.25	21.021	0.000	0.000	0.457		
acetic acid	1:10		−5.88	25.027	0.495	0.864	0.374		
acetic acid	1:100	Gh	−2.30	34.405	0.057	0.571	0.036		
acetic acid	1:1000		−1.42	26.981	0.257	1.000	0.171		
acetic acid	1:10000	Gh Gt	−2.30	44.558	0.002	0.931	0.001		
acetone	0		5.38	17.702	0.727	0.343	0.742		
acetone	1:10		−4.23	23.147	0.372	0.825	0.267		
acetone	1:100	Gh Gt	3.38	38.938	0.037	0.867	0.020		
acetone	1:1000		10.92	13.954	0.825	0.223	0.927		
acetone	1:10000		14.80	27.238	0.144	0.133	0.198		
α-humulene	0		−4.64	26.174	0.243	0.516	0.186	SIPV	van den Boom et al. 2004,
α-humulene	1:10		−2.97	19.575	0.664	0.690	0.558		Maeda and Takabayashi 2001
α-humulene	1:100		−6.65	14.264	0.879	0.470	0.865		
α-humulene	1:1000		−0.33	24.073	0.474	0.930	0.352		
α-humulene	1:10000		−12.94	9.272	0.904	0.189	0.994		
α-pinene	0	Gh Gt	−8.48	35.317	0.034	0.739	0.019		
α-pinene	1:10	Gh Gt	5.29	33.930	0.039	0.283	0.033		
α-pinene	1:100		−5.45	27.387	0.364	0.609	0.279		
α-pinene	1:1000		−9.67	31.294	0.065	0.327	0.053		
α-pinene	1:10000		0.71	8.678	0.997	0.874	0.991		
α-terpinene	0	Gp Gt	−49.70	25.792	0.000	0.000	0.126		
α-terpinene	1:10		−1.89	19.436	0.748	0.690	0.652		
α-terpinene	1:100	Gp Gh Gt	19.04	39.852	0.008	0.035	0.026		
α-terpinene	1:1000		−10.78	18.878	0.420	0.141	0.570		
α-terpinene	1:10000		12.59	24.948	0.139	0.111	0.211		
benzyl benzoate	0	Gp Gh Gt	−17.59	35.880	0.007	0.039	0.019		
benzyl benzoate	1:10		−2.94	14.248	0.943	0.609	0.917		
benzyl benzoate	1:100	Gp	23.45	25.618	0.174	0.030	0.509		
benzyl benzoate	1:1000	Gh Gt	−19.35	44.371	0.000	0.057	0.000		
benzyl benzoate	1:10000		−0.13	31.128	0.126	0.796	0.078		
β-farnesene	0	Gp Gh Gt	−15.58	40.683	0.011	0.049	0.026	SIPV	van den Boom et al. 2004
β-farnesene	1:10		15.23	28.867	0.105	0.095	0.173		
β-farnesene	1:100		−7.02	25.401	0.312	0.292	0.307		
β-farnesene	1:1000	Gh Gt	−8.42	32.653	0.049	0.280	0.043		
β-farnesene	1:10000	Gp	16.75	24.337	0.097	0.046	0.241		
butan-1-ol	0	Gp Gt	−31.42	28.407	0.001	0.000	0.168	SIPV	Krips et al. 1999
butan-1-ol	1:10		−16.05	23.296	0.353	0.166	0.447		

Table 1 (continued)

Compound	Dilution	Significant G values	Average preference index	SEM	*P* value *G* test			Spider mite induced	Reference
					P Gt	*P* Gp	*P* Gh		
butan-1-ol	1:100		−9.28	27.612	0.142	0.227	0.148		
butan-1-ol	1:1000		1.79	22.515	0.566	1.000	0.437		
butan-1-ol	1:10000		11.90	20.433	0.597	0.300	0.621		
cis-3-hexen-1-ol	0	Gp	−23.59	27.464	0.069	0.018	0.296	SIPV	van den Boom et al. 2004
cis-3-hexen-1-ol	1:10		3.57	27.695	0.159	0.881	0.100		
cis-3-hexen-1-ol	1:100	Gp Gt	21.41	24.232	0.049	0.009	0.328		
cis-3-hexen-1-ol	1:1000		10.12	16.646	0.415	0.116	0.608		
cis-3-hexen-1-ol	1:10000	Gh Gt	6.85	38.427	0.020	0.479	0.012		
cis-3-hexenyl-acetate	0	Gp Gt	−68.57	13.092	0.000	0.000	0.614	SIPV	Arimura et al. 2000,
cis-3-hexenyl-acetate	1:10	Gp Gt	−24.70	14.241	0.031	0.001	0.777		Horiuchi et al. 2003
cis-3-hexenyl-acetate	1:100	Gp Gh Gt	−10.07	34.414	0.007	0.037	0.021		
cis-3-hexenyl-acetate	1:1000		−5.87	15.581	0.805	0.503	0.764		
cis-3-hexenyl-acetate	1:10000	Gp Gh Gt	−16.16	30.116	0.006	0.013	0.034		
decan-1-ol	0		5.63	27.480	0.271	0.737	0.188		
decan-1-ol	1:10	Gp	20.39	18.612	0.273	0.032	0.706		
decan-1-ol	1:100	Gp Gh Gt	18.64	38.983	0.002	0.014	0.013		
decan-1-ol	1:1000		9.29	30.110	0.166	0.508	0.122		
decan-1-ol	1:10000		6.79	24.671	0.264	0.374	0.230		
(E)-DMNT	0	Gh Gt	−9.50	43.609	0.003	0.165	0.003	SIPV	Arimura et al. 2001,
(E)-DMNT	1:10		1.06	21.437	0.597	0.876	0.471		Horiuchi et al. 2003
(E)-DMNT	1:100	Gh Gt	15.42	51.064	0.000	0.065	0.000		
(E)-DMNT	1:1000		15.16	23.818	0.182	0.070	0.350		
(E)-DMNT	1:10000		7.91	20.072	0.493	0.245	0.542		
dodecyl-acetate	0		−12.93	21.021	0.905	0.479	0.895		
dodecyl-acetate	1:10		10.09	32.449	0.436	0.170	0.549		
dodecyl-acetate	1:100	Gp	17.11	23.460	0.115	0.245	0.114		
dodecyl-acetate	1:1000		6.20	18.163	0.075	0.038	0.209		
dodecyl-acetate	1:10000		5.97	14.803	0.542	0.329	0.540		
farnesol	0	Gp Gt	−15.55	31.118	0.022	0.023	0.085		
farnesol	1:10	Gp Gt	−25.87	24.490	0.034	0.004	0.382		
farnesol	1:100		−3.17	26.252	0.316	0.631	0.234		
farnesol	1:1000	Gt	13.93	32.012	0.042	0.105	0.063		
farnesol	1:10000		12.55	17.671	0.494	0.088	0.778		
hexan-1-ol	0	Gp Gt	−37.31	29.375	0.000	0.000	0.088		
hexan-1-ol	1:10	Gh Gt	−14.87	35.386	0.017	0.214	0.017		
hexan-1-ol	1:100		−11.90	35.857	0.070	0.393	0.053		
hexan-1-ol	1:1000		−0.03	22.357	0.625	1.000	0.496		
hexan-1-ol	1:10000		−8.93	24.573	0.247	0.458	0.198		
hexyl-acetate	0	Gp Gh Gt	−19.55	43.790	0.000	0.004	0.001	SIPV	van den Boom et al. 2004
hexyl-acetate	1:10		−13.43	12.028	0.746	0.151	0.923		
hexyl-acetate	1:100		8.03	19.609	0.762	0.514	0.710		
hexyl-acetate	1:1000		−10.95	22.756	0.367	0.163	0.470		
hexyl-acetate	1:10000		−17.02	26.315	0.159	0.074	0.298		
(+/-) linalool	0		−6.97	21.019	0.626	0.369	0.613	SIPV	van den Boom et al. 2004,
(+/-) linalool	1:10		−3.26	33.782	0.160	0.922	0.100		Krips et al. 1999 Kant
(+/-) linalool	1:100		−10.72	24.657	0.434	0.344	0.415		et al. 2004
(+/-) linalool	1:1000		4.18	16.197	0.828	0.516	0.787		
(+/-) linalool	1:10000		−13.01	22.873	0.192	0.116	0.286		
MeSA	0	Gp Gt	−71.81	16.270	0.000	0.000	0.188	SIPV	van den Boom et al. 2004,
MeSA	1:10	Gp Gt	−67.05	12.818	0.000	0.000	0.769		Agrawal et al. 2002, Meada
MeSA	1:100	Gp Gt	−32.96	18.756	0.000	0.000	0.609		and Takabayashi 2001, Kant
MeSA	1:1000	Gh Gt	5.85	32.439	0.034	0.324	0.027		et al. 2004, Arimura et al.
MeSA	1:10000	Gp Gt	33.19	21.047	0.000	0.000	0.324		2000, Arimura et al. 2001
Nerolidol	0		1.26	7.664	0.998	0.941	0.994	SIPV	Kant et al. 2004
Nerolidol	1:10		−21.29	33.040	0.805	0.503	0.764		
Nerolidol	1:100		−6.54	23.245	0.805	0.503	0.764		
Nerolidol	1:1000	Gp Gt	−21.89	22.061	0.019	0.003	0.262		

Table 1 (continued)

Compound	Dilution	Significant G values	Average preference index	SEM	*P* value *G* test			Spider mite induced	Reference
					P Gt	*P* Gp	*P* Gh		
Nerolidol	1:10000		−5.46	19.499	0.671	0.547	0.597		
octan-1-ol	0		8.51	28.581	0.217	0.321	0.199		
octan-1-ol	1:10	Gp Gt	38.61	27.403	0.000	0.000	0.206		
octan-1-ol	1:100		10.95	26.854	0.278	0.215	0.310		
octan-1-ol	1:1000		2.33	21.224	0.717	0.785	0.604		
octan-1-ol	1:10000		−8.58	20.986	0.467	0.265	0.496		
propan-1-ol	0		−9.83	20.185	0.379	0.177	0.468		
propan-1-ol	1:10		−7.64	22.954	0.475	0.431	0.424		
propan-1-ol	1:100		13.93	19.908	0.390	0.136	0.538		
propan-1-ol	1:1000	Gt	14.41	32.185	0.039	0.057	0.085		
propan-1-ol	1:10000		3.75	24.897	0.332	0.586	0.253		
propionic acid	0	Gp Gt	−57.94	15.977	0.000	0.000	0.742		
propionic acid	1:10	Gp	17.96	22.287	0.133	0.039	0.352		
propionic acid	1:100	Gp	20.14	17.428	0.158	0.011	0.732		
propionic acid	1:1000	Gp Gh Gt	17.38	34.147	0.002	0.023	0.009		
propionic acid	1:10000		−4.21	26.868	0.519	0.577	0.430		
(S)-(-)-limonene	0	Gp Gt	−31.01	25.992	0.000	0.000	0.087	SIPV	van den Boom et al. 2004,
(S)-(-)-limonene	1:10	Gh	4.98	29.693	0.072	0.649	0.045		Arimura et al. 2000
(S)-(-)-limonene	1:100		14.16	26.283	0.110	0.089	0.187		
(S)-(-)-limonene	1:1000	Gp	−14.89	24.379	0.140	0.037	0.377		
(S)-(-)-limonene	1:10000		5.92	28.631	0.130	0.519	0.092		
(E,E) TMTT	0	Gp Gh Gt	16.22	43.235	0.000	0.037	0.001	SIPV	van den Boom et al. 2004,
(E,E) TMTT	1:10		18.46	21.878	0.348	0.063	0.660		Krips et al. 1999, Meada
(E,E) TMTT	1:100	Gh Gt	5.20	34.916	0.015	0.787	0.008		and Takabayashi 2001,
(E,E) TMTT	1:1000		6.51	37.286	0.086	0.564	0.057		Arimura et al. 2001
(E,E) TMTT	1:10000		−4.87	22.878	0.304	0.768	0.214		
trans-β-ocimene	0	Gp Gt	−12.30	32.780	0.022	0.023	0.089	SIPV	Horiuchi et al. 2003, van den
trans-β-ocimene	1:10	Gp Gt	−20.17	32.144	0.009	0.006	0.090		Boom et al. 2004, Agrawal
trans-β-ocimene	1:100	Gp Gh Gt	−30.60	38.632	0.000	0.000	0.002		et al. 2002, Krips et al. 1999,
trans-β-ocimene	1:1000		8.04	21.053	0.672	0.438	0.633		Kant et al. 2004, Arimura et
trans-β-ocimene	1:10000		13.02	16.434	0.444	0.073	0.761		al. 2000, Arimura et al. 2001
(-) trans-caryophyllene	0		−5.97	22.730	0.385	0.435	0.332	SIPV	Krips et al. 1999, Meada
(-) trans-caryophyllene	1:10		−11.03	21.033	0.449	0.219	0.512		and Takabayashi 2001,
(-) trans-caryophyllene	1:100		−0.24	21.158	0.670	0.871	0.546		van de Boom et al. 2004
(-) trans-caryophyllene	1:1000		−11.97	26.011	0.188	0.117	0.280		
(-) trans-caryophyllene	1:10000		−1.47	31.029	0.161	0.732	0.105		

Each sample was tested with six replicate experiments that each contained about 20 starved mites. Negative average preference indices correspond to cases where the majority of the mites avoided the odor side, whereas positive preference indices correspond to cases where the majority of the mites moved toward the odor source. Average preference indices marked by gray cells correspond to nine moderately attractive samples (G_p* and G_h^{ns}). Just 3 of these nine odors additionally include significance of the total *G* statistic (G_t^*), octan-1-ol, methyl salicylate, and *cis*-3-hexen-1-ol. SIPV indicates that the compound has been reported as a spider-mite-induced plant volatile in de references in the adjacent column.

repellent and only one was attractive. The 10 and 100× diluted samples each contained 10 odors that elicited a response, but here, at the10× dilution, six were attractive whereas only four were repellent. At the 100× dilution, seven odors were repellent and only three were attractive. At the lowest concentrations, the 1,000x dilution yielded only two repellent and one attractive odor, whereas the 10.000× dilution only yielded two repellent and two attractive odors.

Out of 150 samples tested, 18% were significantly more heterogeneous than expected, and sometimes the same set of six replicate experiments revealed statistically significant heterogeneity, whereas the pooled results deviated significantly from an even distribution. In such cases, the pooled significance may depend on one or two extreme replicate experiments. A better indication of the olfactory preference of the population of predatory mites is, thus, found by using more stringent statistical criteria of the replicated *G* test for goodness of fit (Sokal and Rohlf, 1995). By using significance of G_t and G_p and non-significance of G_h as the criteria for choice, 13 repellent compounds at the highest concentration remain; these are 1-*trans*-2-hexenol, 2,3-dimethyl-pyrazine, 2-benzyl-ethanol, acetic acid, α-terpinene, butan-1-ol, *cis*-3-hexenyl acetate, farnesol, hexan-1-ol, methyl salicylate, propionic acid, (*S*)-(-)-

limonene, and β-ocimene. At the 10× diluted samples, only five repellent compounds remain; these are 1-*trans*-2-hexenol, *cis*-3-hexenyl acetate, farnesol, methyl salicylate, and *trans*-β-ocimene. The lowest concentration (10,000×) no longer contains repellent compounds under these statistical criteria, whereas the 100× and 1,000× diluted samples contain only one repellent compound each; these are methyl salicylate and nerolidol. In total, just three samples are attractive based on significance of G_t and G_p and non-significance of G_h, and each at just one of the five tested concentrations. Octan-1-ol was attractive at dilution 10×, *cis*-3-hexen-1-ol at dilution 100×, and methyl salicylate at a dilution of 10,000×.

We also investigated whether typical spider mite-induced compounds are more attractive than other compounds. The set of attractive odor samples, whose preference index does not exceed 39, is only weakly to moderately attractive compared to the repellent samples that often have a preference index smaller than −50, and to the control experiment with a spider mite-infested leaf disc that has a preference index of 68 (Fig. 2). If the statistical criteria for a choice are reduced to the significance of G_p, non-significance of G_h and no restrictions on the significance of G_t are applied, nine out of 150 (30 odors at five concentrations) samples were attractive (indicated by marked preference index in Table 1). This set contains eight out of 30 odors tested (propionic acid was present twice at 10× and 100x dilution). These represent a variety of structurally very different molecules. At the same time, the other set of "non-attractive" odors contains molecules that are structurally similar to some attractive odors. For example, propionic acid was attractive, whereas acetic acid was not; octan-1ol was attractive, whereas 3-octanone was not; *cis*-3-hexen-1-ol was attractive, whereas hexen-1-ol and *cis*-3-hexenyl acetate were not; and octan-1-ol and decan-1-ol were attractive, whereas hexan-1-ol was not. Finally, the attractiveness was highly concentration-dependent. Only propionic acid was attractive at two concentrations and all others at only one. Only three out of these eight attractive odors have been implicated in HIPV produced after spider mite infestation, i.e., methyl salicylate, *cis*-3-hexen-1-ol, and β-farnesene. The other five compounds (propionic acid, octan-1-ol, benzyl benzoate, decan-1-ol, and dodecyl-acetate) have, to the best of our knowledge, never been reported as part of a spider mite-induced odor blend. Thus, starved females of *P. persimilis* are not specifically more attracted by individual spider mite-induced plant volatiles than by volatiles that are not associated with their prey.

Natural Odor Sources If *P. persimilis* does not specifically respond to typical spider mite-induced plant compounds, one may wonder if the mites are able to detect individual HIPV in complex mixtures for which they have no prior experience. This was investigated by using natural plant odors. In this experiment, we were interested not only in the initial choice of the predatory mites, but also in the time spent in the exploration of odor sources that could be associated with prey. To this end, the time that an individual mite spend in each of two odor fields that contained the odor of spider mite-infested tomato (*Lycopersicon esculentum*) (HIPV) or the odor of uninfested bean (*Phaseolus vulgaris*) (no HIPV), was continuously measured for 30 min.

In the absence of experience, the mites invested an equal amount of time in exploring both odor sources (Fig. 4). Subsequently, we experimentally mimicked conditions in which predatory mites explore an uninfested plant for a prolonged time. To this end, the mites were not starved in

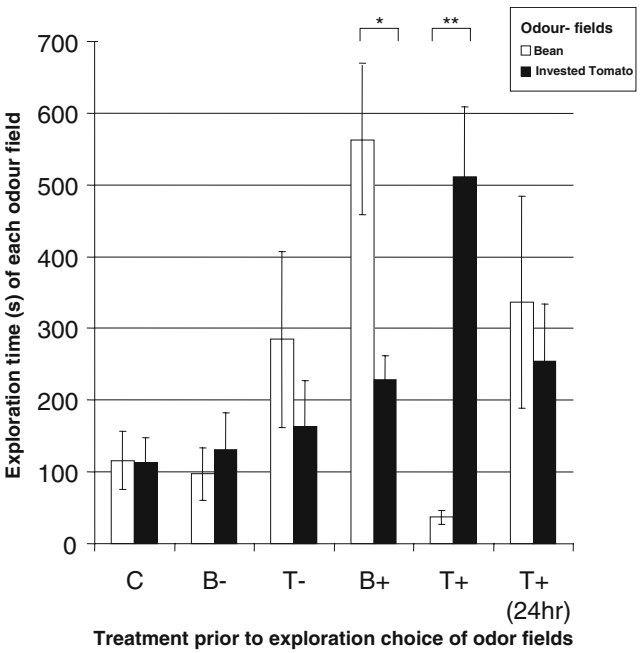

Fig. 4 Innate and acquired preference for spider mite-infested- and uninfested plant odors for which mites have no prior experience. Bars represent the time mites spent in each odor field (during 30 min). Black represents spider mite-infested tomato (HIPV source), white represents uninfested bean (no HIPV source). Significance was tested with a paired sample *t*-test ($^{*}P<0.05$, $^{**}P<0.001$). *C*: without prior experience, predatory mites do not invest more time in the exploration of the HIPV source than in the alternative ($N=11$). *B*−: mites were starved in the arena for 24 hr, while both sides contained bean leaves. There was no evidence of an acquired aversion ($N=10$). *T*−: Starved mites were restrained for 15 min above the infested tomato field without food ($N=8$). There is no evidence of a non-associative acquired response as a result of this treatment. *B* + and *T* + : Starved mites were allowed to feed for 15 min in the presence of either odor, the mites associate the odor with the reward ($N=15$ and $N=16$). T + (24 hr): Mites were first starved, subsequently allowed to forage in the arena, while the tomato patch contained food and the bean patch was unrewarded. Subsequently, the mites were starved for 24 hr until tested ($N=12$). The result suggests that the memory was lost within 24 hr

an Eppendorf tube outside the setup, but instead the starvation took place inside the setup with two odor sources that each contained an uninfested bean leaf. Twenty-four hours later, the mites were transferred to a fresh, identical arena that contained an uninfested bean leaf in one vial, and an infested tomato leaf in the alternative vial. As in the first experiment with naïve individuals, these mites did not spend more time in one of the two fields (Fig. 4). To assess whether the mites were able to associate the odors with food, washed spider mite eggs were offered above one of the odor sources. However, because feeding in the presence of an odor also results in the arrestment of predatory mites in the presence of the odor, we first conducted a control experiment to assess the role of this potential confounding effect. In this control experiment, starved individuals were forced to stay for 15 min in the area above the infested tomato odor (Fig. 4). This treatment did not significantly affect the subsequent olfactory response of the mites, and they still did not prefer one odor over the other. Next, the predatory mites were allowed to feed for 15 min on washed spider mite eggs while experiencing the infested tomato leaf odor or the uninfested bean leaf odor (Fig. 4). This short period of feeding and contact with their food induced a strong behavioral change. After placing the predatory mites in an identical arena without food, they now spent much more time in the odor fields of the odors that were associated with a reward. The mites were able to learn both odors, but they seemed to have slightly more trouble ignoring the infested tomato odor field than the uninfested bean odor field as is evident from the difference in the time spent in both unrewarded odor fields (*t* test: $P<0.05$) (Fig. 4). To analyze the role of memory, we used this acquired response to assess if a prolonged experience with a reward-associated and a non-reward-associated odor would influence the olfactory response of the mites 24 hr after this experience. To this end, the predatory mites were first starved 24 hr, subsequently allowed to forage in the arena for 24 hr where food was available above the infested tomato leaf, and the area above the uninfested bean leaf yielded no food. After this experience, the predatory mites were again starved for 24 hr and subsequently, the time spent in each odor field was measured. After this experience, the mites again spent an equal amount of time in both odor fields (Fig. 4). Hence, we conclude that the type of learning involved in our setup induces only a short-term memory.

Discussion

Several authors have reported on the olfactory responses of *P. persimilis* to individual spider mite-induced plant volatiles (Dicke et al. 1990b; De Boer and Dicke 2004b; De Boer et al. 2004; Kappers et al. 2005). Dicke et al. (1990b) reported on responses elicited by linalool, methyl salicylate, (*E*,*E*)- and (*Z*,*E*) TMTT, *cis*-3-hexen-1-ol, *cis*-3 hexen-1-yl acetate, octan-3-ol, and both (*Z*)- and (*E*)-β-ocimene. In contrast to the experiments presented here, the predatory mites in the experiments by Dicke at al. (1990b) were satiated and thus in a physiological condition that is different from that during long-distance dispersal. Dicke et al. (1990b) reported that, as in our study, methyl salicylate was attractive, but in contrast to our results these authors also reported that (*E*,*E*)-TMTT, linalool, and (*E*)-β-ocimene were attractive. They also assessed the effect of mixing (*E*)- and (*Z*)-β-ocimene, and found only (*E*)-β-ocimene to be attractive, whereas the chemotactic response attenuated when (*Z*)-β-ocimene was added to (*E*)-β-ocimene. We used a racemic mixture of 70% (*E*)-β-ocimene and 30% (*Z*)-β-ocimene, and as in Dicke et al. (1990b) this blend was not attractive. De Boer and Dicke (2004a, 2004b) also reported on the attractiveness of methyl salicylate and additionally showed that it is attractive to both starved and satiated mites. In a second study, de Boer and Dicke (2004b) reported on the attractiveness of 2-butanone, a compound not tested by us. In addition, they found that (*E*,*E*)-TMTT and (*E*)-DMNT did not elicit a significant response from starved *P. persimilis*, although as in Dicke et al. (1990), (*E*)-DMNT was attractive to satiated mites while (*E*,*E*)-TMTT bordered significance. Similarly, these two compounds did not elicit a significant response (G_p^*, G_h^{ns}, G_t^*) from the starved predatory mites in our study, whereas the attractiveness bordered significance at some concentrations. Finally, Kappers et al. (2005) reported on the attractiveness of (*E*)-nerolidol to starved (24 hr) *P. persimilis*, whereas we observed no attraction to this compound.

Behavioral studies are sometimes difficult to compare because starvation times (Dicke et al. 1998; Shimoda and Dicke 2000), wind speeds, the genetic background of the population (Margolies et al. 1997), rearing condition, and previous experience (Takabayashi and Dicke 1992; Krips et al. 1999; Drukker et al. 2000; de Boer and Dicke 2004a, 2005; De Boer et al. 2005) might all vary among experiments from different authors. In this study, we report a comprehensive study on the olfactory responses to a range of individual spider mite-induced plant volatiles in starved *P. persimilis* females. To avoid reporting on the particular preference of a particular strain of *P. persimilis*, a large laboratory population was founded with mites that were collected at various locations along the coast of Sicily (Italy). The results should, thus, be interpreted as responses at the population level. The olfactory response among individual mites could differ as a result of genetic factors (Margolies et al. 1997), and might explain some of the heterogeneity observed among replicate experiments.

The predatory mites responded to a wide range of structurally different molecules. There also was no clearly observable pattern of chemical motifs that elicited a specific response from the mites. It appears that the olfactory system of the mites is not specifically sensitive to a few ecologically relevant compounds. The olfactory system is rather more likely to identify a wide range of chemical motifs. This is not self-evident, especially if we do not take the simplicity of the olfactory system into account (Jagers op Akkerhuis et al. 1985; van Wijk et al. 2006). These results are, however, consistent with observations in insects and vertebrates where olfactory receptor cells possess a broader molecular receptive range, and respond to several, similar odors, particularly at higher concentrations (de Bruyne et al. 2001; Abaffy et al. 2006; Pelz et al. 2006). Moreover, the strength of the response, which odors elicit in predatory mites, increases with increasing concentration. Close examination of the data, however, revealed that this only applies to significantly repellent (G_t^*, G_p^*, and G_h^{ns}) samples. For the nine attractive samples (G_p^* and G_h^{ns}), such a correlation does not exist. Repellence of individual compounds is, thus, largely explained by odor quantity, whereas attraction appears to depend on unique combinations of odor quality and quantity.

The most remarkable result is the low number of significantly attractive compounds among spider mite-induced plant volatiles. Under the most stringent statistical criteria (G_t*, G_p*, and G_h^{ns}) just three samples were attractive, i.e., octan-1-ol, *cis*-3-hexen-1-ol, and methyl salicylate. These odors are only moderately attractive compared to the control experiment that involved spider mite-infested Lima bean (Fig. 1), and the results indicate that individual compounds hardly induce a chemotactic response. Octan-1-ol is—to the best of our knowledge—not involved in indirect plant defense. *Cis*-3-hexen-1-ol is known as a green leaf volatile, and is induced in many plants upon spider mite infestation (Van Den Boom et al. 2004), but also in direct response to wounding of plant tissue (Arimura et al. 2001). Like *cis*-3-hexen-1-ol, methyl salicylate is often induced by spider-mite feeding (e.g., Ament et al. 2004; De Boer et al. 2004; Kant et al. 2004; Van Den Boom et al. 2004). Methyl salicylate is, however, already induced in plants when only aspects of the mechanical damage caused by chewing insects are simulated (Mithofer et al. 2005). Thus, it is by no means a spider mite-specific signal.

The absence of a strong chemotactic response to individual typical HIPV indicates that these stimuli have no *a priori* meaning to predatory mites. This suggests that the ability to perceive the individual compounds in mixtures will be of little survival value. Consequently, mites are unlikely to possess the ability to discriminate between odors from herbivore-infested and clean plants on the basis of one or a few attractive compounds when they have never experienced these odors before. The results indicate that naïve mites are not able to discern infested tomato odor from uninfested bean odor (Fig. 4). The infested tomato is known to produce several typical spider mite-induced volatiles, among which is methyl salicylate (Ament et al. 2004; Kant et al. 2004), a compound that is attractive to the mites. With experience, the predatory mites were capable of differentiating between these odors. These data are, thus, not consistent with the hypothesis that mites possess an elemental odor perception that enables them to respond to the presence of specific HIPV that they identify in mixtures. The results also suggest that mites do not possess an innate preference for a combination of HIPV that could help them to select without prior experience the infested tomato leaf. Similar results were reported by Drukker et al. (2000) who found that *P. persimilis* reared in the absence of plant odors did not prefer the odors of infested plants over those of uninfested plants. These predatory mites only acquired a preference after prolonged (24 hr) feeding in the presence of either the uninfested- or infested plant odor. In a similar way, the mites in our experiment were able to associate both odors, irrespective of the HIPV contents of the blend, with the presence of food (Fig. 4).

The predatory mites require only a short learning period of less than 15 min to acquire a preference for an odor, a period much shorter than previously reported for the mites. Prolonged experience with odors that were not associated with food (24 hr) did not induce avoidance, in contrast to the results reported by Drukker et al. (2000). This result, however, is similar to those reported by de Boer et al. (2004), who also found no evidence of an acquired aversion as a result of starvation in the presence of odors. With respect to learning and memory, we conclude that the mites were able to associate odors with the presence of prey after a brief learning experience of less than 15 min, but the same learning experience of 24 hrs was insufficient to induce a long-term memory.

In summary, the mites responded to a wide range of structurally different molecules, suggesting that their small olfactory system is not sensitive to a few specific ecologically relevant compounds. This is further supported by the fact that the mites were not more attracted to spider mite-induced plant volatiles than to volatiles that are not associated with spider mites. This indicates that these individual spider mite-induced plant volatiles have no *a priori* meaning and that the ability to identify these compounds in mixtures is of little use to the mites. As a consequence, mites are not expected to use the presence of these compounds in odor mixtures, to select a spider mite-infested plant, for which they have no prior experience. Results of our study confirm that the predatory mites were not able to innately identify a spider mite-infested plant

odor, even though it emits methyl salicylate, a compound that was attractive to *P. persimilis*. We, thus, conclude that *P. persimilis* females possess a limited ability to identify individual spider mite-induced plant volatiles in odor mixtures. Instead, the mites learn to respond to prey-associated odor mixtures.

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References

ABAFFY, T., MATSUNAMI, H., and LUETJE, C. W. 2006. Functional analysis of a mammalian odorant receptor subfamily. *J. Neurochem.* 97:1506–1518.

AGRAWAL, A. A., JANSSEN, A., BRUIN, J., POSTHUMUS, M. A., and SABELIS, M. W. 2002. An ecological cost of plant defense: attractiveness of bitter cucumber plants to natural enemies of herbivores. *Ecol. Lett.* 5:377–385.

AMENT, K., KANT, M. R., SABELIS, M. W., HARING, M. A., and SCHUURINK, R. C. 2004. Jasmonic acid is a key regulator of spider mite-induced volatile terpenoid and methyl salicylate emission in tomato. *Plant. Physiol.* 135:2025–2037.

ARIMURA, G., KOST, C., and BOLAND, W. 2005. Herbivore-induced, indirect plant defenses. *Biochim. Biophys. Acta Mol. Cell Biol. Lipids.* 1734:91–111.

ARIMURA, G., OZAWA, R., HORIUCHI, J., NISHIOKA, T., and TAKABAYASHI, J. 2001. Plant–plant interactions mediated by volatiles emitted from plants infested by spider mites. *Biochem. Syst. Ecol.* 29:1049–1061.

ARIMURA, G., OZAWA, R., SHIMODA, T., NISHIOKA, T., BOLAND, W., and TAKABYASHI, J. 2000. Herbivory-induced volatiles elicit defense genes in lima bean leaves. *Nature* 406:6795512–515.

BERNAYS, E. A. 2001. Neural limitations in phytophagous insects: Implications for diet breadth and evolution of host affiliation. *Annu. Rev. Entomol.* 46:703–727.

BOLLAND, H. R., GUTIERREZ, J., and FLECHTMANN, C. H. W. 1998. World catalogue of the spider mite family. Brill, Leiden, The Netherlands.

DE BOER, J. G., and DICKE, M. 2004a. Experience with methyl salicylate affects behavioral responses of a predatory mite to blends of herbivore-induced plant volatiles. *Entomol. Exp. Appl.* 110:181–189.

DE BOER, J. G., and DICKE, M. 2004b. The role of methyl salicylate in prey searching behavior of the predatory mite Phytoseiulus persimilis. *J. Chem. Ecol.* 30:255–271.

DE BOER, J. G., and DICKE, M. 2005. Information use by the predatory mite *Phytoseiulus persimilis* (Acari: Phytoseiidae), a specialised natural enemy of herbivorous spider mites. *Appl. Entomol. Zool.* 40:1–12.

DE BOER, J. G., POSTHUMUS, M. A., and DICKE, M. 2004. Identification of volatiles that are used in discrimination between plants infested with prey or nonprey herbivores by a predatory mite. *J. Chem. Ecol.* 30:2215–2230.

DE BOER, J. G., SNOEREN, T. A. L., and DICKE, M. 2005. Predatory mites learn to discriminate between plant volatiles induced by prey and nonprey herbivores. *Anim. Behav.* 69:869–879.

DE BRUYNE, M., FOSTER, K., and CARLSON, J. R. 2001. Odor coding in the *Drosophila* antenna. *Neuron* 30:537–552.

DE MORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DEGEN, T., DILLMANN, C., MARION-POLL, F., and TURLINGS, T. C. J. 2004. High genetic variability of herbivore-induced volatile emission within a broad range of maize inbred lines. *Plant Physiol.* 135:1928–1938.

DICKE, M. 1994. Local and systemic production of volatile herbivore-induced terpenoids—their role in plant–carnivore mutualism. *J. Plant Physiol.* 143:465–472.

DICKE, M., and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth. J. Zool.* 38:148–165.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990a. Plant strategies of manipulating predator–prey interactions through allelochemicals—prospects for application in pest-control. *J. Chem. Ecol.* 16:3091–3118.

DICKE, M., TAKABAYASHI, J., POSTHUMUS, M. A., SCHUTTE, C., and KRIPS, O. E. 1998. Plant–phytoseiid interactions mediated by herbivore-induced plant volatiles: variation in production of cues and in responses of predatory mites. *Exp. Appl. Acarol.* 22:311–333.

DICKE, M., VANBEEK, T. A., POSTHUMUS, M. A., BENDOM, N., VANBOKHOVEN, H., and DEGROOT, A. E. 1990b. Isolation and identification of volatile kairomone that affects acarine predator–prey interactions—involvement of host plant in its production. *J. Chem. Ecol.* 16:381–396.

DRUKKER, B., BRUIN, J., JACOBS, G., KROON, A., and SABELIS, M. W. 2000. How predatory mites learn to cope with variability in volatile plant signals in the environment of their herbivorous prey. *Exp. Appl. Acarol.* 24:12881–895.

EGAN, S. P., and FUNK, D. J. 2006. Individual advantages to ecological specialization: insights on cognitive constraints from three conspecific taxa. *Proc. R. Soc. Lond. B. Biol. Sci.* 273:843–848.

EIGENBRODE, S. D., DING, H., SHIEL, P., and BERGER, P. H. 2002. Volatiles from potato plants infected with potato leafroll virus attract and arrest the virus vector, *Myzus persicae* (Homoptera: Aphididae) (vol 269, pg 455, 2002). *Proc. R. Soc. Lond. B. Biol. Sci.* 269:2603–2603.

GOUINGUENE, S. P., and TURLINGS, T. C. J. 2002. The effects of abiotic factors on induced volatile emissions in corn plants. *Plant Physiol.* 129:1296–1307.

HORIUCHI, J., ARIMURA, G., OZAWA, R., SHIMODA, T., TAKABAYASHI, J., and NISHIOKA, T. 2003. A comparison of the responses of Tetranychus urticae (Acari: Tetranychidae) and Phytoseiulus persimilis (Acari: Phytoseiidae) to volatiles emitted from lima bean leaves with different levels of damage made by T-urticae or Spodoptera exigua (Lepidoptera: Noctuidae). *Appl. Entomol. Zool.* 38:109–116.

JACKSON, G. J. 1974. Chaetotaxy and setal morphology of the palps and first tarsi of *Phytoseiulus persimilis* A. -H (Acarina: Phytoseiidae). *Acarologia* 16:583–594.

JAGERS OP AKKERHUIS, G., SABELIS, M. W., and TJALLINGII, W. F. 1985. Ultrastructure of chemical receptors on the pedipalps and first tarsi of *Phytoseiulus persimilis*. *Ex.p Appl. Acarol.* 1: 235–251.

JIMENEZ-MARTINEZ, E. S., BOSQUE-PEREZ, N. A., BERGER, P. H., ZEMETRA, R., DING, H. J., and EIGENBRODE, S. D. 2004. Volatile cues influence the response of *Rhopalosiphum padi* (Homoptera: Aphididae) to barley yellow dwarf virus-infected transgenic and untransformed wheat. *Environ. Entomol.* 33:1207–1216.

KANT, M. R., AMENT, K., SABELIS, M. W., HARING, M. A., and SCHUURINK, R. C. 2004. Differential timing of spider mite-induced direct and indirect defenses in tomato plants. *Plant Physiol.* 135:483–495.

KANT, M. R., SABELIS, M. W., HARING, M. A., and SCHUURINK, R. C. 2008. Intrapsecific variation in a generalist herbivore accounts for differential induction and impact of host plant defenses. *Proc. R. Soc., B.* 275:443–452.

KAPPERS, I. F., AHARONI, A., VAN HERPEN, T., LUCKERHOFF, L. L. P., DICKE, M., and BOUWMEESTER, H. J. 2005. Genetic engineering of terpenoid metabolism attracts, bodyguards to Arabidopsis. *Science* 309:2070–2072.

KRIPS, O. E., WILLEMS, P. E. L., GOLS, R., POSTHUMUS, M. A., and DICKE, M. 1999. The response of *Phytoseiulus persimilis* to spider mite-induced volatiles from gerbera: influence of starvation and experience. *J. Chem. Ecol.* 25:2623–2641.

MAEDA, T., and TAKABAYASHI, J. 2001. Production of herbivore-induced plant volatiles and their attractiveness to *Phytoseius persimilis* (Acari: Phytoseiidae) with changes of *Tetranychus urticae* (Acari: Tetranychidae) density on a plant. *Appl. Entomol. Zool.* 36:47–52.

MARGOLIES, D. C., SABELIS, M. W., and BOYER, J. E. 1997. Response of a Phytoseiid predator to herbivore-induced plant volatiles: selection on attraction and effect on prey exploitation. *J. Insect Behav.* 10:695–709.

MITHOFER, A., WANNER, G., and BOLAND, W. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137:1160–1168.

PELZ, D., ROESKE, T., SYED, Z., DE BRUYNE, M., and GALIZIA, C. G. 2006. The molecular receptive range of an olfactory receptor in vivo (*Drosophila melanogaster* Or22a). *J. Neurobiol.* 66:1544–1563.

SABELIS, M. W., and AFMAN, B. P. 1994. Synomone-induced suppression of take-off in the phytoseiid mite *Phytoseiulus persimilis* Athias-Henriot. *Exp. Appl. Acarol.* 18:711–721.

SABELIS, M. W., AFMAN, B. P., and SLIM, P. J. 1984a. Location of distant spider-mite colonies by Phytoseiulus persimilis: Localisation and extraction of a kairomone , pp. 431–440, in D. A. Griffiths, and C. E. Bowman (eds.). Acarology VI, vol. 1John Wiley and Sons, New York.

SABELIS, M. W., JANSSEN, A., PALLINI, A., VENZON, M., BRUIN, J., DRUKKER, B., and SCUTAREANU, P. 1999. Behavioral responses of predatory and herbivorous arthropods to induced plant volatiles: From evolutionary ecology to agricultural applications, in A. Agrawal, S. Tuzun and E. Bent (eds.). Induced Plant Defenses against Pathogens and Herbivores. The American Phytopathological Society (APS) Press, St. Paul, Minnesota.

SABELIS, M. W., TAKABAYASHI, J., JANSSEN, A., KANT, M. R., VAN WIJK, M., SZNAJDER, B., ARATCHIGE, N., LESNA, I., BELLIURE, B., and SCHUURINK, R. C. 2006. Ecology meets plant physiology: Herbivore-induced plant responses and their indirect effects on arthropod communities pp. 188–217, in T. Ohgushi, T. P. Craig and P. W. Price. (eds.). Ecological Communities: Plant Mediation in Indirect Interaction Webs. Cambridge University Press, Cambridge.

SABELIS, M. W., and VAN DER BAAN, H. E. 1983. Location of distant spider mite colonies by phytoseiid predators: demonstration of specific kairomones emitted by *Tetranychus urticae* and *Panonychus ulmi*. *Exp. Appl. Acarol.* 33:303–314.

SABELIS, M. W., VERMAAT, J. E., and GROENEVELD, A. 1984b. Arrestment responses of the predatory mite, *Phytoseiulus persimilis*, to steep odor gradients of a kairomone. *Physiol. Entomol* 9:437–446.

SHIMODA, T., and DICKE, M. 2000. Attraction of a predator to chemical information related to nonprey: when can it be adaptive. *Behav. Ecol.* 11:606–613.

SOKAL, R. R., and ROHLF, F. J. 1995. Biometry. W. H. Freedman and Company, New York.

TAKABAYASHI, J., and DICKE, M. 1992. Response of predatory mites with different rearing histories to volatiles of uninfested plants. *Entomol. Exp. Appl.* 64:187–193.

TAKABAYASHI, J., DICKE, M., TAKAHASHI, S., POSTHUMUS, M. A., and VANBEEK, T. A. 1994. Leaf age affects composition of herbivore-induced synomones and attraction of predatory mites. *J. Chem. Ecol.* 20:373–386.

VALLAT, A., GU, H. N., and DORN, S. 2005. How rainfall, relative humidity and temperature influence volatile emissions from apple trees in situ. *Phytochemistry* 66:1540–1550.

VAN DEN BOOM, C. E. M., VAN BEEK, T. A., and DICKE, M. 2002. Attraction of *Phytoseiulus persimilis* (Acari: Phytoseiidae) towards volatiles from various *Tetranychus urticae*-infested plant species. *Bull. Entomol. Res.* 92:539–546.

VAN DEN BOOM, C. E. M., VAN BEEK, T. A., POSTHUMUS, M. A., DE GROOT, A., and DICKE, M. 2004. Qualitative and quantitative variation among volatile profiles induced by *Tetranychus urticae* feeding on plants from various families. *J. Chem. Ecol* 30: 69–89.

VAN WIJK, M., WADMAN, W. J., and SABELIS, M. W. 2006. Morphology of the olfactory system in the predatory mite *Phytoseiulus persimilis*. *Exp. Appl. Acarol.* 40:217–229.

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Protein Expression Changes in Maize Roots in Response to Humic Substances

Paolo Carletti · Antonio Masi · Barbara Spolaore · Patrizia Polverino De Laureto · Mariangela De Zorzi · Loris Turetta · Massimo Ferretti · Serenella Nardi

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Abstract Humic substances are known to affect plant metabolism at different levels. We characterized humic substances extracted from earthworm feces by diffuse reflectance infrared Fourier transform (DRIFT) spectroscopy and used them to treat corn, *Zea mays* L., seedlings to investigate changes in patterns of root protein expression. After root plasma membrane extraction and purification, proteins were separated by two-dimensional gel electrophoresis, and differential spot intensities were evaluated by image analysis. Finally, 42 differentially expressed proteins were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The majority of them were downregulated by the treatment with humic substances. The proteins identified included malate dehydrogenase, ATPases, cytoskeleton proteins, and different enzymes belonging to the glycolytic/gluconeogenic pathways and sucrose metabolism. The identification of factors involved in plant responses to humic substances may improve our understanding of plant–soil cross-talk, and enable a better management of soil resources.

Keywords DRIFT spectroscopy · Humic substances · LC-MS-MS · Proteomics · Sucrose metabolism · Two-dimensional gel electrophoresis · *Zea mays* L.

P. Carletti · A. Masi · M. De Zorzi · L. Turetta · M. Ferretti · S. Nardi (✉)
Department of Agricultural Biotechnology, University of Padua, Agripolis, Strada Romea, 16-35020 Legnaro, Padova, Italy
e-mail: serenella.nardi@unipd.it

B. Spolaore · P. Polverino De Laureto
Centro di Ricerca Interdipartimentale per le Biotecnologie Innovative (CRIBI), University of Padua, viale G. Colombo 3, 35121 Padova, Italy

Introduction

Humic substances (HS) consist of a mixture of different organic compounds resulting from the decomposition of plant and animal residues (MacCarthy 2001). They are found in all terrestrial and aquatic environments and constitute one of the most abundant forms of organic matter on the surface of the Earth (MacCarthy 2001). HS interact with the organic component of soil and the root apparatus of plants in the soil matrix, where HS can have a fundamental influence not only on overall soil fertility and conservation, but also on plant physiology (Nardi et al. 2002). Under laboratory and field conditions, HS enhance plant growth, as measured in terms of an increase in length or in the fresh and dry weights of shoots and roots. HS also result in the production of higher leaf chlorophyll concentrations, more lateral root initials, an improved micro- and macronutrient uptake, and many other biological effects (Nardi et al. 2002). These effects have been attributed partly to the complexing properties of HS, which increase the availability of micronutrients from scarcely soluble hydroxides, and particularly to the maintenance of sufficient levels of Fe and Zn in solution (Cesco et al. 2000).

Other targets of HS on plant roots are the enzymes associated with the plasma membrane (PM) (Canellas et al. 2002). At the molecular level, the effects of HS have been demonstrated on the expression of specific genes, such as the two H^+-ATPase isoforms *Mha1* and *Mha2* (Quaggiotti et al. 2004). Muscolo et al. (2007b) showed that HS, and particularly those with a low molecular mass, are taken up by plant cells and are able to affect plant metabolism. In this sense, a hormone-like activity has been suggested for several humic fractions (Zandonadi et al. 2007) whose biological action appears to mimic the responses induced by gibberellic and indole-3-acetic acid (IAA). The metabolic

pathways involved in these responses have been only partially described, and the whole mechanism implicated is far from being elucidated.

The PM represents the site for the exchange of information and substances between the cell and its environment. Proteins associated with root cell PMs can thus be reasonably assumed to be a target for HS, and changes in their expression may be seen as the primary reactions leading to the biological responses reported so far. Since protein analysis is the most direct approach for studying the dynamics of plant cell metabolism, valuable information on plant cell and HS interactions can be obtained from a proteomic assay.

We extracted HS from earthworm feces, and used the extract to treat 11-d-old seedlings of corn, *Zea mays*. Next, we used a proteomics approach to assess changes in protein expression in PM-enriched root extracts in response to HS treatment. The biochemical reaction of *Z. mays* to HS at the level of root proteins is shown here for the first time. The main aim of this study was to establish which proteins are differentially regulated after exposure to HS, and to provide new insight on the molecular basis of the response of *Z. mays* PM to these compounds.

Methods and Materials

Preparation of Humic Extract The feces of *Nicodrilus* [=*Allolobophora* (Eisen)=*Aporrectodea* (Oerley)] *caliginosus* (Savigny) and *Allolobophora rosea* (Savigny) (Minelli et al. 1995) were collected from the Ah horizon of an uncultivated couchgrass, *Agropyron repens* L., growing in soils classified as Calcaric Cambisol (CMc-F.A.O. classification) (FAO-UNESCO 1990). Earthworm culture conditions, HS extraction, and extract purification were conducted as reported in Quaggiotti et al. (2004). HS extraction and purification were performed with 0.1 N KOH. The extract was desalted by using 14 kDa cut-off dialysis Visking (Medicell, London, UK) tubing against distilled water. Subsequently, the extract was desalted on ion exchange Amberlite IR-120 (H^+ form), assessed for organic carbon content, and lyophilized before conducting the following analyses.

Diffuse Reflectance Infrared Fourier Transform Spectroscopy DRIFT spectroscopy was performed with a Nicolet Impact 400 FT-IR Spectrophotometer (Nicolet Instruments, Madison, WI, USA) fitted with an apparatus for diffuse reflectance (Spectra-Tech. Inc., Stamford, CT, USA). SiC disks (320-grid-Carb paper obtained from Spectra-Tech) were used to collect 5 mg of lyophilized sample. Spectra were obtained by combining at least 200 scans at a resolution of 4 cm^{-1}. The background was obtained on the abrasive SiC disk. Spectral data were analyzed with Grams 386 spectral software (Galactic Industries, Salem, NH, USA).

Plant Material and Growth Conditions Maize (*Zea mays* L. cv. DKc 5783, Dekalb, Monsanto Agricoltura SpA, Lodi, Italy) seeds were soaked for one night in running water and germinated in the dark at 27°C on filter paper wetted with 1 mM $CaSO_4$ for 96 hr (Nardi et al. 2000b). Seedlings were grown in pools containing 40 l of Hoagland solution for 11 d as follows: 16 hr of light at 25°C and 60% relative humidity, 8 hr of dark at 18°C and 80% relative humidity. For the treatment, a batch of plants was moved for 16 hr to a nutrient solution with HS added at a concentration of 1 mg/l carbon (C), while controls were moved in fresh medium without HS. The HS concentration was chosen according to Nardi et al. (2000b) and in light of preliminary experiments (data not published). At the end of the treatment time, about 120 g of plant roots were collected, snap frozen in liquid nitrogen, and immediately treated for extraction.

Protein Extraction from Root Tissues and Two-Dimensional Polyacrylamide Gel Electrophoresis A total of eight independent samples, i.e., biological replicates (four control and four HS-treated), were extracted. Plasma membrane enriched fractions were obtained by two-phase partitioning (Sandelius and Morrè 1990; Ephritikhine et al. 2004). The sensitivity of the H-ATPase (Mg-dependent) activity to vanadate was used as a marker of PM purity. The activity that was sensitive to vanadate amounted to 83.3% (N=8) of the total ATPase activity, which was similar to the purity reported in other studies (Santoni et al. 1998). PM-enriched samples were submitted to phenol extraction followed by methanolic ammonium acetate precipitation, according to the method of Hurkman and Tanaka (1986). Proteins were then solubilized in 7 M urea, 2 M thiourea, 2% CHAPS, 2% DTT, and a protease inhibitor cocktail. The amount of protein in the PM fraction was assayed with the Biorad (Hercules, CA, USA) protein quantitation kit following the manufacturer's instructions.

After solubilization, isoelectric focusing (IEF) was performed with IPG strips (11 cm, pH range 3–10) by using IPGphor instrumentation following the manufacturer's instructions (GE Healthcare Bio-Science AB, Uppsala, Sweden). IPG strips were loaded with 100 μg protein, 2% ampholytes (pH range 3–10, GE Healthcare), 60 mM DTT, and bromophenol 121 blue (as a tracer) in 210 μl final volume. Rehydration was done overnight. Next day, a pre-focalization program was run at low voltage (300 V) for 2 hr to enable salt removal, then focalization was performed according to the manufacturer's instructions (GE Healthcare), followed by reduction and alkylation with

iodoacetoamide and equilibration in a solution containing 2% SDS, 15 mM DTT, 62 mM TRIS–HCl pH 6.8, and 10% glycerol. Strips were then placed on top of precast gels and sealed with agarose. Separation on the second dimension was done on polyacrylamide gradient precast gels (8–16%, Biorad) in electrophoretic chambers accommodating up to 12 gels at once (Criterion DodecaCel, Biorad). Running conditions: buffer 25 mM Tris-Base, 192 mM glycine, and 0.1% SDS; 20 mA per gel. Proteins were fixed at least 30 min in 10% methanol + 7% acetic acid. The fixing solution was changed at least twice to wash the gel. Gels were then incubated in undiluted Sypro Ruby (Biorad) stain overnight and rinsed with water. Each of the eight biological replicates was analyzed through three separate gels (technical replicates) for a total of 24 gels. Gel images were acquired with the Chemidoc system (Biorad).

Image and Data Analysis Gel images were analyzed with the Imagemaster 2D Platinum 6.0 software (GE Healthcare Amersham Biosciences AB, Uppsala, Sweden,) To select only high-quality protein spots for expression profiling, the following threshold criteria were applied: Each protein spot had to be present in at least three biological replicate gels per class (treated or untreated) and detected in at least six gels per class. Only spots with over 1.5-fold changes in volume after normalization between the two classes were defined as altered and further validated with the nonparametric Wilcoxon–Mann–Whitney test (Imagemaster 2D Platinum 6.0 software with either $P<0.05$ or $P<0.01$) as recommended in Wilkins et al. (2006). Spots appearing in only one class on visual inspection were also defined as newly induced or suppressed by the treatment.

In-Gel Digestion, Protein Identification and Database Search Excised from the gel spots were washed with 50% *v/v* acetonitrile (ACN) in 0.1 M NH_4HCO_3, and vacuum-dried. The gel fragments were reduced for 45 min at 55°C in 10 mM DTT in 0.1 M NH_4HCO_3. After cooling, the DTT solution was immediately replaced with 55 mM iodoacetamide in 0.1 M NH_4HCO_3. After washing with 50% ACN in 0.1 M NH_4HCO_3, the dried gel pieces were swollen in a minimum volume of 10 µl digestion buffer containing 25 mM NH_4HCO_3 and 12.5 ng/l trypsin (Promega, Madison, WI, USA) and incubated overnight at 37°C. Tryptic-digested peptides were extracted according to the protocol described by Kim et al. (2004).

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analyses were performed on a Micromass CapLC unit (Waters, Milford, MA, USA) interfaced with a Micromass Q-TOF Micro mass spectrometer (Waters) equipped with a nanospray source. For each 2D gel spot, 6.4 µl of tryptic digest were injected at a flow rate of 20 µl/min into an Atlantis dC18 Trap Column. After valve switching, the sample was separated in an Atlantis dC18 NanoEase column (Waters, 0.075×150 mm, 3-µm particle size) at a flow rate 200 nl/min with a gradient from 10% B to 55% B in 35 min (solvent A: 95% H_2O, 5% ACN, 0.1% formic acid; solvent B: 5% H_2O, 95% ACN, 0.1% formic acid). Instrument control, data acquisition, and processing were performed with MassLynx V4.0 software (Waters, Milford, MA, USA). Database searching was done with the on-line version of the Mascot program (www.matrixscience.com) by using the following parameters: one missed cleavage; 0.8 and 0.6 Da mass accuracy allowed for parent and fragment ions, respectively; carbamidomethyl as fixed modification; oxidized methionine as variable modification; 100 hits allowed. Proteins identified with at least two peptides, both showing a molecular weight search (MOWSE) score (Perkins et al. 1999) higher than 40, were considered as matched. For proteins identified by only one peptide with a MOWSE score higher than 40, the peptide sequence was routinely checked manually. All database searches were performed in both the National Center for Biotechnology Information (NCBI) complete database with no species specified, and in the NCBI Viridiplantae-specified database.

Results and Discussion

DRIFT Spectroscopy The DRIFT spectrum of the HS considered in this study (Fig. 1) shows an intense and broad band centered at 3,282 cm^{-1} attributed to water bridging of OH stretching of the phenolic hydroxyl groups or to stretching of the hydrogen-bonded OH and NH groups (Niemeyer et al. 1992); the shoulder at 3,089 cm^{-1} might be attributable to aromatic CH stretching, whose weak relative intensity may be due to the extensive substitution of aromatic rings in HS macromolecules and/or to masking from the broad band of the OH stretching. The peak at 2,930 cm^{-1} and the weaker shoulder at 2,850 cm^{-1} are attributable to CH asymmetric and symmetric stretching in aliphatic groups, respectively (Montecchio et al. 2006). The broad band at 2,626 cm^{-1} was attributed to the formation of intermolecular hydrogen bonding between OH groups in oxygenated compounds (Niemeyer et al. 1992). The sharp, strong band at 1,717 cm^{-1} is caused by C=O stretching vibration, mainly of COOH. This band assigned to C—O stretching and OH deformation and the band at 1,231 cm^{-1} are the most clearly distinct bands of the COOH group vibrations (Ding et al. 2002). In addition, the band at 1,231 cm^{-1} might also be due to C—OH stretching of the phenolic groups (Francioso et al. 2002). The strong band at 1,655 cm^{-1} might denote the presence of C=O stretching in quinonic and ketone (Gressel et al. 1995) or amide groups (Niemeyer et al. 1992). The peak at 1,542 cm^{-1} is due to secondary amide deformation (Gressel et al. 1995) and to

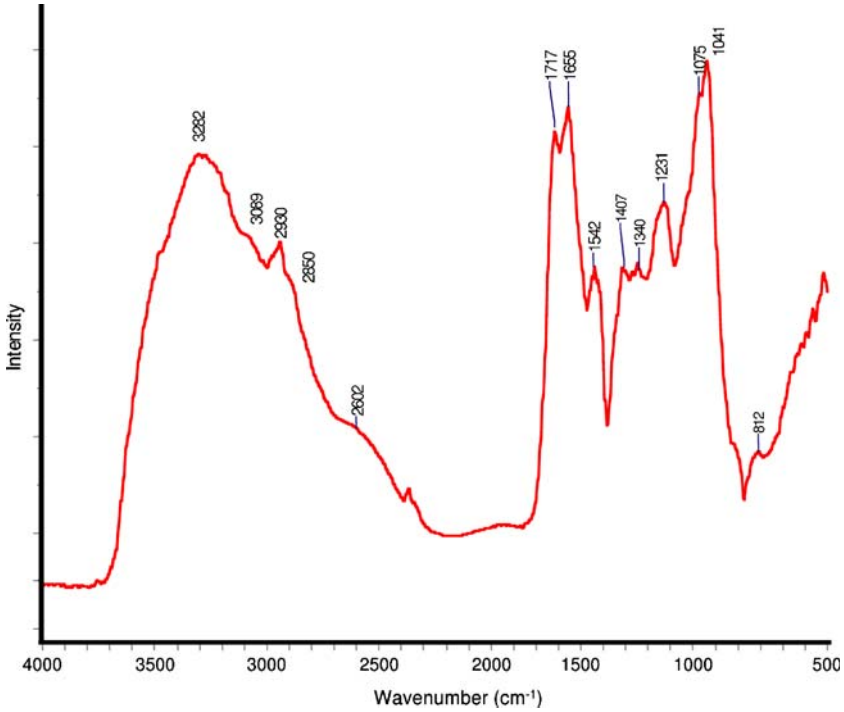

Fig. 1 Diffuse reflectance infrared Fourier Transform (DRIFT) spectroscopy spectrum for Humic Subtances (HS) extracted from earthworm feces

C=C stretching vibrations of aromatic moieties. The region between 1,400–1,300 cm^{-1} is assigned to CH_2 and CH_3 bending, C—OH deformation of COOH, and COO- symmetric stretching (Ding et al. 2002). The intense peak at around 1,040 cm^{-1} is attributed to C—O stretching of polysaccharide-like components. Taken together, these findings show that this humic fraction is endowed with the characteristic structural network described for most HS isolated from different sources of organic matter (Clapp and Hayes 1999).

Proteomic Analysis In many cases, plant gene expression modifications are induced by changes in nutrient availability. In particular, Ca^{++} affects the activity of many actin-binding proteins, and HS are able to chelate cations. In our case study, the Ca^{++} was provided in the nutrient solution as 0.2 mmol/L of $Ca(NO_3)_2$. Both treated and untreated plants were grown in the same medium and HS ash content was very low (<1%). Thus, the reported effects in the proteome could be regarded neither as Ca-dependent nor as related to improved nutrient contents following HS addition.

Image analysis of the 24 sypro-stained gels enabled the detection of approximately 480–550 spots on each gel. By using the threshold criteria (see Methods and Materials), a total of 325 protein spot groups were matched, manually validated, and quantified. Sixty-three spots were found affected by HS treatment, representing more then 10% of the total proteins detected on each gel. Twenty-two spots were upregulated by the HS treatment, whereas 38 were downregulated (Fig. 2). The most upregulated spot (ID 370) represented a 2.92-fold enhancement, whereas the most downregulated protein (ID 1272) resulted in a 2.53-fold diminishment (Table 1). Moreover, two spots appeared to be suppressed by HS treatment, whereas one spot was detected only in gels from treated plants. These 63 spots (Fig. 3) were excised from the gels, digested with trypsin, and the peptides were analyzed by LC-MS-MS, thus enabling the identification of 42 protein species (some of which are illustrated in Fig. 4). In most cases, the database search revealed that the experimental p*I* and M_r values of the identified proteins matched their theoretical values (Table 1). In a few cases, a discrepancy was observed, but this happens frequently in proteomic analyses and is probably a consequence of posttranslational modification, proteolytic cleavage, or matching proteins from different species. In this expression study, proteins were isolated from plasma membrane-enriched fractions. Although the purity was good and in line with other studies (Santoni et al. 1998), some contamination from other cell components certainly occurred. Even if some of the identified protein species have been previously described from other subcellular compartments, the reproducibility and statistical analysis of the expression data indicate that the differences depended on HS treatment.

According to the FunCat functional classification (Ruepp et al. 2004), the proteins identified were grouped into the following categories: energy and metabolism; cellular transport, transport facilitation and transport routes;

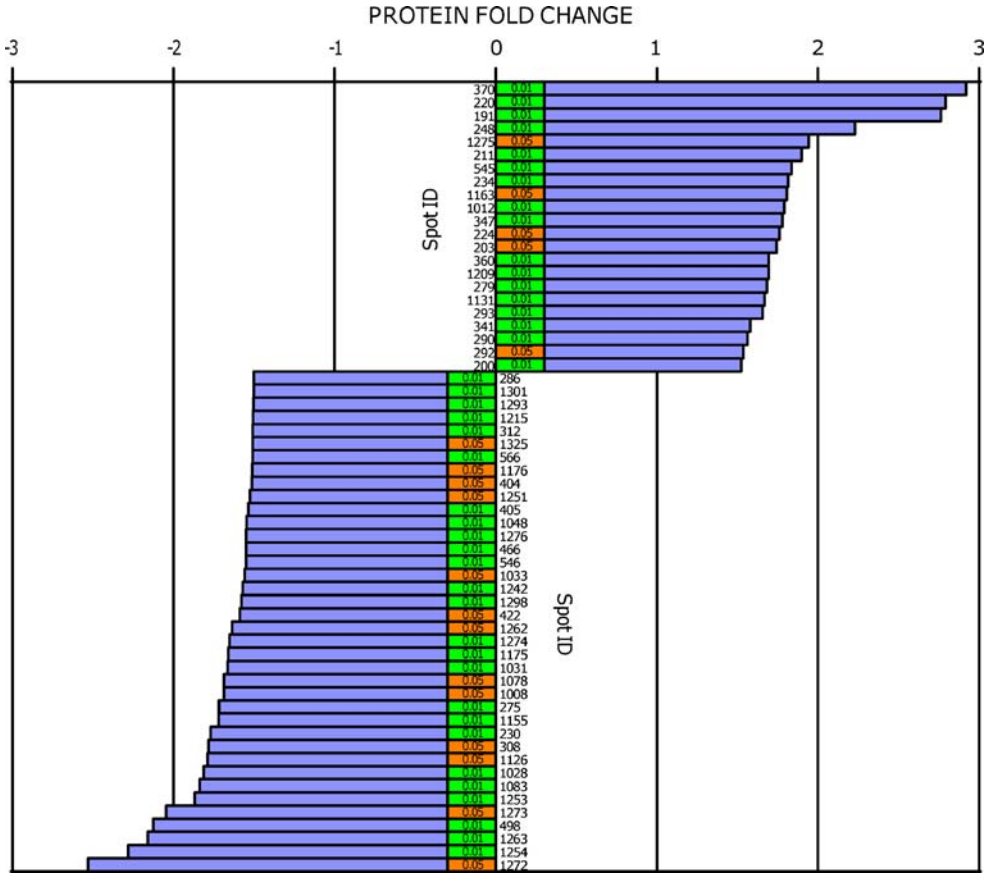

Fig. 2 Relative intensities, spot ID, and *P* values (Wilcoxon–Mann–Whitney test) for the 60 differentially expressed protein spots

interaction with the environment, signaling, defense, cell rescue; and unclassified proteins (Table 1). Below, we briefly discuss the possible significance of these protein groups and the interpretation of changes in abundance of the protein species.

Energy and Metabolism Glycolytic enzymes have long been considered to exist as soluble proteins in the cytosolic compartment, but in recent years it has been suggested that the cytoskeleton and/or membranes contribute to this compartmentalization (Holtgrawe et al. 2005). Protein–protein interactions exist between actin or tubulin and a number of glycolytic enzymes and enzymes of sucrose metabolism (Holtgrawe et al. 2005) that have also been found to co-localize (Anderson and Carol 2005), so it is hardly surprising to find these enzymes in PM-enriched fractions (Santoni et al. 1998, 1999b). In the present study, the enzymes identified were triosophosphate isomerase (TIM; EC 5.3.1.1), glyceraldehyde-3-P dehydrogenase (GAPD; EC 1.2.1.12), phosphoglycerate mutase (PGAM; EC 5.4.2.1), fructose biphosphate aldolase (FBA; EC 4.1.2.13), 2-phosphoglycerate dehydratase (enolase, ENO; EC 4.2.1.11), phosphoglucomutase (PGM; EC 5.4.2.2), and 3-phosphoglycerate kinase (PGK; EC 2.7.2.3). In our experimental conditions, all of the above-mentioned enzymes appear to be downregulated after HS treatment except for TIM, which showed a 69% increase, and GAPD, which maintained the same level of expression in treated and untreated samples. An effect of HS on enzymes involved in the glycolytic/gluconeogenic pathways has already been reported (Muscolo et al. 2007a), and it was thought to impair these pathways. This impairment may affect starch and sucrose metabolism. Three spots (IDs 1251; 1253; 1254) match with sucrose synthase (SUS; EC 2.4.1.13). Spot 546 has been identified as fructokinase-1 (FRK; EC 2.7.1.4) and spot 1298 as PGM, representing the link between glycolysis and starch synthesis pathways. These two proteins were downregulated by HS treatment. SUS can associate with actin, the cytoskeleton, and the plasma membrane, where it is thought to funnel uridine diphosphoglucose (UDP-glucose) to cellulose synthase to produce cellulose (Amor et al. 1995). Merlo et al. (1991) reported that SUS activity appeared to be lower in HS-treated maize leaves. The depletion of these enzymes after HS treatment in our experimental conditions may indicate a slackening of the energy machinery.

Malate has a central role in the energetics of the plant cell, and malate dehydrogenase (MDH; EC 1.1.1.37) is one of the key enzymes in malate metabolism. Sukalovic et al. (1999) found that purified PMs isolated from maize roots contain a

Table 1 HS-induced differentially expressed maize root PM proteins identified by LC-MS/MS analysis

Spot ID	Protein fold change[a]	Accession Number[b]	Identified Protein[c]	SC (%)[d]	PM[e]	Theoretical *M*r/p*I*	Experimental *M*r/p*I*	Protein Classification	Peptide sequences[f]
370	+2.92	YP_588408	ATPase subunit 1	10	3	55.4/5.8	53.0/5.8	Cellular transport, transport facilitation and transport routes	VVDALGVPIDGK AAELTTLLESR NILSTINPELLK
545	+1.83	CAA66901	Annexin p35	13	1	35.5/6.8	36.5/6.5	Cellular transport, transport facilitation and transport routes	TPAQLFAVK (*m*/*z*=487.7368; z=2; error (Da)=−0.1006)[i]
1012	+1.79	CAA66901	Annexin p35	17	5	35.5/6.8	35.5/6.8	Cellular transport, transport facilitation and transport routes	TPAQLFAVK LLVPLVSAYR AQLLATFNSYK AVILWTLDPAER DYEDI*M*LALLGAE
224	+1.76	P49608	Aconitate hydratase, cytoplasmic (ACO) (*Cucurbita maxima*)	3	2	98.6/5.7	99.8/5.8	Energy and metabolism	SDETVS*M*IEAYLR SNLVG*M*GIIPLCFK
1209	+1.69	P12863	Triosephosphate isomerase, cytosolic (TIM)	24	5	27.2/5.5	27.0/5.5	Energy and metabolism	VAYALSQGLK TNASPEVAESTR VIACVGETLEQR ALLGESNEFVGDK EAGST*M*DVVAAQTK
1131	+1.67	CAN68719	Hypothetical protein (*Vitis vinifera*)	13	2	34.5/6.3	35.1/6.1	Unclassified proteins	AVQEEDELPPEK LINPQT*MM*LGQEPR
341	+1.58	P49087	Vacuolar ATP synthase catalytic subunit A	27	10	62.2/5.9	59.8/5.7	Cellular transport, transport facilitation and transport routes	SGDVYIPR FDPDFIDIR LYDDLTTGFR LAADTPLLTGQR KLYDDLTTGFR EDDLNEIVQLVGK TTLVANTSN*M*PVAAR EASIYTGITIAEYFR LAE*M*PADSGYPAYLAAR ISYIAPAGQYNLQDTVLELEFQGIK
290	+1.56	CAA31663	Heat shock protein (HSP) 70 (*Petunia x hybrida*)	4	2	71.1/5.1	71.1/4.6	Interaction with the environment, signalling, defense, cell rescue	VEIIANDQGNR NQVA*M*NPINTVFDAK
488	+1.01	Q09054	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 2 (GAPD)	31	3	36.6/6.4	41.3/6.4	Energy and metabolism	VVISAPSK AASFNIIPSSTGAAK VPTVDVSVVDLTVR

286	−1.50	AAL40137	Phenylalanine ammonia-lyase (PAL)	20	11	75.3/6.5	72.2/5.9	Interaction with the environment, signalling, defense, cell rescue	ELISAIDR VFVGISQGK KVDAAEAFK VGQVAAVASAK NPSLDYGFK DGPALQVELLR VNELDPLLKPK TKDGPALQVELLR EVNSVNDNPVIDVHR DASGVAVELDEEARPR EAVFTYAEDAASASLPL*M*QK
1301	−1.50	P49101	Calcium-dependent protein kinase 2 (CDPK)	2	1	58.3/6.1	54.8/5.7	Interaction with the environment, signalling, defense, cell rescue	DIVGSAYYVAPEVLR (m/z=826.4362; z=2; error (Da)=−0.0039)[i]
1293	−1.50	P30792	Phosphoglycerate mutase (PGAM)	5	2	60.7/5.3	72.2/5.6	Energy and metabolism	LDQLQLLLK YLVSPPEIDR
1215	−1.51	AAL57038	UDP-glucosyltransferase BX9	22	6	50.5/5.2	47.2/5.2	Unclassified proteins	LSALLAAEGR TDLTDLVDLIK ALSVPVFAVAPLNK GFESGALPDGVEDEVR VDTSDLEEFAELLAR LLASEDIAAIVTTLNASCDAPFR
312	−1.51	P49087	Vacuolar ATP synthase catalytic subunit A (V-ATPase)	33	10	62.2/5.9	65.9/5.4	Cellular transport, transport facilitation and transport routes	LASFYER SGDVYIPR TVISQALSK FDPDFIDIR LYDDLTTGFR LAADTPLLTGQR EDDLNEIVQLVGK TTLVANTSN*M*PVAAR D*M*GYNVS*MM*ADSTSR EASIYTGITIAEYFR NIIHFNTLANQAVER LVSQKFEDPAEGEEALVGK EVLQREDDLNEIVQLVGK
1325	−1.51	P08440	Fructose-bisphosphate aldolase, cytoplasmic isozyme (FBA)	17	3	39.0/7.5	43.7/6.2	Energy and metabolism	NAAYIGTPGK VTPEVIAEYTVR IGPNEPSQLAIDLNAQGLAR
566	−1.51	AAF71261	Beta-glucosidase aggregating factor (BGAF)	6	1	31.9/6.1	33.6/5.9	Unclassified proteins	AGVYLDALGVYVR (m/z=698.3304; z=2; error (Da)=−0.1095)[i]
404	−1.51	Q02245	Tubulin alpha chain (Alpha-tubulin)	18	4	50.5/4.9	49.9/5.2	Cellular transport, transport facilitation and transport routes	DVNAAVATIK EIVDLCLDR LISQIISSLTTSLR

Table 1 (continued)

Spot ID	Protein fold change[a]	Accession Number[b]	Identified Protein[c]	SC (%)[d]	PM[e]	Theoretical *M*r/p*I*	Experimental *M*r/p*I*	Protein Classification	Peptide sequences[f]
									AIFVDLEPTVIDEVR
		P18025	Tubulin beta-chain (Beta tubulin)	11	3	50.5/4.8	49.9/5.2		YLTASA*M*FR FPGQLNSDLR EVDEQ*M*LNVQNK
1251	−1.53	P49036	Sucrose synthase 2 (SUS)	22	9	93.5/6.0	87.1/6.1	Energy and metabolism	SLSALQGALR A*M*ENE*M*LLR ASALLVDFFDK NLTGLVELYGR LKDGAFEDVLR VNVSELAVEELR LLPDATGTTCGQR T*M*ASTVPLAVEGEPSSK IGDSLSAHPNELVAVFTR
		P04712	Sucrose synthase 1 (SUS) (Shrunken-1)	17	5	92.1/6.0	87.1/6.1		ALENE*M*LLR *M*YSLIDEYK YIE*M*FYALK AADILVNFFDK VIGTEHTDIIR
405	−1.54	Q02245	Tubulin alpha-5 chain (Alpha-5 tubulin)		5	50.2/4.9	48.9/5.2	Cellular transport, transport facilitation and transport routes	EDAANNFAR EIVDLCLDR LISQIISSLTTSLR AIFVDLEPTVIDEVR AVC*M*ISNNTAVAEVFSR
		P33627	Tubulin alpha-6 chain (Alpha-6 tubulin)		5	50.2/4.9	48.9/5.2		EDAANNFAR EIVDLCLDR LISQIISSLTTSLR AIFVDLEPTVIDEVR AVC*M*ISNNTAVAEVFSR
		P14640	Tubulin alpha-1 chain (Alpha-1 tubulin)		4	50.2/4.9	48.9/5.2		EDAANNFAR LISQIISSLTTSLR AIFVDLEPTVIDEVR AVC*M*ISNNTAVAEVFSR
1048	−1.55	P49106	14-3-3-like protein GF14-6	49	9	29.7/4.7	27.4/4.8	Interaction with the environment, signalling, defense, cell rescue	NLLSVAYK DSTLI*M*QLLR DAAENT*M*VAYK KDAAENT*M*VAYK TVDSEELTVEER LLETHLVPSSTAPESK AAQDIALAELAPTHPIR

									QAFDEAISELDTLSEESYK
1276	−1.55	Q08062	Malate dehydrogenase, cytoplasmic (MDH)	13	3	35.9/5.8	39.9/5.7	Energy and metabolism	DNLTLWTSDISEDPAEEIR LNVQVSDVK IVQGLPIDEFSR VLVVANPANTNALILK
466	−1.55	P26301	Enolase 1 (ENO) (2-phosphoglycerate dehydratase 1)	3	1	48.2/5.2	43.0/5.7	Energy and metabolism	VQIVGDDLLVTNPTR (m/z-820.4318; z-2; error (Da)=−0.0451)[i]
546	−1.55	Q6XZ79	Fructokinase-1 (FRK)	8	1	34.8/4.9	36.5/5.0	Energy and metabolism	APGGAPANVAIAVSR (m/z=675.8791; z=2; error (Da)=0.0021)[i]
1033	−1.56	P49076	ADP-ribosylation factor (ARF)	16	2	20.7/6.3	17.7/5.9	Interaction with the environment, signalling, defense, cell rescue	DAVLLVFANK IL*M*VGLDAAGK
1298	−1.58	P93804	Phosphoglucomutase, cytoplasmic 1 (Glucose phosphomutase 1) (PGM)	22	8	63.3/5.4	66.6/5.6	Energy and metabolism	GATIVVSGDGR LVTVEDIVR LSGTGSVGATIR S*M*PTSAALDVVAK YDYENVDAGAAK DSQDALAPLVDVALK ATTPFDGQKPGTSGLR YN*M*GNGGPAPESVTDK
		P93805	Phosphoglucomutase, cytoplasmic 2 (Glucose phosphomutase 2) (PGM)	20	7	63.2/5.4	66.6/5.6		GATIVVSGDGR LVTVEDIVR LSGTGSVGATIR S*M*PTSAALDVVAK YDYENVDAGAAK DSQEALAPLVDVALK YN*M*GNGGPAPESVTDK
422	−1.59	AAL57038	UDP-glucosyltransferase BX9	24	6	50.5/5.2	46.9/5.3	Unclassified proteins	LSALLAAEGR FVPVTVEADPK TDLTDLVDLIK VGTELVGEQLER GFESGALPDGVEDEVR VDTSDLEEFAELLAR
1262	−1.64	ABC59687	Lipoxygenase (LOX)	20	10	100.5/6.2	100.5/6.2	Interaction with the environment, signalling, defense, cell rescue	EADAVFIR *M*PAALVPYR DTLNINALAR *M*SDFLGYSLK ETLAGVNPVLIK AAHLEEAVVSLK QTLINADGIFER VFFANDTYLPSK GVAVPDQSSPYGVR

Table 1 (continued)

Spot ID	Protein fold change[a]	Accession Number[b]	Identified Protein[c]	SC (%)[d]	PM[e]	Theoretical *M*r/p*I*	Experimental *M*r/p*I*	Protein Classification	Peptide sequences[f]
									INELEGNFIYASR
1274	−1.65	AAO32643	Cytosolic 3-phosphoglycerate kinase (PGK)	33	5	31.6/5.0	46.2/5.7	Energy and metabolism	IGVIESLLAK ELDYLVGAVANPK GVSLLLPTDIVVADK GVTTIIGGGDSVAAVEK LAAALPEGGVLLLENVR
1031	−1.66	P80639	Eukaryotic translation initiation factor 5A (eIF-5A)	10	2	17.7/5.6	19.0/5.7	Unclassified proteins	LPTDETLVAQIK DDLRLPTDETLVAQIK
1078	−1.69	AAT42176	Putative 3-glucanase	10	4	52.4/4.8	52.5/4.8	Unclassified proteins	SVAPGNFER YA*M*DLSGQGR ALNDAGFGDTIK ATVPLNADVYNSPK
275	−1.72	AAL33589	Methionine synthase (MS)	39	20	84.7/5.7	80.2/5.7	Energy and metabolism	YLFAGVVDGR IPSAEEIADR FALESFWDGK ISEEEYVTAIK FETCYQIALAIK IPSAEEIADRIDK DEAYFAANAAAQASR GTQTLGLVTSAGFPAGK YGAGIGPGVYDIHSPR YTEVKPALTN*M*VSAAK G*M*LTGPVTILNWSFVR TLTSLSSVTAYGFDLVR KYTEVKPALTN*M*VSAAK ISEEEYVTAIKEEINK LQEELDIDVLVHGEPER ALAGQKDEAYFAANAAAQASR LNLPILPTTTIGSFPQTVELR LVVSTSCSL*M*HTAVDLVNETK KLNLPILPTTTIGSFPQTVELR YIPSNTFSYYDQVLDTTA*M*LGAVPER
1155	−1.72	AAR33048	Allene oxide synthase (AOS)	9	3	53.2/6.5	51.3/6.2	Interaction with the environment, signalling, defense, cell rescue	VTLAAVER QALDTAEGLGLSR VFGDTAGDFVPDR
230	−1.77	P49608	Aconitate hydratase, cytoplasmic (ACO) [Cucurbita maxima]	5	3	98.5/5.7	99.0/5.9	Energy and metabolism	TSLAPGSGVVTK SDETVS*M*IEAYLR SNLVG*M*GIIPLCFK

308	−1.78	P49087	Vacuolar ATP synthase catalytic subunit A (V-ATPase)	23	5	62.2/5.9	66.6/5.4	Cellular transport, transport facilitation and transport routes	FDPDFIDIR LYDDLTTGFR LAADTPLLTGQR TTLVANTSN*M*PVAAR LAE*M*PADSGYPAYLAAR
1083	−1.84	P49106	14-3-3-like protein GF14-6	17	4	29.7/4.7	29.8/4.8	Interaction with the environment, signalling, defense, cell rescue	NLLSVAYK DSTLI*M*QLLR DAAENT*M*VAYK AAQDIALAELAPTHPIR
1253	−1.87	P04712	Sucrose synthase 1 (SUS) (Shrunken-1)	5	2	92.1/6.0	87.1/6.1	Energy and metabolism	ALENE*M*LLR AADILVNFFDK
498	−2.13	Q08062	Malate dehydrogenase, cytoplasmic (MDH)	19	2	35.9/5.8	41.1/5.7	Energy and metabolism	LNVQVSDVK *M*ELVDAAFPLLK
1263	−2.16	ABC59687	Lipoxygenase (LOX)	10	7	100.5/6.2	100.5/6.1	Interaction with the environment, signalling, defense, cell rescue	DTLNINALAR STTDGETVYR ETLAGVNPVLIK QTLINADGIFER VFFANDTYLPSK GVAVPDQSSPYGVR INELEGNFIYASR
1254	−2.28	P49036	Sucrose synthase 2 (SUS)	10	3	93.5/6.0	87.7/6.0	Energy and metabolism	A*M*ENE*M*LLR VNVSELAVEELR N*M*TGLVE*M*YGK
544	N[g]	CAA66900	Annexin p33	19	3	35.7/7.13	36.6/6.2	Cellular transport, transport facilitation and transport routes	SITDEISGDFER AYAEAYGEELLR QAIAGLGTDENSLTR
		CAA66901	Annexin p35	18	2	35.5/6.8	36.6/6.2		TPAQLFAVK LLVPLVSAYR
1356	ST[h]	CAN75785	Hypothetical protein (*Vitis vinifera*)	10	3	48.4/6.5	52.4/6.2	Unclassified proteins	NPLDLLPPSK YNDENTVSFVTLNK VPVLETPDGPVFESNAIAR
1369	ST[h]	AAB40105	Actin	4	1	37.2/5.5	55.6/4.7	Cellular transport, transport facilitation and transport routes	SYELPDGQVITIAADR (*m*/*z*=874.3623; z=2; error (Da)=−0.1688)[i]

[a] Increased or decreased (±) compared to the control.
[b] NCBI accession number.
[c] Proteins have been identified in *Zea mays* L. protein database when no species is reported.
[d] Sequence coverage. Peptides with individual ion MOWSE scores lower then the threshold indicating identity or extensive homology ($P<0.05$) have not been reported, but have been considered in sequence coverage calculation.
[e] Number of peptide matches.
[f] Underscored methionine in sequences reported indicate oxidation.
[g] Newly induced.
[h] Suppressed by the treatment.
[i] In instances where proteins were identified based on a single peptide, precursor mass, charge, and mass error observed are provided.

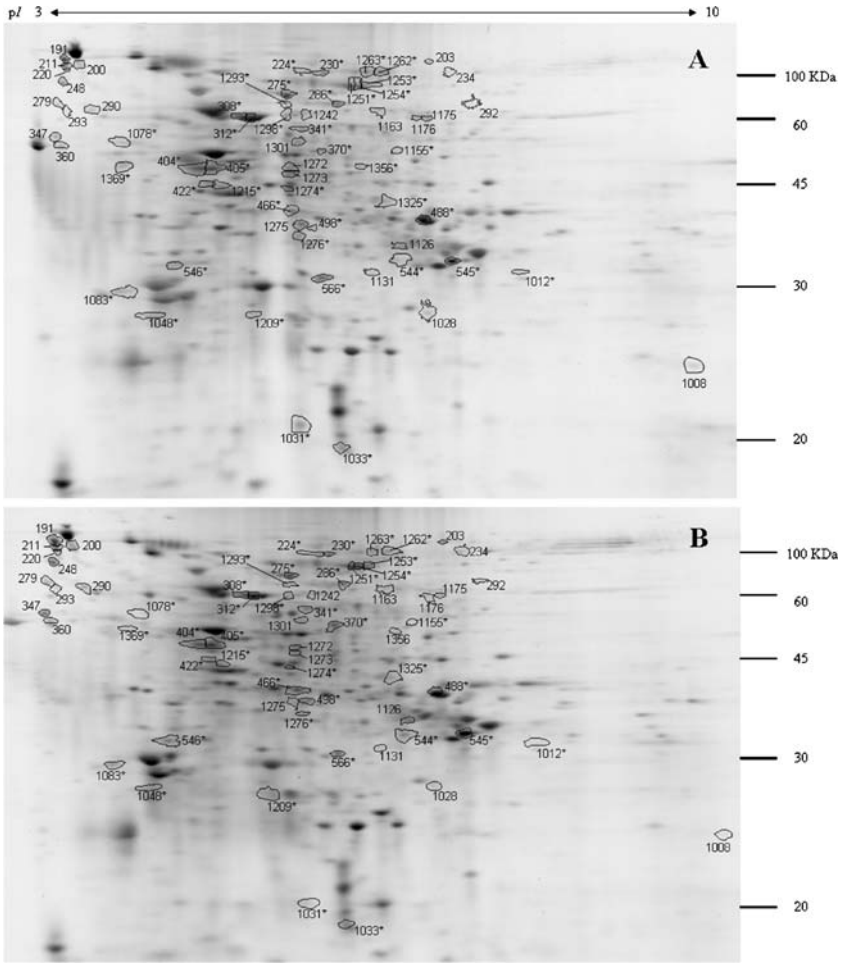

Fig. 3 Two-dimensional polyacrylamide gel electrophoresis (2-DE) analysis of maize, *Zea mays*, root plasma membrane proteins after HS-treatment. One hundred micrograms of protein were extracted and separated by 2-DE, as described in "Methods and Materials" and visualized by Sypro-Ruby staining. A and B show 2-DE gel patterns of protein samples prepared from control and HS-treated roots, respectively. Numbers indicate differentially expressed proteins subjected to LC-MS-MS analysis. Numbers marked with asterisk indicate identified proteins

closely associated, deeply buried, NAD^+-dependent MDH, and this enzyme was also identified in *Arabidopsis* PM-enriched fractions (Santoni et al. 1998). This enzyme is involved in the plasma membrane redox system (Berczi and Moller 2000). Spots 498 and 1276 identified as MDH, showed downregulation after HS treatment in both cases. An *in vitro* inhibition of this enzyme has already been reported after treatment with HS (Pflug and Ziechmann 1981), whereas activity was reportedly stimulated in carrot cell cultures and, more recently, in maize leaves (Nardi et al. 2007).

Aconitase (ACO; EC 4.2.1.3) catalyzes the reversible isomerization of citrate to isocitrate via the intermediate product *cis*-aconitate. There are two isoforms of ACO; the mitochondrial isoform is a component of the citric acid cycle, whereas the cytosolic isoform participates in the glyoxylate cycle (Moeder et al. 2007). We identified two adjacent protein spots as cytosolic ACO from *Cucurbita maxima*, one showing downregulation (spot 230, −77%) and the other upregulation (spot 224, +79%). This result is suggestive of a posttranslational modification of this enzyme. Citric acid is a component of root exudates and plays an important role in disaggregating HS (Nardi et al. 2000a). ACO expression may induce changes in root excretion participating in the rhizospheric cross-talk between plant and soil. Methionine synthase (MS, EC 2.1.1.14) was found as a downregulated protein: the function of this enzyme is the *de novo* synthesis of methionine on one hand and, on the other, the regeneration of the methyl group of *S*-adenosyl methionine from *S*-adenosyl homocysteine after methylation reactions (Hesse et al. 2004). Although cytosolic and plastid isoforms have been reported, this enzyme has also been identified in PM extracts (Santoni et al. 1999b). Muscolo and Nardi (1997) found that HS-treated plants showed no change in methionine concentrations.

Cellular Transport, Transport Facilitation and Transport Routes Annexin, actin and tubulin isoforms were identified in our samples. These proteins have been reported already in studies on the *Arabidopsis* plasma membrane proteome (Santoni et al. 1999a, 2000; Marmagne et al. 2004). Actin and tubulin are major components of the cytoskeleton, forming microfilaments and microtubules, respectively. The cytoskeleton coordinates all aspects of growth in plant cells, including exocytosis of the membrane and wall components

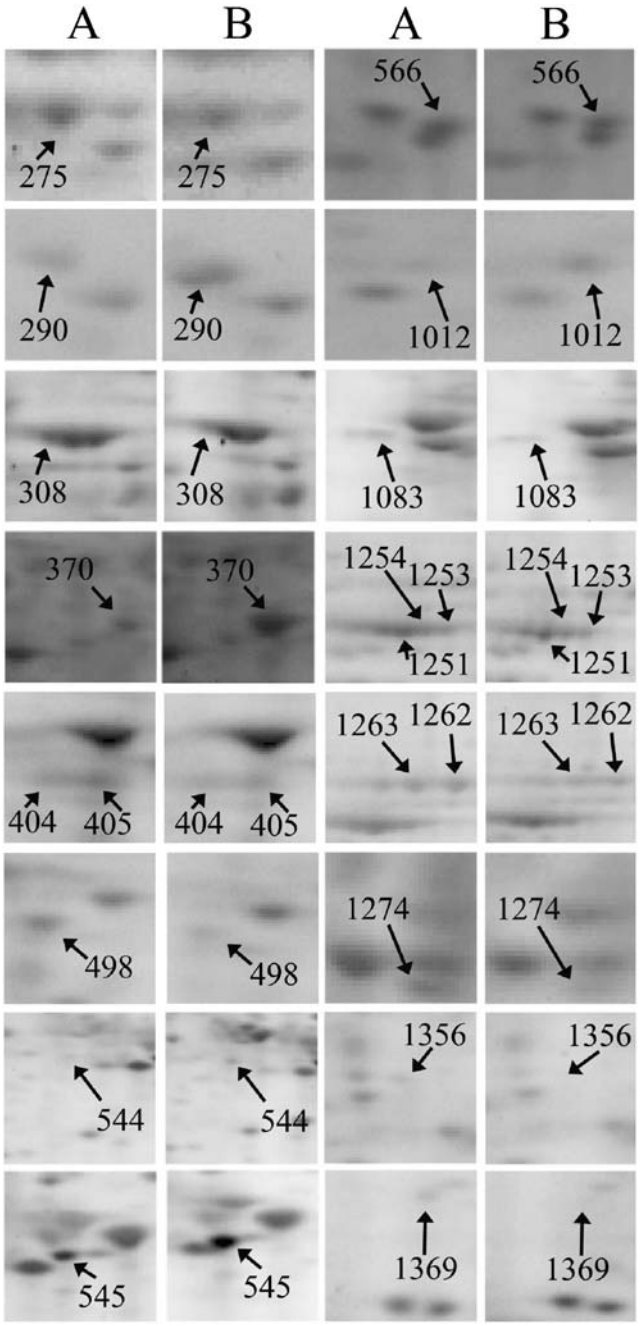

Fig. 4 Magnified views of some differentially expressed proteins in maize roots plasma membrane in response to HS treatment. Plants were controls (column A) or treated with HS for 16 hr (column B) (see "Methods and Materials")

during cell expansion. Both proteins are reported to be associated with plasma membranes and also an interaction between the two proteins has been demonstrated by Wasteneys and Galway (2003). It is generally assumed that annexins are implicated in several processes related to membranes, including the regulation of membrane organization, membrane trafficking, interactions with the cytoskeleton, and secretion (Konopka-Postupolska 2007), but the primary physiological function of annexins has yet to be elucidated. Certain proteins from this family were identified as actin binding, making them ideal mediators in cell membrane and cytoskeleton interactions (Konopka-Postupolska 2007). In this study, the protein spots 545 and 1012, identified as annexin, were upregulated by about 80%. Spot 544, also identified as annexin, was detectable in 10 of the 12 gels generated from treated roots and in none of the gels obtained from control samples. The fact that multiple spots in the same gel area are recognized as annexin may be due to posttranslational modifications, e.g., phosphorylation has been reported for maize annexin (Delmer and Potikha 1997). Due to the role of annexins in cell exocytosis and cell expansion, their upregulation may be correlated with modifications in plant metabolism leading to humic substances-induced stimulation of plant growth (Canellas et al. 2002). Spot 1369, identified as actin, was detectable in seven of the gels obtained from control samples and in none of the HS-treated ones: this indicates that PM-associated actin is low in untreated plants and drops to below the detection limits in treated plants. Spots identified as tubulin showed a depletion of about 53% in the gels obtained from treated plants. Alpha tubulin expression was reported to be modulated by cell sugar content (Datta and Chourey 2001), so this result may correlate with the previously shown modifications in the enzymes of the sucrose pathway. These effects point to an involvement of the cytoskeleton proteins in the response to HS. The responsiveness of the cytoskeleton to environmental cues has been widely documented (for a review, see Wasteneys and Galway 2003).

ATPases are membrane-bound ion transporters that couple ion movement through a membrane with the synthesis or hydrolysis of ATP. Different forms of membrane-associated ATPases have evolved over time to meet specific cell demands. V-ATPases hydrolyze ATP to drive a proton pump but cannot work in reverse to synthesize ATP. They are involved in a variety of vital intra- and intercellular processes such as receptor-mediated endocytosis, protein trafficking, and active transportation of metabolites. The F-ATPases ($F_{(0)}F_{(1)}$-ATPases) can synthesize ATP by using a H^+ gradient, and they work in reverse to create a H^+ gradient by using the energy gained from the hydrolysis of ATP. Subunits belonging to either the V_1 vacuolar sector or the F_1 mitochondrial complex of ATPase were found among the differentially expressed proteins in this research: these proteins are classically thought to reside in the tonoplast (Nelson and Harvey 1999) and in the mitochondrial membrane, respectively, although they were found in the PM in other organisms (Nelson and Harvey 1999). The presence of these two proteins either reveals a true contamination caused by technical problems or it reflects

functional interactions between the PM and these particular structures (Marmagne et al. 2004). Moreover, V-ATPase subunits have been identified already in highly purified plasma membrane extracts (Santoni et al. 1998; Marmagne et al. 2004). In the case of the regulatory alpha subunit of mitochondrial ATP synthase that shows an almost three-fold relative increase in the treated samples, this effect may be caused by an enhanced metabolic energy demand in response to HS. In the case of vacuolar ATPase, the result is apparently contradictory, given the presence of two down-regulated spots (308 and 312, −78% and −51%, respectively) and one upregulated spot (341, +58%). This variation may result from posttranslational modifications of the protein that could not be resolved in this work. In most of the previous cases, the results were attributed to the plasma membrane E_1-E_2-type ATPase, but changes in vacuolar (Pinton et al. 1992) and mitochondrial (Visser 1987) ATPases have also been found.

Others A number of the differentially expressed proteins are involved in the plant's interaction with the environment, while the functions of others have yet to be clarified. The interpretation of the changes in their abundance after the HS treatment is more challenging because of the complex network of signaling pathways. In some cases, the changes observed might reflect interesting aspects of plant metabolism that deserve further study. For example, beta-glucosidase-aggregating factor (BGAF) (spot 566, lowered by the treatment) interacts with beta-glucosidases (Kittur et al. 2007), which may release IAA from its glycosidic form, thus influencing IAA availability and possibly inducing a hormonal effect (Pizzeghello et al. 2001). Phenylalanine ammonia-lyase (PAL) expression was also affected by HS. As it links the primary metabolism of aromatic amino acids with the secondary metabolism of the phenylpropanoid pathway, its downregulation can be interpreted as forcing carbon skeletons toward energy metabolism.

To the best of our knowledge, this is the first proteomic analysis of changes induced in maize root plasma membranes after exposure to HS. A total of 42 differentially expressed proteins were identified. We focused on the proteins that relate to energy and metabolism, and cellular transport, transport facilitation, and transport routes. The majority of the proteins identified were downregulated. HS appeared to affect sucrose metabolism, malate dehydrogenase, ATPases, and cytoskeletal proteins. In some cases, our data better illustrate effects already reported in more simple models (i.e., *Nicotiana tabacum* callus), whereas in other cases, they enable a more detailed description of previous findings, or help to explain earlier results reported on the level of enzyme activity.

HS concentration and time of incubation are major parameters in unraveling plant responses to HS. Given the theoretical and practical limitations of proteomics, future investigations should consider these issues. Moreover, to investigate completely the HS/maize system, changes on the m-RNA level in HS-treated plants should also be considered in future research.

The proteins analyzed in this study are only a small part of the maize proteome; many other HS target proteins remain to be identified. However, the HS-responsive proteins in the root PM of *Z. mays* reported in this work can serve as a platform for future work investigating plant reactions to HS.

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References

AMOR, Y., HAIGLER, C. H., JOHNSON, S., WAINSCOTT, M., and DELMER, D. P. 1995. A membrane-associated form of sucrose synthase and its potential role in synthesis of cellulose and callose in plants. *Proc. Natl. Acad. Sci. USA* 92:9353–9357.

ANDERSON, L. E., and CAROL, A. A. 2005. Enzyme co-localization in the pea leaf cytosol: 3-P-glycerate kinase, glyceraldehyde-3-P dehydrogenase, triose-P isomerase and aldolase. *Plant Sci.* 169:620–628.

BERCZI, A., and MOLLER, I. M. 2000. Redox enzymes in the plant plasma membrane and their possible roles. *Plant Cell Environ.* 23:1287–1302.

CANELLAS, L. P., OLIVARES, F. L., OKOROKOVA-FACANHA, A. L., and FACANHA, A. R. 2002. Humic acids isolated from earthworm compost enhance root elongation, lateral root emergence, and plasma membrane H+-ATPase activity in maize roots. *Plant Physiol.* 130:1951–1957.

CESCO, S., ROMHELD, V., VARANINI, Z., and PINTON, R. 2000. Solubilization of iron by water-extractable humic substances. *J. Plant Nutr. Soil Sci.* 163:285–290.

CLAPP, C. E., and HAYES, M. H. B. 1999. Sizes and shapes of humic substances. *Soil Sci.* 164:777–789.

DATTA, R., and CHOUREY, P. S. 2001. Sugar-regulated control of alpha-tubulin in maize cell suspension culture. *Plant Cell Rep.* 20:262–266.

DELMER, D. P., and POTIKHA, T. S. 1997. Structures and functions of annexins in plants. *Cell. Mol. Life Sci.* 53:546–553.

DING, G., NOVAK, J. M., AMARASIRIWARDENA, D., HUNT, P. G., and XING, B. S. 2002. Soil organic matter characteristics as affected by tillage management. *Soil Sci. Soc. Am. J.* 66:421–429.

EPHRITIKHINE, G., FERRO, M., and ROLLAND, N. 2004. Plant membrane proteomics. *Plant Physiol. Biochem.* 42:943–962.

FAO-UNESCO. 1990. World Soil Map, Revised Legend. FAO, Rome, Italy.

FRANCIOSO, O., SANCHEZ-CORTES, S., CASARINI, D., GARCIA-RAMOS, J. V., CIAVATTA, C., and GESSA, C. 2002. Spectroscopic study of humic acids fractionated by means of tangential ultrafiltration. *J. Mol. Struct.* 609:137–147.

GRESSEL, N., INBAR, Y., SINGER, A., and CHEN, Y. 1995. Chemical and spectroscopic properties of leaf-litter and decomposed organic-matter in the Carmel Range, Israel. *Soil Biol. Biochem.* 27:23–31.

HESSE, H., KREFT, O., MAIMANN, S., ZEH, M., and HOEFGEN, R. 2004. Current understanding of the regulation of methionine biosynthesis in plants. *J. Exp. Bot.* 55:1799–1808.

HOLTGRAWE, D., SCHOLZ, A., ALTMANN, B., and SCHEIBE, R. 2005. Cytoskeleton-associated, carbohydrate-metabolizing enzymes in maize identified by yeast two-hybrid screening. *Physiol. Plant.* 125:141–156.

HURKMAN, W. J., and TANAKA, C. K. 1986. Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. *Plant Physiol.* 81:802–806.

KIM, S. T., KIM, S. G., HWANG, D. H., KANG, S. Y., KIM, H. J., LEE, B. H., LEE, J. J., and KANG, K. Y. 2004. Proteomic analysis of pathogen-responsive proteins from rice leaves induced by rice blast fungus, *Magnaporthe grisea*. *Proteomics* 4:3569–3578.

KITTUR, F. S., LALGONDAR, M., YU, H. Y., BEVAN, D. R., and ESEN, A. 2007. Maize beta-glucosidase-aggregating factor is a polyspecific jacalin-related chimeric lectin, and its lectin domain is responsible for beta-glucosidase aggregation. *J. Biol. Chem.* 282:7299–7311.

KONOPKA-POSTUPOLSKA, D. 2007. Annexins: putative linkers in dynamic membrane-cytoskeleton interactions in plant cells. *Protoplasma* 230:203–215.

MACCARTHY, P. 2001. The principles of humic substances. *Soil Sci.* 166:738–751.

MARMAGNE, A., ROUET, M. A., FERRO, M., ROLLAND, N., ALCON, C., JOYARD, J., GARIN, J., BARBIER-BRYGOO, H., and EPHRITIKHINE, G. 2004. Identification of new intrinsic proteins in *Arabidopsis* plasma membrane proteome. *Mol. Cell. Proteomics* 3:675–691.

MERLO, L., GHISI, R., RASCIO, N., and PASSERA, C. 1991. Effects of humic substances on carbohydrate-metabolism of maize leaves. *Can. J. Plant Sci.* 71:419–425.

MINELLI, A., OMODEO, P., ROTA, E., and SAMBUGAR, B. 1995. Anellida Clitellata, Aphanomeura, pp. 12–13, in A. Minelli, S. Rufo, and S. La Posta (eds.). Checklist delle Specie della Fauna ItalianaCalderoni, Bologna.

MOEDER, W., DEL POZO, O., NAVARRE, D., MARTIN, G., and KLESSIG, D. 2007. Aconitase plays a role in regulating resistance to oxidative stress and cell death in *Arabidopsis* and *Nicotiana benthamiana*. *Plant Mol. Biol.* 63:273–287.

MONTECCHIO, D., FRANCIOSO, O., CARLETTI, P., PIZZEGHELLO, D., CHERSICH, S., PREVITALI, F., and NARDI, S. 2006. Thermal analysis (TG-DTA) and drift spectroscopy applied to investigate the evolution of humic acids in forest soil at different vegetation stages. *J. Therm. Anal. Calorim.* 83:393–399.

MUSCOLO, A., and NARDI, S. 1997. Auxin or auxin-like activity of humic matter, pp. 987–992, in J. Drozd, S.S. Gonet, N. Senesi, and J. Werber (eds.). The Role of Humic Substances in the Ecosystems and Environmental ProtectionPolish Society of Humc Substances, Wroclaw, Poland.

MUSCOLO, A., SIDARI, M., ATTINA, E., FRANCIOSO, O., TUGNOLI, V., and NARDI, S. 2007a. Biological activity of humic substances is related to their chemical structure. *Soil Sci. Soc. Am. J.* 71:75–85.

MUSCOLO, A., SIDARI, M., FRANCIOSO, O., TUGNOLI, V., and NARDI, S. 2007b. The auxin-like activity of humic substances is related to membrane interactions in carrot cell cultures. *J. Chem. Ecol.* 33:115–129.

NARDI, S., CONCHERI, G., PIZZEGHELLO, D., STURARO, A., RELLA, R., and PARVOLI, G. 2000a. Soil organic matter mobilization by root exudates. *Chemosphere* 41:653–658.

NARDI, S., PIZZEGHELLO, D., GESSA, C., FERRARESE, L., TRAINOTTI, L., and CASADORO, G. 2000b. A low molecular weight humic fraction on nitrate uptake and protein synthesis in maize seedlings. *Soil Biol. Biochem.* 32:415–419.

NARDI, S., PIZZEGHELLO, D., MUSCOLO, A., and VIANELLO, A. 2002. Physiological effects of humic substances on higher plants. *Soil Biol. Biochem.* 34:1527–1536.

NARDI, S., MUSCOLO, A., VACCARO, S., BAIANO, S., SPACCINI, R., and PICCOLO, A. 2007. Relationship between molecular characteristics of soil humic fractions and glycolytic pathway and Krebs cycle in maize seedlings. *Soil Biol. Biochem.* 39: 3138–3146.

NELSON, N., and HARVEY, W. R. 1999. Vacuolar and plasma membrane proton-adenosinetriphosphatases. *Physiol. Rev.* 79:361–385.

NIEMEYER, J., CHEN, Y., and BOLLAG, J. M. 1992. Characterization of humic acids, composts, and peat by diffuse reflectance Fourier-transform infrared-spectroscopy. *Soil Sci. Soc. Am. J.* 56:135–140.

PERKINS, D. N., PAPPIN, D. J. C., CREASY, D. M., and COTTRELL, J. S. 1999. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* 20:3551–3567.

PFLUG, W., and ZIECHMANN, W. 1981. Inhibition of malate dehydrogenase by humic acids. *Soil Biol. Biochem.* 13:293–299.

PINTON, R., VARANINI, Z., VIZZOTTO, G., and MAGGIONI, A. 1992. Soil humic substances affect transport-properties of tonoplast vesicles isolated from oat roots. *Plant Soil* 142: 203–210.

PIZZEGHELLO, D., NICOLINI, G., and NARDI, S. 2001. Hormone-like activity of humic substances in *Fagus sylvaticae* forests. *New Phytol.* 151:647–657.

QUAGGIOTTI, S., RUPERTI, B., PIZZEGHELLO, D., FRANCIOSO, O., TUGNOLI, V., and NARDI, S. 2004. Effect of low molecular size humic substances on nitrate uptake and expression of genes involved in nitrate transport in maize (*Zea mays* L.). *J. Exp. Bot.* 55:803–813.

RUEPP, A., ZOLLNER, A., MAIER, D., ALBERMANN, K., HANI, J., MOKREJS, M., TETKO, I., GULDENER, U., MANNHAUPT, G., MUNSTERKOTTER, M. et al. 2004. The FunCat, a functional annotation scheme for systematic classification of proteins from whole genomes. *Nucleic Acids Res.* 32:5539–5545.

SANDELIUS, A. S., and MORRÈ, D. J. 1990. Plasma membrane isolation, pp. 45–75, in C. Larsson, and I. M. Møller (eds.). The Plant Plasma MembraneSpringer-Verlag, Berlin.

SANTONI, V., ROUQUIE, D., DOUMAS, P., MANSION, M., BOUTRY, M., DEGAND, H., DUPREE, P., PACKMAN, L., SHERRIER, J., PRIME, T. et al. 1998. Use of a proteome strategy for tagging proteins present at the plasma membrane. *Plant J.* 16:633–641.

SANTONI, V., DOUMAS, P., ROUQUIE, D., MANSION, M., RABILLOUD, T., and ROSSIGNOL, M. 1999a. Large scale characterization of plant plasma membrane proteins. *Biochimie* 81:655–661.

SANTONI, V., RABILLOUD, T., DOUMAS, P., ROUQUIE, D., MANSION, M., KIEFFER, S., GARIN, J., and ROSSIGNOL, M. 1999b. Towards the recovery of hydrophobic proteins on two-dimensional electrophoresis gels. *Electrophoresis* 20:705–711.

SANTONI, V., KIEFFER, S., DESCLAUX, D., MASSON, F., and RABILLOUD, T. 2000. Membrane proteomics: Use of additive main effects with multiplicative interaction model to classify plasma membrane proteins according to their solubility and electrophoretic properties. *Electrophoresis* 21:3329–3344.

SUKALOVIC, V. H. T., VULETIC, M., IGNJATOVIC-MICIC, D., and VUCINIC, Z. 1999. Plasma-membrane-bound malate dehydrogenase activity in maize roots. *Protoplasma* 207:203–212.

VISSER, S. A. 1987. Effect of humic substances on mitochondrial respiration and oxidative phosphorylation. *Sci. Total Environ.* 62: 347–354.

WASTENEYS, G. O., and GALWAY, M. E. 2003. Remodelling the cytoskeleton for growth and form: An overview with some new views. *Annu. Rev. Plant Biol.* 54:691–722.

WILKINS, M. R., APPEL, R. D., VAN EYK, J. E., CHUNG, M. C. M., GORG, A., HECKER, M., HUBER, L. A., LANGEN, H., LINK, A. J., PAIK, Y. K. et al. 2006. Guidelines for the next 10 years of proteomics. *Proteomics* 6:4–8.

ZANDONADI, D., CANELLAS, L., and FAÇANHA, A. 2007. Indolacetic and humic acids induce lateral root development through a concerted plasmalemma and tonoplast H^+ pumps activation. *Planta* 225:1583–1595.

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Letter from the Editor-in-Chief

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This is the fifth Special Issue of the Journal of Chemical Ecology since 2000. Previous topics were: Allelopathy—September 2000; Aquatic Chemical Ecology—October 2002; Molecular Chemical Ecology—December 2004; and Mammalian Chemical Ecology—June 2006. This issue on "Olfactory Ecology" is largely the result of efforts by John Hildebrand and Jeffrey Riffell both in the Division of Neurobiology at the University of Arizona. It resulted from discussions at the International Society of Chemical Ecology meeting in 2006.

As well as contributing as authors, Hildebrand and Riffell established a conceptual framework for this issue, solicited review papers, had each paper peer reviewed, and contributed to the editing. As stated in their following preface, they have bridged the field of chemical ecology, olfactory neurobiology, and animal behavior. The focus on both vertebrates and invertebrates is this regard is unique. I suspect that these papers will both enlighten many workers in diverse research areas and assist others in focusing future work.

The issue includes three other major contributions: two modeling papers by Gould et al. (with a separate introductory Preface by Marcel Dicke) and a review article by Thomas Baker based on his Silverstein/Simeone Award Lecture at an International Society for Chemical Ecology meeting in Hamburg. While not directly linked to the "olfactory ecology" symposium effort, these papers seem to be well placed here.

I express particular thanks to John Hildebrand for the original idea and the intellectual stimulation and to Jeffrey Riffell for hours and hours of drudge work, diplomacy, and collaborative goodwill; to Marcel Dicke for graciously helping us place some perspective on the modeling work; and especially to the authors of these stimulating and important contributions.

John T. Romeo
University of South Florida

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Preface

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This special issue of the *Journal of Chemical Ecology* focuses on the olfactory ecology of insects and other vertebrate and invertebrate taxa. The impetus for this project arose in part from discussions at the Annual Meeting of the International Society of Chemical Ecology in Barcelona in 2006 and in connection with a symposium on sensory ecology at the FENS Forum in Vienna in the same year. Eight invited review papers, two contributed papers, and an invited paper by a Silverstein/Simeone honoree explore important aspects of the physical nature of behaviorally relevant olfactory stimuli in the natural environment and the adaptedness of the olfactory system to detect and process them in mediating behavioral responses.

Our goal was to bridge the fields of chemical ecology, olfactory neurobiology, and animal behavior, and to highlight synergies. Much is known about olfaction in vertebrates and invertebrates, and the field has been vigorous and productive in the last two decades. Chemical ecologists have clarified the chemistry of natural mediators of interactions among organisms and associated behavioral responses to those stimuli for many taxa. Most studies of olfaction, however, emphasize the neurobiology of the olfactory system but pay relatively less attention to natural chemical stimuli and their role in the natural environment—that is, the stimuli for which the olfactory system is adapted. We believe that there is a need for chemical ecology to inform olfactory research and *vice versa*, and that the emerging field of sensory ecology offers a fruitful context for such bridge-building.

We have focused on "olfactory ecology" as a specific case of sensory ecology. In our view, research on insects has been in the vanguard in making the connections we hope to foster, while other taxa often provide interesting contrasts and comparisons with what has been learned from insects. Thus, this issue offers four articles on insects as exemplars, each paired with a companion article that focuses on other, vertebrate or invertebrate, taxa.

Explanation of olfaction-mediated search strategies requires understanding of the spatial and temporal properties of natural chemical stimuli. Indeed, olfaction-mediated navigation is exquisitely governed by physical–environmental processes and their effects on natural chemical stimuli. In the introductory paper, Zimmer and Zimmer discuss the need for accurate simulation of both chemical and physical processes for accurate determination of how air- and waterborne chemical signals mediate ecological interactions. The following paper, by Riffell, Abrell, and Hildebrand, suggests techniques for measurement of the physical properties of chemical stimuli in the environment for studies of odor-modulated behavior of insects. The articles by Cardé and Willis and by DeBose and Nevitt review the behavioral mechanisms by which insects, birds, and fish navigate in turbulent odor plumes. The similarity of behavioral strategies employed by both aquatic and terrestrial animals—vertebrates and invertebrates alike—suggests that the challenges associated with olfaction-mediated searching for sources of chemical stimuli are universal and may be reflected in similar neural mechanisms in diverse taxa.

Olfactory-receptor responses and olfactory coding in peripheral sensory organs provide examples of ways in which olfactory systems track rapidly varying, natural stimuli and encode specific cues while sampling the complex chemical landscape that guides innate and adaptive behaviors. With the recent sequencing of insect genomes, a wealth of information has emerged about molecular and neural mechanisms of receptor-cell responses to chemical stimuli. The paper by de Bruyne and Baker provides insight into the ecological importance of natural chemical cues while enabling phylogenetic comparisons among insect

species. In their companion paper, Derby and Sorenson address the complexity of the olfactory systems of aquatic animals and of the chemical stimuli they must analyze, relative to the situation for terrestrial insects. Together, these two papers highlight exciting research opportunities that combine genetic and molecular with behavioral and analytical approaches. Moreover, the articles demonstrate that while chemical stimuli that mediate interactions among aquatic animals and terrestrial animals differ, olfactory receptor cells in fish, crustaceans, and insects can sample the chemical environment rapidly while encoding diverse and complex stimuli.

An understanding of how sensory information about natural chemical stimuli is processed by the olfactory system is essential for unraveling mechanisms by which complex odors evoke innate and adaptive behaviors. In their paper, Lei and Vickers examine some of the mechanisms by which the primary and higher-order processing centers of the olfactory system in insects may encode information about odor mixtures. In a review of olfactory processing in vertebrates, Gelperin examines the computational means by which the higher-order olfactory system encodes complex stimuli. For both insects and vertebrates, single odorants predominantly have been used to examine how the olfactory system encodes odor information. In nature, however, olfactory stimuli typically are mixtures. The mechanisms by which such complex olfactory stimuli are discriminated, recognized, and elicit behavior currently are under investigation, but recent evidence suggests that behaviorally important mixtures may be encoded by the olfactory system as a singular object, whereas odors of lesser significance may be represented by the dominant constituents.

Finally, as the eight review articles in this special issue provide an overview of the processes that underlie olfaction-mediated behaviors and ecological interactions, we hope that this collection will stimulate chemical ecologists, investigators of animal behavior, and olfactory neurobiologists to cooperate and collaborate in future research.

John G. Hildebrand
Jeffrey A. Riffell
ARL Division of Neurobiology
University of Arizona
Tucson, Arizona

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REVIEW ARTICLE

Dynamic Scaling in Chemical Ecology

Richard K. Zimmer · Cheryl Ann Zimmer

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Abstract Natural rates of chemical production, release, and transport of fluid-borne molecules drive fundamental biological responses to these stimuli. The scaling of the field signaling environment to laboratory conditions recreates essential features of the dynamics and establishes ecological relevance. If appropriately scaled, laboratory simulations of physical regimes, coupled with natural rates of chemical cue/signal emission, facilitate interpretation of field results. From a meta-analysis of papers published in 11 journals over the last 22 years (1984–1986, 1994–1996, 2004–2006), complete dynamic scaling was rare in both field and laboratory studies. Studies in terrestrial systems often involved chemical determinations, but rarely simulated natural aerodynamics in laboratory wind tunnels. Research in aquatic (marine and freshwater) systems seldom scaled either the chemical or physical environments. Moreover, nearly all research, in all environments, focused on organism-level processes without incorporating the effects of individual-based behavior on populations, communities, and ecosystems. As a result, relationships between chemosensory-mediated behavior and ecological function largely remain unexplored. Outstanding exceptions serve as useful examples for guiding future research. Advanced conceptual frameworks and refined techniques offer exciting opportunities for identifying the ecological significance of chemical cues/signals in behavioral interactions and for incorporating individual effects at higher levels of biological organization.

Keywords Chemical cue · Chemical signal · Chemical ecology · Dynamic scaling · Odor plume

R. K. Zimmer (✉) · C. A. Zimmer
Department of Ecology and Evolutionary Biology,
University of California,
Los Angeles, CA 90095-1606, USA
e-mail: z@biology.ucla.edu

R. K. Zimmer
Neurosciences Program and Brain Research Institute,
University of California,
Los Angeles, CA 90095-1606, USA

Introduction

The nature of chemical cues and signals varies considerably over time and in space. Behavioral activation is a unique property of specific stimulus conditions that characterizes each microhabitat. Responses of chemosensory receptors, cells, organs, and hence, whole, integrated organisms are scaled according to the number of molecular contacts per unit time (Hildebrand 1995; Mead 2002). Larger values usually reflect higher rates of contact. The intensity of a given reaction to an ecologically relevant chemical cue, therefore, determines whether or not a behavior is effective. Appropriate scaling of the field signaling environment to the laboratory simulates the essential characteristics of the dynamics (Nowell and Jumars 1987; Zimmer and Butman 2000). Such scaling enables analysis of constraints imposed by natural physicochemical phenomena on biological responses at individual, population, and community levels.

Whereas many studies have contributed significantly to current knowledge of dynamic scaling, two watershed contributions, published in 1984, are particularly seminal. Elkinton et al. (1984) presented the first experiments to test predictions of the Gaussian plume model within a complex natural habitat. They provided unequivocal evidence that

time-averaged, Gaussian descriptions of airborne attractants could not account for observed behavioral responses. Nowell and Jumars (1984) offered a fresh, new conceptual framework, along with compelling examples, of hydrodynamic effects on organisms living in boundary-layer flow environments. Together, these studies showed that (1) tracking scents depends on fine details of near-bed, turbulent fluid motions, and (2) mean and fluctuating terms of velocity (i.e., Reynolds stresses) determine the outcomes of basic ecological processes, including courtship and mating, habitat colonization, and predator–prey interactions.

In the present synthesis, research on contact and fluid-borne chemical cues/signals is assessed relative to the basic principles of dynamic scaling. There are two, complementary, components to the paper. The first section is a discussion of conceptual issues, and the second evaluates, quantitatively, recent history and current research trends in the literature (Tables 1 and 2). By using a meta-analysis of papers published in 11 journals, this critique evaluates the pervasiveness of dynamic scaling in studies of chemical cues and signals. The contribution of a research field cannot be determined solely by the number of papers published in specific subdisciplines. Nevertheless, this assessment of status and trends is a valuable starting point, and suggests fruitful avenues for future research.

Table 1 Meta-analysis[a]: For 11 journals, papers were included in the analysis if published as research articles, notes, or communications over three, 3-yr blocks (1984–1986, 1994–1996, and 2004–2006)

Subject area	Periodical name (abbreviation)
Chemical ecology	Journal of Chemical Ecology (JCE)
	Chemoecology (C)
General ecology	Ecology (E)[b]
	Ecological Monographs (EM)[b]
	Ecology Letters (EL)
	Oecologia (O)
Marine biology/ ecology	The Biological Bulletin (BB)
	Marine Ecology Progress Series (MEPS)
	Marine Biology (MB)
	Journal of Experimental Marine Biology and Ecology (JEMBE)
	Limnology and Oceanography (LO)

[a] While not exhaustive, this select list was a reasonable representation of each field. Chosen journals were ranked highly by impact factor (ISI 2006). The present analysis did not take into account general biological and scientific journals (e.g., Science, Nature, PNAS, and PLoS Biology), because they devoted <3% of all published manuscripts to overall topics in ecology, as indicated by a more limited survey (1986, 1996, 2006). Far fewer papers were dedicated to chemical cues/signals.

[b] Manuscripts appearing in Ecology and Ecological Monographs were pooled prior to analysis, because they are essentially the same journal, published by the Ecological Society of America.

Dynamic Scaling: Conceptual Issues

Chemical, physical, and biological variables are rarely static in nature. Experiments, thus, require 'dynamic scaling'—the simulation of essential chemical and physical features of a changing native signaling environment—to establish ecological relevance. Moreover, in chemical ecology, the dynamics of individual-based performance must be scaled up to understand basic population, community, and system structure and function.

Field studies are designed to capture the innate spatiotemporal properties of cue/signal distributions and experimentally evaluate cause and effect. Complementary field measurements also can set boundary conditions for dynamically scaled laboratory experiments. In the lab, separate effects of biological, chemical, or physical factors often can be analyzed more clearly in experiments with individual organisms, small populations, or even multiple species at different trophic levels. Whereas laboratory studies may offer insights about processes that influence community-wide and system-level mechanisms, they lack the complexity of native field environments. Studies of how ecological relationships are mediated by chemical cues/signals may profit from an approach that combines natural field studies with dynamically scaled laboratory experiments.

Scaling the Chemistry The physical and chemical properties of habitats can determine the nature and success of ecological interactions. In terrestrial environments, for example, compounds with high vapor pressures facilitate chemical transport in air. Many signal molecules thus are low molecular weight (less than 20 carbon atoms) and hydrophobic (alcohols, aldehydes, or ester acetates; Roelofs 1995; Ando et al. 2004). In contrast, aqueous solubility imparted mainly by electronic charge or hydrophilicity, rather than gaseous volatility, may constrain the types of substances that act principally as waterborne chemical agents (Carr 1988). Peptides, free amino acids, carbohydrates, organic nitrogen bases, nucleotides and nucleosides, and fatty acids are all putative, chemical cues/signals that mediate ecological processes in aquatic environments (Howe and Sheikh 1975; Pawlik 1992; Painter et al. 1998; Krug and Manzi 1999; Hardege et al. 2004; Cummins et al. 2005; Kicklighter et al. 2007). Even compounds with low solubilities, being small and hydrophobic, or large proteins, can provide effective signals in water when suspended and transported by fluid motion.

Chemical identities are essential for studies that link behavior, chemosensory physiology, and receptor function. Structural elucidations are required to distinguish unique natural products from more common metabolites that serve a variety of functions. The courtship and mating attractants

Table 2 Meta-analysis: major goals, rationales and qualifications

Goal	Rationale and qualification
1. Establish, for each journal, the relative percentage of all papers published on topics pertaining to chemical cues and chemical signals (Fig. 1)	A. Manuscripts on marine and freshwater systems were analyzed separately within categories of 'chemical ecology' and 'general ecology' B. Studies pertaining to the use of chemical defense compounds and allelopathics were not included with cues and signals, unless direct evidence was presented for chemosensory-mediated processes. Antifeedants, toxins, and related molecules often target physiological processes unrelated directly to behavior.
2. Of those papers published on chemical cues/signals, establish the percentage associated with a given habitat type (terrestrial insects, other terrestrial, and aquatic [freshwater + marine] organisms; Fig. 2)	A. The terrestrial habitat group was broken into two sub-groups, because of a preponderance of studies on insects in some journals.
3. Establish the percentage of papers that focused on a particular behavior and/or ecological relationship (habitat [or host] selection, trophic relations, courtship and mating, other social interactions, and miscellaneous; Fig. 3)	A. Categorization, according to infochemical terminology (e.g., pheromone, allomone, synomone, kairomone; Dicke and Sabelis 1988), was avoided because these names relate only costs and benefits to cue/signal producing and receiving organisms without specifying the type of ecological interaction.
4. Establish the percentage of papers that reported results using either partially-, fully-, or un-identified chemical cue/signal molecules (Figs. 4, 5, and 6)	A. The 'fully identified' cue/signal group was broken into two sub-groups: (a1) substances identified via bioassay-guided fractionations of natural materials, and (a2) compounds taken directly off-the-shelf without reference to a native source.
5. Establish the percentage of papers that reported results using either partially-, fully-, or un-characterized flow environments (Fig. 7)	A. Field and lab investigations, with measurements of mean and fluctuating components of velocity, were classified as 'fully'. In contrast, studies having any type of determination, such as mean flow speed, were labeled as 'partially'.
6. Establish the percentage of papers having as a principal focus individual organisms, populations, communities, or ecosystems (Fig. 8)	A. The 'individual organism' group was divided into three subgroups of 'molecular biology/biochemistry', 'physiology', and 'behavior and/or ecology'.

of lepidopteran insects are especially useful examples. They present a wide range of novel compounds and mixture blends that confer species-specific recognition. The molecular basis for attractant biosynthesis has revealed much about the evolution of chemical communication systems and the explosive radiation of lepidopteran species (e.g., Mayer and McLaughlin 1991; Roelofs 1995; Roelofs and Rooney 2003). Notably, a single point mutation can cause activation of a nonfunctional desaturase gene transcript, alter attractant structure, and lead to insipient speciation within a single generation (Roelofs et al. 2002). Ordinary molecules also have extraordinary effects. Histamine, for instance, initiates local immune responses (Jutel et al. 2006; Sugata et al. 2007), regulates receptor-mediated physiological processes in the gut (Filippova and Nozdrachev 2007), acts as a neurotransmitter (Jones 2005; Stuart et al. 2007), and stimulates habitat colonization by organisms of highly divergent species (Swanson et al. 2004, 2006). Such assorted communication functions arise from convergent evolution. Studies on the comparative biology of signaling systems across taxa, therefore, can lead to improved appreciation for mechanisms that create and maintain biodiversity.

Laboratory experiments dynamically scaled to field environments simulate natural patterns of contact between signal molecules and biological sensors. The type and magnitude of behavioral responses depend on rates of signal production and release, and hence, on stimulus encounter frequencies. Chemical identification is the first step towards achieving dynamic scaling, and enables matching of natural concentrations and fluxes among study environments. As an example, the relative effects of chemical composition (of an attractant mixture), concentration, mean volume flow rate (of chemical input), and flux (concentration × volume flow rate) were evaluated for search behavior of marine snails (Zimmer et al. 1999). In separate field tests, only flux was significantly correlated with the number of animals attracted to the site of chemical emission. Behavioral responses were more tightly coupled to chemical release and transport dynamics than to specific properties of any single attractant molecule.

Scaling the Physics For organisms living in air or water, fluid motion is a characteristic property of the environment. Even in the absence of noticeable wind or water flow (such as in small ponds), differential heating causes convection

and induces movement. Fluid velocity is characterized by mean and random, fluctuating terms. Whereas the mean flow moves fluid along one principal axis (such as downwind or down current), velocity fluctuations generate shear forces that potentially distribute momentum in three spatial dimensions (Nowell and Jumars 1984; Denny 1988; Weissburg 2000). The rate at which fluid disperses dissolved chemicals away from a principal axis, as a consequence of random velocity fluctuations, is called turbulent (or eddy) diffusion. Just as a biologist measures the mean and variation of a morphological, physiological, or behavioral trait, a chemical ecologist needs to quantify the mean and variation of chemical transport due to fluid flow. Scaling between native field environments and laboratory regimes (i.e., in wind tunnels and flumes) involves matching key dimensionless parameters that embody the essential physics of the research system (Miller et al. 1984; Nowell and Jumars 1987; Peterson and Hastings 2001). The creation of dynamic similarity is usually straight forward and facilitates the extrapolation of laboratory results to the field.

For macroscopic organisms, distributions of chemical cues are patchy, both temporally and spatially, as a consequence of turbulent diffusion (Murlis and Jones 1981; Zimmer-Faust et al. 1988; Murlis et al. 1992, 2000; Mylne et al. 1996; Finelli et al. 1999). The magnitude of signal variation ultimately depends, however, on the relative strength of mean versus fluctuating terms of fluid motion. These two quantities reflect the unique properties of inertial forces that put fluid into motion, of structural features that distinguish natural habitats (e.g., heights and densities of plants or trees, topographies of seafloor or stream bottom sedimentary environments), and of morphological features that characterize respondent organisms. In combination, such features establish complex sets of spatiotemporal dynamics in the chemical cues/signals affecting behavior of different organisms in various ways.

Effects of turbulence can be experienced by organisms of all sizes. At the smallest scales (bacteria, sperm, many protozoa), adjacent layers of fluid slide past each other without being mixed (called "laminar flow"; Karp-Boss et al. 1996). Much like a pot of honey stirred by a spoon, the fluid moves along lines of constant velocity and quickly stops when the force ceases. Within the smallest turbulent structures (called "eddies"), speed increases from the center towards the perimeter, characterizing the laminar shear flow within. Whether suspended in a moving fluid or attached to a wall (e.g., blood vessel), at any fluid–surface interface, single cells experience shear stresses on their membrane surfaces.

Behavior is an important process that mediates recruitment of sperm to conspecific eggs (Miller 1985; Ward et al. 1985; Riffell et al. 2002; Yoshida et al. 2002; Kaupp et al. 2006). Relative to high shears, cells swim significantly faster and orient more directly towards eggs at low shears and in still water. In fact, even though male gametes swim at the same speed in low shears and still water, sperm navigation is significantly enhanced at low shears (Riffell and Zimmer 2007). Accordingly, fertilization success peaks in these slow flows. Low shear/slow flow creates fluid-dynamic conditions that are highly conducive for broadcasting chemical signals. Egg attractant is released and transported by flow with minimal dilution. The result is a behaviorally active attractant plume. Plume length and active volume peak at the lowest shears tested, and then decay rapidly thereafter, reflecting precisely the patterns described for sperm recruitment and fertilization success (Riffell and Zimmer, unpublished data). As for macroscopic organisms, dynamic scaling of flow relative to nature is required to understand the ecological and evolutionary consequences of chemosensory-mediated behavior of single cells.

Scaling up from Individuals Services rendered by an ecosystem reflect the composite attributes of its inhabitants. System-level processes, such as total primary productivity, efficiency of nutrient use, disease dynamics, invasions by exotic species, and stability in the face of natural climate forcing and human perturbations have profound global implications (Tilman 1999; Loreau et al. 2001; Duffy and Stachowicz 2006). These properties are, in turn, highly dependent on networks of interactions among individuals both within and between species. Relying on sensory information to shape decisions, individuals exploit beneficial and avoid detrimental interactions. In fact, stimulus-response dynamics potentially influence every other level of organization, from population to ecosystem.

Because cellular mechanisms of chemosensory reception are highly converged across taxa (Ache 1994; Hildebrand 1995; Hildebrand and Shepherd 1997), the same bioactive compounds can be informative for phylogenetically diverse species. One, or only a few, "infochemicals" potentially have system-level consequences. A well-documented case of scaling up chemosensory-mediated interactions is found in the open ocean. Here, single-celled plants ("phytoplankton") create dense blooms at convergent zones where high nutrient levels occur in surface ocean waters. These cells have extremely high concentrations of dimethylsulfoniopropionate (DMSP) that equalize osmotic pressure between the cytoplasm and external ocean environment (Dacey et al. 1987; Matrai and Keller 1994). When phytoplankton cells burst open during herbivore ("zooplankton") grazing, DMSP is released into seawater and enzymatically cleaved to dimethylsulfide (DMS), acrylic acid, and a proton (Dacey and Wakeham 1986). In seawater, acrylic acid acts as a chemical deterrent against protozoan grazers (Wolfe et

al. 1997; Wolfe 2000). Alternatively, DMSP serves as a chemoattractant to biodegradatory bacteria (Zimmer-Faust et al. 1996). Whereas dissolved DMS stimulates zooplankton foraging (Steinke et al. 2006), atmospheric DMS guides seabirds over kilometers to rich zooplankton feeding grounds (Nevitt et al. 1995; Nevitt 2000). Thus, a combination of three closely related compounds (DMS, DMSP, and acrylic acid) produces effects that cascade down four trophic levels, thus connecting microbial loop dynamics (protozoans, bacteria) with apex predation (seabirds).

Dynamic Scaling: Recent History and Current Trends

The evaluation of recent history and current trends of dynamic scaling in studies of chemical cues/signals provides a judicious retrospective of ecological context. Papers published in 11 journals over the last 22 years were classified according to various attributes (Tables 1 and 2). Having identified the percentage of studies on chemical cues/signals, research was further broken down by habitat type, ecological interaction, chemical identification, flow characterization, and level of ecological organization. Specific goals and details of the meta-analysis procedure are provided in Table 2. Where appropriate, differences in literature trends were tested for significance by using Students *t* tests, or analysis of variance (ANOVAs) and post hoc Scheffé tests, with percentages arc-sine transformed before analyses. When data were pooled across 3-year intervals (1984–1986, 1994–1996, and 2004–2006), we applied *G* tests in determining significance levels among proportions of published articles between specified categories.

Meta-Analysis: Chemical Cues/Signals For chemical ecology journals, mean percentages of published papers on chemical cues/signals decreased over time [Fig. 1A and one-way ANOVA $F_{2,6}=31.32$, $P<0.001$ for Journal of Chemical Ecology (JCE)]. During the last 22 years, JCE devoted ~50–75% of all articles per year to this topic. Similar percentages, although based on much lower numbers, were published by Chemoecology (C), since its inception in 1990. Still, overall, total number of articles printed on cues/signals varied considerably from 240–310/year in JCE to 16–24/year in C. Both periodicals dedicated a majority of these papers to work highlighting terrestrial insects, and thus, were relatively limited in scope (Fig. 2, and two-way ANOVA followed by Scheffé tests: $P<0.001$ for JCE).

Likely, more articles were published on chemical cues/signaling concerning insects simply because more work was done on this topic. Increased access to model organisms and habitats for experimental manipulations, availability of funding, and the focus of entire academic departments and research labs on entomology, per se, all could have contributed to proportionally more research articles on insects relative to other taxa. By using JCE as a case study, no significant difference was found in the ratio of manuscripts submitted to those accepted for publication on insects relative to other taxa (J. Romeo, personal communication). Thus, there is no inherent bias for, or against, any specific taxonomic group.

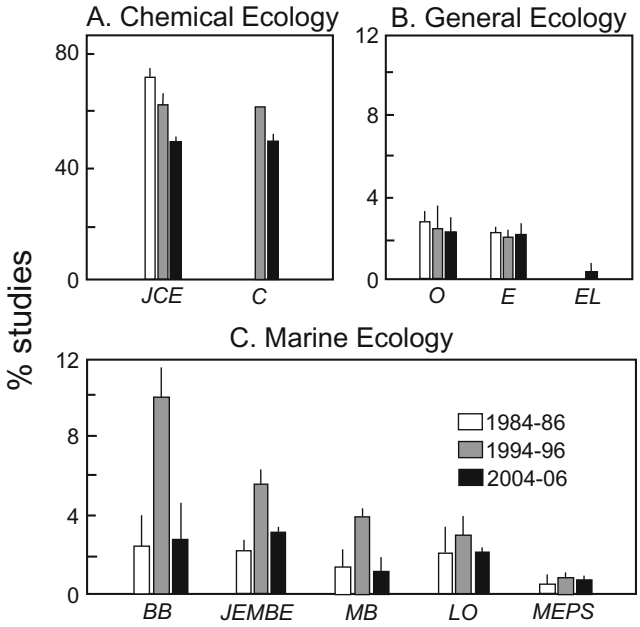

Fig. 1 Relative percentages (mean ± SEM) of all papers published on topics pertaining to chemical cues and chemical signals. Abbreviations: JCE (Journal of Chemical Ecology); C (Chemoecology); O (Oecologia); E (Ecology and Ecological Monographs, combined); EL (Ecology Letters); BB (Biological Bulletin); JEMBE (Journal of Experimental Marine Biology and Ecology); MB (Marine Biology); LO (Limnology and Oceanography); MEPS (Marine Ecology Progress Series)

A different trend was identified for general ecology journals (Fig. 1B). Mean percentages of papers on chemical cues/signals were nearly constant, but extremely low [2–3% per year for Ecology (E) and Oecologia (O)] over the survey period (one-way ANOVAs: $F_{2,6}\leq 0.61$, $P\geq 0.59$, both tests). A third journal, Ecology Letters (EL), appeared in 1998 and published only 0.25% of papers on cues/signals from 2004 to 2006. Over 1984–1986, rates of publication in E and O were nearly identical for chemical cue/signal research (2.3–3.0%) and for chemical defense/allelopathic research (2.6–3.1%) (Students *t* tests: $t_4\leq 0.37$, $P\geq 0.72$, for E and O). The values for defenses/allelopathics increased precipitously to 4.1–5.2% during 1994–1996, and remained steady through 2004–2006. By comparison, percentages of papers on chemical cues/signals decreased slightly (to 2.0–2.4%) from 1984–1986 to 2004–2006 (*t* tests: $t_4\geq 5.43$, $P\leq$

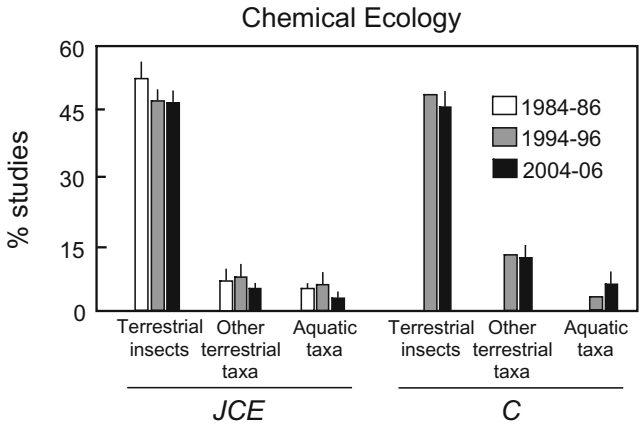

Fig. 2 Chemical ecology journals: Relative percentages (mean ± SEM) of all papers published on topics pertaining to chemical cues/signals for terrestrial insects, other terrestrial organisms, or aquatic (freshwater + marine) organisms. Abbreviations: JCE (Journal of Chemical Ecology) C (Chemoecology)

0.008, both tests, when comparing separately data for 2004–2006 in O and in E). These trends may reflect heightened interest in chemical defense/allelopathics relative to chemical cues/signals.

In fact, ecologists have championed community-wide impacts of defenses and allelopathics. Historically, these natural products were known as direct contributors to competition and predator–prey interactions, two biological processes that regulate strongly community dynamics (Muller et al. 1964; Feeny 1976; Atsatt and O'Dowd 1976; Hay and Fenical 1988; Hämback and Beckerman 2003; Agrawal et al. 2006). Research on cues and signals, in contrast, has focused almost entirely at the organism level, without much attention to higher levels of biological organization (see details in subsequent sections). There is, however, growing appreciation for chemical cues and signals in determining trait-mediated interactions of animals, plants, and microbes. Community-level consequences of such chemically mediated behavioral and morphological responses by organisms are a newly emerging topic of growing interest (Alborn et al. 1997; De Moraes et al. 1998; Peacor and Werner 2001; Trussell et al. 2002, 2006; Pohnert et al. 2007). The ratio of published articles on defense/allelopathy to cues/signaling, therefore, may tend more towards unity over the next 5 to 10 years.

For marine journals, a third publication pattern distinguishes it from other periodicals (Fig. 1C). Except for Marine Ecology Progress Series (MEPS) and Limnology and Oceanography (LO) all surveyed publications exhibited a major spike in percentages of papers on chemical cues/signals during 1994–1996 (two-way ANOVA, journal × time: $F_{4,30}=9.63$, $P<0.001$; Scheffé tests: $P<0.05$), although it was not sustained by The Biological Bulletin (BB), Journal of Experimental Marine Biology and Ecology (JEMBE), and Marine Biology (MB). Mean percentages showed a slight, but nonsignificant, increase from 1984–1986 to 2004–2006 (Scheffé tests: $P>0.20$).

Repeatedly, over these two decades, marine chemical ecology was declared an exciting, emergent field (Bakus et al. 1986; Hay 1996, 2002; McClintock and Baker 2001). Yet, this enthusiasm is not reflected in the percentage of research articles dedicated each year to marine chemical cues/signals. Many factors may contribute to the current paucity of research on marine species. First, chemical ecologists have had limited ability (or interest?) to scale-up findings from organism-based research to populations and communities. In particular, there have been relatively few successful efforts to isolate and identify the complete structures of marine natural products that act as dissolved chemical cues and signal molecules (see review of Zimmer and Butman 2000). Second, critical properties of fluid dynamic environments have been only rarely measured within natural habitats or applied appropriately in scaling laboratory experiments that investigate chemical-mediation of ecological interactions among plants and animals. These limitations in scaling chemistry and physics are detailed more fully in subsequent sections of this paper.

Either marine biologists are not talking with chemists and physicists, or their messages are not being heard. In the absence of adequate chemical and physical determinations, cues and signals from laboratory and field experiments cannot be interpreted within natural historical contexts. With recent, seminal advances in theory and technology, especially over the last 10–15 years, however, future research on marine organisms should progress more quickly and consistently towards natural-product identifications and hydrodynamics characterizations (see, for example: Painter et al. 1998; Crimaldi et al. 2002; Webster et al. 2003; Swanson et al. 2004; Dreanno et al. 2006; Jackson et al. 2007; Kicklighter et al. 2007).

Meta-Analysis: Ecological Interactions The breadth of publications in chemical ecological journals has increased over time (Fig. 3A, and G test: $G_8=16.11$, $P=0.05$). From 1984–1986, investigations focused primarily on structural elucidations of mating and courtship attractants. An exciting period, there was a major paradigm shift away from single attractant molecules towards novel blends of attractant mixtures that confer species specificity in communication between prospective mates. Later (1994–1996 and 2004–2006), percentages of publications were more evenly distributed among trophic relations, other social interactions, and mating and courtship. During these intervals, research emphasized, for example, feeding attractants of specialized insect herbivores, chemically mediated relations between predators (or parasitoids), herbivores and host plants, conspecific attraction and aggregation (such as

those in pine bark beetles), courtship and mating attraction, as well as intra- and interspecific communication among eusocial insects (e.g., Camacho et al. 1994; Agelopoulos et al. 1995; Loughrin et al. 1995; Hamilton et al. 2005; Gammans et al. 2006).

Historically, ecologists have emphasized predator–prey interactions as seminal factors that structure populations, communities, and ecosystems (Paine 1966; Murdoch 1969; Connell 1970). Trophic relations, thus, have dominated journal publications on chemical cues/signals in general ecology (G test: G_8=27.71, P<0.001, comparing among each topic category and each 3-year interval). There was, however, a compelling reorganization of research priorities over time (Fig. 3B). For E and O, 78% of cue/signal publications during 1984–1986 focused on prey selection by consumers (e.g., Inouye and Waller 1984; Marden 1984). This research largely involved tests of optimal foraging theory. In contrast, a significant percentage (68%) of more recent articles (during 2004–2006, and G test: G_1=8.12, P=0.004 for E and O combined) focused on the consequences of prey alarm and avoidance behavior, and on prey morphological responses to predator presence (e.g., Blaustein et al. 2004; Winder et al. 2004; Brodin et al. 2006). Thus, contributions of chemical cues/signals to trophic interactions were explored sequentially by general ecologists from both predator and prey perspectives.

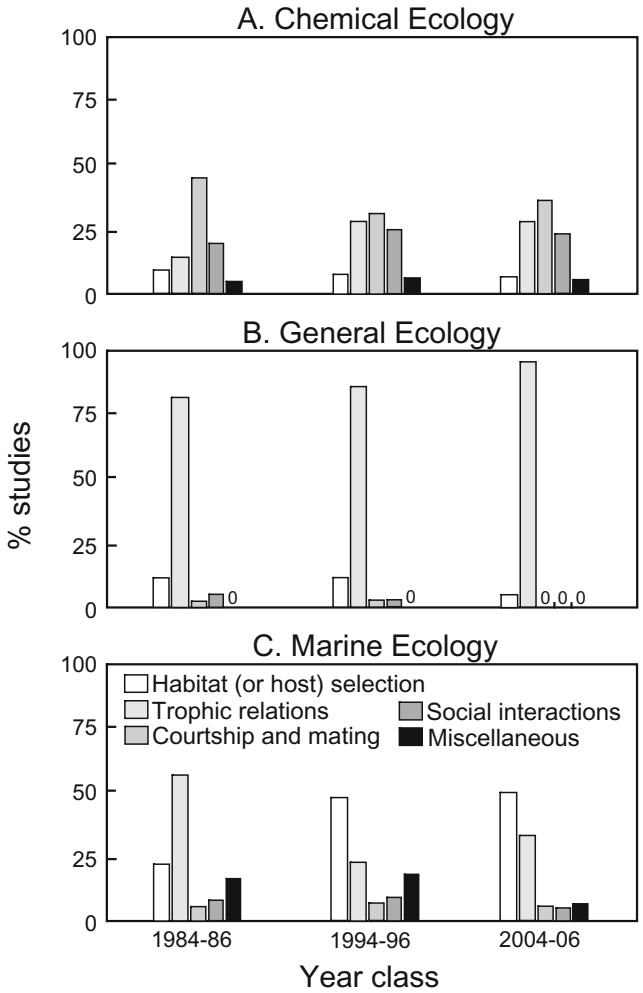

Fig. 3 Of those papers published on chemical cues/signals, the relative percentage that focused on a particular ecological interaction (see legend in **c**.). Data were pooled for chemical ecology (JCE and C), general ecology (O, E, and EL), and marine biology/ecology (BB, JEMBE, MB, LO, MEPS) journals over indicated time intervals

Research published by marine journals likewise evolved over the last 22 years (Fig. 3C). Investigations were dominated by trophic interactions from 1984–1986, with an emphasis on feeding attraction and stimulation (e.g., Trott and Robertson 1984; Johnson and Atema 1986; Sakata et al. 1986). This trend shifted in 1994–1996, when studies concentrated more on habitat colonization (G test: G_1=6.81, P=0.01). Considerable research has targeted effects of surface-adsorbed and waterborne chemical cues in mediating larval settlement behavior and metamorphosis (e.g., Gibson and Chia 1994; Boettcher and Targett 1996; Toonen and Pawlik 1996). Such cues are generally associated with hospitable habitats that provide valuable food resources for newly metamorphosed juveniles and adults, or that improve survivorship and/or mating. Responses, therefore, establish an irreversible commitment by larvae to colonize a given habitat, potentially driving rates of immigration to open marine populations, with profound consequences for demographics and community structure.

Over the last 10–15 years, there has been an explosive growth in the field of microbial chemical ecology (Crespi 2001; Fenchel 2002; Redfield 2002; Strack et al. 2003; Visser and Jackson 2004; Keller and Surette 2006; Hense et al. 2007). This work, however, has been published primarily in periodicals that target basic and applied microbiology. Positive interactions, and especially mutualisms, are gaining increased attention from general ecologists for their roles in mediating community and system dynamics (Bertness and Callaway 1994; Stachowicz 2001; Bruno et al. 2003). Many of these positive interactions are the products of relationships among microbes, or between microbes and eukaryotes. Corals, for example, can live in unproductive, oligotrophic waters because of their relationship with endosymbiotic single-celled algae. The algae produce organic carbon substrates through photosynthesis, and then pass on large proportions of these energy-rich molecules in response to chemical signals from their coral hosts (Gates et al. 1995, 1999). Similarly, sedimentary environments of terrestrial plant roots are characterized by diverse communities of microorganism species. These microbes are of singular importance in making essential nutrients bioavailable. Through quorum sensing and diffusion signaling pathways, chemical cues/signals ultimately regulate many microbial and symbiotic interactions, and

thus, have profound consequences in structuring both prokaryotic and eukaryotic communities (Perret et al. 2000; Hirsch et al. 2003; Jones et al. 2007). Future trends hopefully will see a stronger integration of research on microbial cues/signaling into leading chemical ecological, general ecological, and marine biological journals.

Meta-Analysis: Chemical Identifications Over the entire survey interval, investigations on terrestrial organisms in chemical–ecological journals have regularly included structural identities of cue/signal molecules (Fig. 4, and *G* test: $G_2=14.13$, $P<0.001$, showing a significant difference between bioassay-guided fractionations and all other cue/signal categories combined). Starting at 70–79% during 1984–1986, percentages of papers that identified molecules increased to 90–94% during 2004–2006 (*G* test: $G_1=12.36$, $P<001$, for data pooled among each 3-year interval). There has been a strong commitment to bioassay-guided fractionation, with direct tests of known bioactive compounds.

The general ecology journals demonstrated different patterns altogether (Fig. 5). Publications on terrestrial versus aquatic (marine + freshwater) systems also showed divergent temporal trends. Most terrestrial studies used unidentified chemical cues/signals during 1984–1986 and 1994–1996, whereas many cues were fully identified by 2004–2006. Percentages of cue/signal molecules, fully identified through bioassay-guided fractionation, increased from 18% to 74% over this 22-year interval (*G* test: $G_1=5.21$, $P=0.02$, comparing 1984–1986 to 2004–2006). In contrast, studies on aquatic systems rarely, partially, or fully, identified cues/signals over the entire meta-analysis period. Of all aquatic investigations evaluated, 86–98% used unidentified, while only 0–4% used fully identified cues/signals over the last 22 years (*G* test: $G_1=1.03$, $P=0.39$).

As potential sources of cues and signals, targeted aquatic organisms frequently were homogenized, and their filtered, crude extracts were bioassayed for effects on respondents.

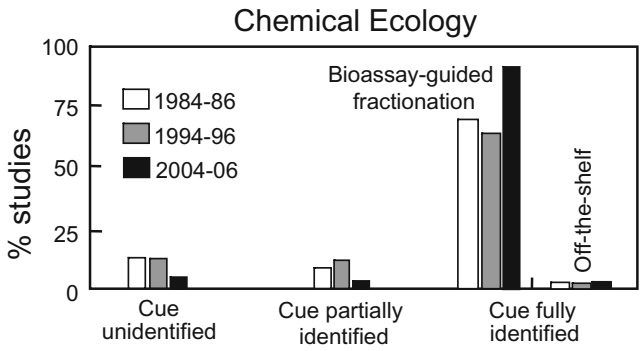

Fig. 4 Of those papers published on chemical cues/signals in chemical ecology journals (JCE and C), the relative percentage that reported results using either partially-, fully-, or unidentified compounds. Data were pooled over indicated time intervals

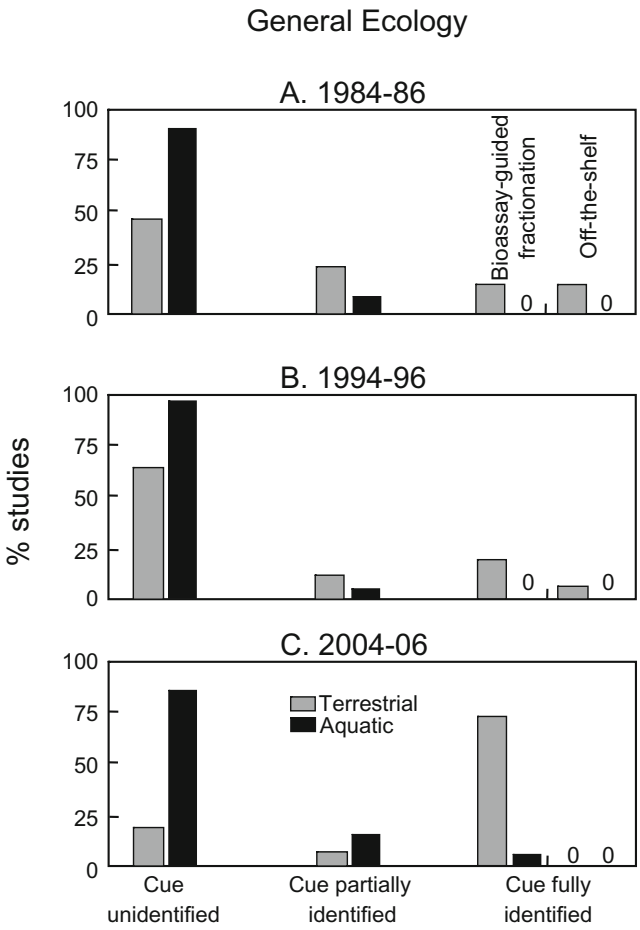

Fig. 5 Of those papers published on chemical cues/signals in general ecology journals (O, E, and EL), the relative percentage that reported results using either partially-, fully-, or unidentified compounds. Data were pooled over indicated time intervals

These procedures would be appropriate only in studies of phagostimulation. In other applications, such preparations are vulnerable to considerable artifacts (Velez et al. 2007; Nylund et al. 2007). Homogenate molecules, elaborated through invasive cell disruption, can differ substantially from those released naturally from live, intact organisms.

Alternatively, water-conditioned stimuli used in aquatic studies often were produced by bathing a targeted organism (i.e., for release of bioactive compounds). Although conditioned water is preferable over a homogenate, immersion periods often were unrealistically long (one to many [24–48] h), and in questionably small water volumes (milliliters to liter). Concentrations of combined secreta, excreta, and other effluents from targeted organisms may have been appreciably higher, or lower, than those encountered by respondents in nature. Moreover, placements of targeted organisms in running water would allow cues/signals to accumulate at natural levels only if densities and flow conditions (advection, turbulent mixing) matched actual field environments. Thus, ecological relevance is

equivocal without chemical identifications and known concentrations (and fluxes) of applied stimuli.

The probability of chemical cue/signal identification was smallest in studies published in marine journals (Fig. 6). In the early studies (1984–1986), for example, only 5% used fully identified cue/signal molecules (i.e., via bioassay-guided fractionation). In fact, cues typically were not identified: 62% (1984–1986) to 73% (2004–2006) of the investigations (*G* test: G_1=1.41, P=0.33). These results differ from those for terrestrial systems, where chemical structural elucidations have become the accepted norm.

Meta-Analysis: Physical Environments Across research environments, there have been several compelling studies that demonstrate chemical signaling effects as a function of flow. In air, the behavioral basis and neural control of odor plume navigation by flying and walking insects has superseded that for any other taxonomic group. Unprecedented knowledge of navigational mechanisms now extends from primary, olfactory receptor neurons to the brain (Vickers et al. 2001; Bau et al. 2005), and from brain to behavior (Mafra-Neto and Cardé 1994a, b; Vickers and Baker 1992, 1994). In the sea, essential properties of the field have been scaled in several laboratory flumes (Pawlik and Butman 1993; Weissburg and Zimmer-Faust 1994; Moore and Crimaldi 2004). As a consequence, fundamental relationships have been established between the mechanics of search behavior and dynamics of turbulent odor plumes (Weissburg and Zimmer-Faust 1993; Smee and Weissburg 2006). These principles were extended to field experiments (Zimmer-Faust et al. 1995; Finelli et al. 2000; Smee et al. 2008), enabling predictions of chemosensory-mediated behaviors to populations and communities.

Still, ecological significance is contingent on dynamic similarity between field and flume/wind tunnel environments (Nowell and Jumars 1987). If not, results are generic, at best, and may or may not have direct relevance to the field. As an overall group, studies in air and water showed little tendency to dynamically scale physical environments between field and laboratory (wind tunnel or flume). Mean wind speeds usually were reported in chemical ecology journals, but no investigation (over 1984–1986, 1994–1996, or 2004–2006) described the structure of turbulent flow within an apparatus or scaling to the field. Moreover, in general ecology journals, all but one investigation (Smee and Weissburg 2006) did not characterize mean and fluctuating components of natural flow environments. Over the last 22 years, marine papers exhibited growing awareness of physical controls/constraints on ecological processes (Nowell and Jumars 1984; Butman 1987; Denny 1988). A few studies, thus, involved fully characterized flows (8% in 2004–2006; Fig. 7). Still, far more of these papers (78% over 2004–2006) included no flow determinations at all (*G* test: G_1=24.11, $P<0.001$, comparing "no flow" to all other flow categories combined).

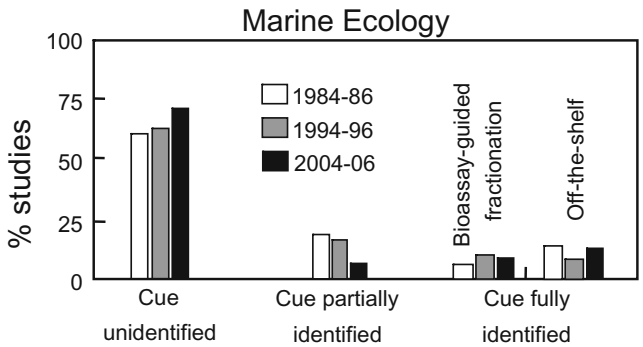

Fig. 6 Of those papers published on chemical cues/signals in marine biology/ecology journals (BB, JEMBE, MB, LO, and MEPS), the relative percentage that reported results using either partially, fully, or un-identified compounds. Data were pooled over indicated time intervals

Meta-Analysis: Scaling up from Individuals There is strong convergence among journals in publication patterns at the four levels of biological organization (individual, population, community, ecosystem). Each of the 11 surveyed periodicals on chemical ecology, general ecology, and marine biology printed, almost exclusively (≥97%, over 1984–1986, 1994–1996, and 2004–2006), articles that summarized interactions among individual organisms only. For chemical ecology journals, this trend revealed an emphasis on molecular and physiological mechanisms of chemosensory-mediated behavior, on the evolutionary basis for chemical communication and on chemical determinants of immediate trophic relations (Fig. 8, and *G* test: $G_2 \geq 7.71$, $P \leq 0.02$, showing a significant difference between molecular/biochemistry, physiology, and behavior categories among each of the 3-year intervals). Moreover, a majority of papers highlighted details of biosynthetic pathways, molecular receptive fields, and cue/signal structural elucidations. Consequently, behavior was mostly a bioassay tool, aiding the discovery of active chemical moieties. With a few notable exceptions across all journal categories (e.g., Philips et al. 2003; Kunert et al. 2005; Trussell et al. 2006, Torto et al. 2007), clear connections have yet to be established between individual-based behavior and ecological interactions at other levels. Outstanding opportunities await.

Synthesis and Conclusions

Chemical cues/signals present an extraordinary opportunity for broadening knowledge of ecological processes. The extent to which future research advances the field depends, in part, on appreciation for conceptual and methodological

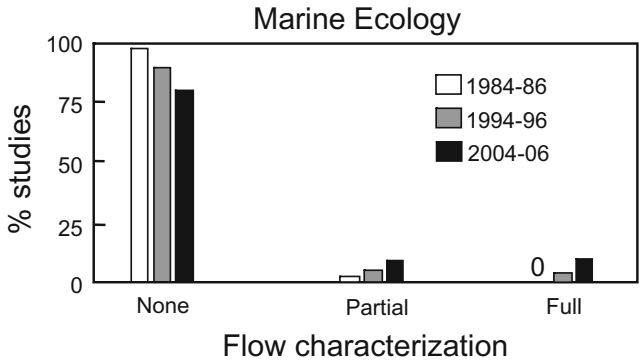

Fig. 7 Of those papers published on chemical cues/signals in marine biology/ecology journals (BB, JEMBE, MB, LO, and MEPS), the relative percentage that reported results using partially, fully, or uncharacterized flow environments. Data were pooled over indicated time intervals

issues of the past. Increased attention to combined chemical and physical determinations will improve the ecological significance of future investigations. Such advancements will be especially welcome in aquatic systems, where cue/signal molecules only rarely have been identified. Moreover, progress in ecology will proceed more rapidly from extending research beyond individual organisms to encompass populations, communities, and even ecosystems.

Current emphasis on individual-based studies is largely a research legacy. Historically, work on chemical cues/signals began in earnest through pioneering efforts of notable ethologists. Karl von Frisch (1941), for example, investigated behavioral reactions of European minnows to alarm substances from skin of damaged conspecifics. These compounds are emitted only after successful predatory attack and serve as an early warning to other, yet-unharmed, minnows. Such investigations ultimately inspired physiologists to understand the cellular basis of behavior. Today, research that links chemistry, physiology, and behavior flourishes, with a common goal of understanding the molecular basis and evolutionary ecology of chemical communication systems.

New, broad integrative approaches will be challenging, but can bear significant fruit. No longer is there a clear distinction between bioactive molecules acting either in communication or in defense. Many herbivorous animal species exhibit resistance, borrowing plant toxins for their own defense and using these same compounds in courtship and mate attraction (Shulz et al. 1993; Matsumura 1995; Zimmer and Ferrer 2007). Moreover, toxins act as feeding stimulants for consumer species that are resistant to their adverse effects (Camacho and Thacker 2006; Glendenning 2007). Similarly, plants use volatile chemical constituents for communicating with each other at a distance (Agrawal 2000; Baldwin et al. 2006; Izaguirre et al. 2006; Frost et al. 2007). Airborne substances in response to herbivore attack are carried downwind, reaching plants not yet in contact with the predator and activating inducible defenses in their tissues. Thus, lines are becoming blurred between experimental studies on animal and plant taxa, and between chemical communication and chemical defense.

Outstanding examples can guide future research. Historically, research has emphasized, for instance, the roles of chemical cues/signals in mediating behavioral interactions among single predator and single prey systems. Yet, some studies have failed to capture the more realistic ecological scenario in which prey must contend with multiple predators. Today, new investigations are increasingly incorporating three, or more, species that comprise simple food webs of top-, mid-, and bottom-level consumers with, or without, primary producers (Bernot and Turner 2001; Werner and Peacor 2003; Preisser et al. 2005). The complexity of trait-mediated (behavioral and morphological) interactions among, and between, individuals within small trophic networks can reveal the roles of chemical cues/signals under more realistic ecological conditions (Peacor and Werner 2001; Trussel et al. 2006).

Findings of diverse studies also can be assembled in creating a composite view on how chemical cues/signals influence species across multiple trophic levels, and hence, influence population-, community-, and system-wide dynamics. Pyrrolizidine alkaloids, for instance, function in plant chemical defense, mate attraction, and the stimulation of feeding among resistant insect and vertebrate consumers (Dussourd et al. 1989; Eisner and Eisner 1991; Trigo et al. 1996; Weller et al. 1999; Eisner et al. 2000; Bernays et al. 2002a,b). Thus, they fundamentally impact species at multiple trophic levels. The marine alkaloid, saxitoxin (STX), likewise, has varied, opposing physiological effects with strong, but contrasting, ecological consequences. Its presence in phytoplankton behaviorally cues habitat selec-

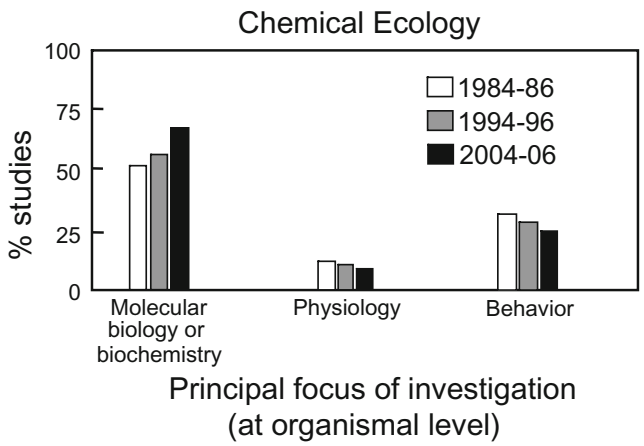

Fig. 8 Of those papers published on chemical cues/signals at the organismal level in chemical ecology journals (JCE and C), the relative percentage focused on molecular mechanisms and biochemical identifications, physiology, or behavior. Data were pooled over indicated time intervals

tion and prey choices of higher-order consumers, significantly structuring coastal ocean communities (Kvitek 1991; Kvitek and Bretz 2004, 2005). Combined behavioral and toxic effects mediated by STX also modify primary plant–herbivore relationships and regulate trophic cascades between benthic and pelagic environments (Carpenter et al. 1985; Myers and Worm 2003; Bruno and O'Connor 2005).

As exemplified by studies on pyrrolizidines, STX, and trait-mediated interactions among suites of species, the scaling up of research on individuals to higher levels of organization can paint an integrated picture of ecological processes. Chemical cues and signals do not operate in isolation. Discovering their full biological impacts will require interdisciplinary studies on multiple scales over time and in space. There now are vast opportunities for ascertaining how organisms react to chemical cues/signals under environmentally realistic conditions. The incorporation of these findings within a larger ecological framework should lead to improved understanding of natural physicochemical phenomena that establish communities and determine ecosystem dynamics.

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References

ACHE, B. W. 1994. Towards a common strategy for transducing olfactory information. *Semin. Cell. Biol.* 5:55–63.

AGELOPOULOS, N. G., DICKE, M., and POSTHUMUS, M. A. 1995. Role of volatile infochemicals emitted by feces of larvae in host-searching behavior of parasitoid *Cotesia rubecula* (Hymenoptera: Braconidae): A behavioral and chemical study. *J. Chem. Ecol.* 21:1789–1811.

AGRAWAL, A. A. 2000. Communication between plants: This time it's real. *Trends Ecol. Evol.* 15:446.

AGRAWAL, A. A., LAU, J. A., and HAMBÄCK, P. A. 2006. Community heterogeneity and the evolution of interactions between plants and insect herbivores. *Q. Rev. Biol.* 81:349–376.

ALBORN, H. T., TURLINGS, T. C. J., JONES, T. H., STENHAGEN, G., LOUGHRIN, J. H., and TUMLINSON, J. H. 1997. An elicitor of plant volatiles from beet armyworm oral secretion. *Science* 276:945–949.

ANDO, T., INOMATA, S., and YAMAMOTO, M. 2004. Lepidopteran sex pheromones. *Topics Curr. Chem.* 239:51–96.

ATSATT, P. R., and O'DOWD, D. J. 1976. Plant defense guilds: many plants are functionally independent with respect to their herbivores. *Science* 193:24–29.

BAKUS, G. J., TARGETT, N. M., and SCHULTE, B. 1986. Chemical ecology of marine organisms: An overview. *J. Chem. Ecol.* 12:951–987.

BALDWIN, I. T., HALITSCHKE, R., PASCHOID, A., VON DAHL, C. C., and PRESTON, C. A. 2006. Volatile signaling in plant-plant interactions: "Talking trees" in the genomics era. *Science* 311: 812–815.

BAU, J., JUSTUS, K. A., LOUDON, C., and CARDÉ, R. T. 2005. Electroantennographic resolution of pulsed pheromone plumes in two species of moths with bipectinate antennae. *Chem. Senses* 30:771–780.

BERNAYS, E. A., CHAPMAN, R. F., and HARTMANN, T. 2002a. A highly sensitive taste receptor cell for pyrrolizidine alkaloids in the lateral galeal sensillum of a polyphagous caterpillar, *Estigmene acrea*. *J. Comp. Physiol. A.* 188:715–723.

BERNAYS, E. A., CHAPMAN, R. F., and HARTMANN, T. 2002b. A taste receptor neuron dedicated to the perception of pyrrolizidine alkaloids in the medial galeal sensillum of two polyphagous arctiid caterpillars. *Physiol. Entomol.* 27:312–321.

BERNOT, R. J., and TURNER, A. M. 2001. Predator identity and trait-mediated indirect effects in a littoral food web. *Oecologia* 129:139–146.

BERTNESS, M. D., and CALLAWAY, R. 1994. Positive interactions in communities. *Ecology* 9:191–193.

BLAUSTEIN, L., KIFLAWI, M., EITAM, A., MANGEL, M., and COHEN, J. E. 2004. Oviposition habitat selection in response to risk of predation in temporary pools: Mode of detection and consistency across experimental treatments. *Oecologica* 138: 300–305.

BOETTCHER, A. A., and TARGETT, N. M. 1996. Induction of metamorphosis in queen conch, *Strombus gigas* Linnaeus, larvae by cues associated with red algae from their nursery grounds. *J. Exp. Mar. Biol. Ecol.* 196:29–52.

BRODIN, T., MIKOLAJEWSKI, D. J., and JOHANSSON, F. 2006. Behavioral and life history effects of predator diet cues during ontogeny in damselfly larvae. *Oecologia* 148:162–169.

BRUNO, J. F., and O'CONNOR, M. L. 2005. Cascading effects of predatory diversity and omnivory in a marine food web. *Ecol. Letters* 8:1048–1056.

BRUNO, J. F., STACHOWICZ, J. J., and BERTNESS, M. D. 2003. Inclusion of facilitation into ecological theory. *Trends Ecol. Evol.* 18:119–125.

BUTMAN, C. A. 1987. Larval settlement of soft-sediment invertebrates: The spatial scales of pattern explained by active habitat selection and the emerging role of hydrodynamical processes. *Oceanogr. Mar. Biol. Annu. Rev.* 25:113–165.

CAMACHO, A. D., PIERCE, H. D., and BORDEN, J. H. 1994. Aggregation pheromones in *Dryocetes affaber* (Mann) (Coleoptera, Scolytidae): Stereoisomerism and species specificity. *J. Chem. Ecol.* 20:111–124.

CAMACHO, F. A., and THACKER, R. W. 2006. Amphipod herbivory on the freshwater cyanobacterium *Lyngbya wollei*: Chemical stimulants and morphological defenses. *Limnol. Oceanogr.* 51:1870–1875.

CARPENTER, S. R., KITCHELL, J. F., and HODGSON, J. R. 1985. Cascading trophic interactions and lake productivity. *Bioscience* 35:634–639.

CARR, W. E. S. 1988. The molecular nature of chemical stimuli in the aquatic environment, pp. 3–27, in J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). The Sensory Biology of Aquatic Animals. Springer-Verlag, New York, N.Y.

CONNELL, J. H. 1970. A predator-prey system in marine intertidal region: I. *Balanus glandula* and several predatory species of *Thais*. *Ecol. Monogr.* 40:49–78.

CRESPI, B. J. 2001. The evolution of social behavior in microorganisms. *Trends Ecol. Evol.* 16:178–183.

CRIMALDI, J. P., WILEY, M. B., and KOSEFF, J. R. 2002. The relationship between mean and instantaneous structure in turbulent passive scalar plumes. J. Turbulence Article 014.

CUMMINS, S. E., SCHEIN, C. H., XU, Y., BRAUN, W., and NAGLE, G. T. 2005. Molluscan attractins: A family of water-borne protein pheromones with interspecific attractiveness. *Peptides* 26: 121–129.

DACEY, J. W. H., KING, G. M., and WAKEHAM, S. G. 1987. Factors controlling emission of dimethylsulfide from salt marshes. *Nature* 330:643–645.

DACEY, J. W. H., and WAKEHAM, S. G. 1986. Oceanic dimethylsulfide: Production during zooplankton grazing on phytoplankton. *Science* 233:1314–1316.

DE MORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Science* 393:570–573.

DENNY, M. W. 1988. Biology and the Mechanics of the Wave-Swept Environment. Princeton University Press, Princeton, N. J.

DICKE, M., and SABELIS, M. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.* 2:131–139.

DREANNO, C., MATSUMURA, K., DOHMAE, N., TAKIO, K., HIROTA, H., KIRBY, R. R., and CLARE, A. S. 2006. An α_2-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. *Proc. Natl. Acad. Sci., USA* 39:14396–14401.

DUFFY, J. E., and STACHOWICZ, J. J. 2006. Why biodiversity is important to oceanography: Potential roles of genetics, species, and trophic diversity in pelagic ecosystem processes. *Mar. Ecol. Prog. Ser.* 311:179–189.

DUSSOURD, D. E., HARVIS, C. A., MEINWALD, J., and EISNER, T. 1989. Pheromonal advertisement of a nuptial gift by a male moth (*Utetheisa ornatrix*). *Proc. Natl. Acad. Sci. USA* 88: 9224–9227.

EISNER, T., and EISNER, M. 1991. Unpalatability of the pyrrolizidine alkaloid-containing moth *Utetheisa ornatrix* and its larva to wolf spiders. *Psyche* 98:111–118.

EISNER, T., EISNER, M., ROSSININ, C., IYENGAR, V. K., ROACH, B. L., BENEDIKT, E., and MEINWALD, J. 2000. Chemical defense against predation in an insect egg. *Proc. Natl. Acad. Sci. USA* 97:1634–1639.

ELKINTON, J. S., CARDÉ, R. T., and MASON, C. J. 1984. Evaluations of time-average dispersion models for estimating pheromone concentration in a deciduous forest. *J. Chem. Ecol.* 10:1081–1108.

FEENY, P. P. 1976. Plant apparency and chemical defense, pp. 1–40, in J. W. Wallace, and R. L. Mansell (eds.). Biochemical Interactions between Plants and Insects. Plenum, New York, N.Y.

FENCHEL, T. 2002. Microbial behavior in a heterogeneous world. *Science* 296:1068–1071.

FILIPPOVA, L. V., and NOZDRACHEV, A. D. 2007. The role of visceral receptors in the mechanisms of neuroimmune interactions in mammalian small intestine. *Biol. Bull.* 34:277–285.

FINELLI, C. M., PENTCHEFF, N., ZIMMER, R. K., and WETHEY, D. S. 2000. Physical constraints on ecological processes: A field test of odor-mediated foraging. *Ecology* 81:784–797.

FINELLI, C. M., PENTCHEFF, N. D., ZIMMER-FAUST, R. K., and WETHEY, D. S. 1999. Odor transport in turbulent flows: Constraints on animal navigation. *Limnol. Oceanogr.* 44: 1056–1071.

FROST, C. J., APPEL, H. M., CARLSON, J. E., DE MORAES, C. M., MESCHER, M. C., and SCHULTZ, J. C. 2007. Within-plant signaling via volatiles overcomes vascular constraints on systemic signaling and primes responses against herbivores. *Ecol. Lett.* 10:490–498.

GAMMANS, N., BULLOCK, J. M., GIBBONS, H., and SCHONROGGE, K. 2006. Reaction of mutualistic and granivorous ants to *Ulex elaiosome* chemicals. *J. Chem. Ecol.* 32:1935–1947.

GATES, R. D., BIL, K. Y., and MUSCATINE, L. 1999. The influence of anthozoan "host factor" on the physiology of a symbiotic dinoflagellate. *J. Exp. Mar. Biol. Ecol.* 232:241–259.

GATES, R. D., HOEGH-GULDBERG, O., MCFALL-NGAI, M. J., BIL, K. Y., and MUSCATINE, L. 1995. Free amino acids exhibit anthozoan "host factor" activity: They induce the release of photosynthate from symbiotic dinoflagellates in vitro. *Proc. Natl. Acad. Sci., USA* 92:7430–7434.

GIBSON, G. D., and CHIA, F. S. 1994. A metamorphic inducer in the opisthobranch *Haminaea callidegenita*: Partial purification and biological activity. *Biol. Bull.* 187:133–142.

GLENDENNING, J. 2007. How do predators cope with chemically defended foods? *Biol. Bull.* 213:252–266.

HÄMBACK, P. A., and BECKERMAN, A. P. 2003. Herbivory and plant resource competition: A review of two interacting interactions. *Oikos* 101:26–37.

HAMILTON, J. G. C., HALL, D. R., and KIRK, W. D. J. 2005. Identification of a male-produced aggregation pheromone in the western flower thrips *Frankliniella occidentalis*. *J. Chem. Ecol.* 31:1369–1379.

HARDEGE, J., BARTELS-HARDEGE, H., MULLER, C. T., and BECKMANN, M. 2004. Peptide pheromones in female *Nereis succinea*. *Peptides* 9:1517–1522.

HAY, M. E. 1996. Marine chemical ecology: What's known and what's next? *J. Exp. Mar. Biol. Ecol.* 200:103–134.

HAY, M. E., and FENICAL, W. 1988. Marine plant–herbivore interactions: The ecology of chemical defense. *Annu. Rev. Ecol. Syst.* 19:111–145.

HAY, M. E. 2002. The next wave in aquatic chemical ecology. *J. Chem. Ecol.* 28:1897–1899.

HENSE, B. A., KUTTLER, C., MÜLLER, J., ROTHBALLER, M., HARTMANN, A., and KREFT, J.-U. 2007. Does efficiency sensing unify diffusion and quorum sensing? *Nature Rev. Microbiol.* 5:230–239.

HILDEBRAND, J. G. 1995. Analysis of chemical signals by nervous systems. *Proc. Natl. Acad. Sci., USA* 92:67–74.

HILDEBRAND, J. G., and SHEPHERD, G. M. 1997. Mechanisms of olfactory discrimination: Convergent evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20:595–631.

HIRSCH, A. M., BAUER, W. D., BIRD, D. M., CULLIMORE, J., TYLER, B., and YODER, J. I. 2003. Molecular signals and receptors: Controlling rhizosphere interactions between plants and other organisms. *Ecology* 84:858–868.

HOWE, N. R., and SHEIKH, Y. M. 1975. Anthopleurine: A sea anemone alarm pheromone. *Science* 189:386–388.

INOUYE, D. W., and WALLER, G. D. 1984. Responses of honeybees (*Apis mellifera*) to amino acid solutions mimicking floral nectars. *Ecology* 65:618–625.

IZAGUIRRE, M. M., MAZZA, C. A., BIONDINI, M., BALDWIN, I. T., and BALLARE, C. I. 2006. Remote sensing of future competitors: Impacts on plant chemical defenses. *Proc. Natl. Acad. Sci., USA* 103:7170–7174.

JACKSON, J. L., WEBSTER, D. R., RAHMAN, S., and WEISSBURG, M. J. 2007. Bed-roughness effects on boundary-layer turbulence and consequences for odor-tracking behavior of blue crabs (*Callinectes sapidus*). *Limnol. Oceanogr.* 52:1883–1897.

JOHNSON, B. R., and ATEMA, J. 1986. Chemical stimulants for a component of feeding behavior in the common gulf weed shrimp, *Leander tenuicornis* (Say). *Biol. Bull.* 170:1–10.

JONES, B. E. 2005. From waking to sleeping: Neuronal and chemical substrates. *Trends Pharm. Sci.* 26:578–586.

JONES, K. M., KOBAYASHI, H., DAVIES, B. W., TAGA, M. E., & WALKER, G. C. 2007. How rhizobial symbiants invade plants: the *Sinorhizobium-Medicago* model. *Nature Rev. Microbiol* 5:619–633.

JUTEL, M., BLASER, K., and AKDIS, C. A. 2006. Histamine receptors in immune regulation and allergen-specific immunotherapy. *Immunol.* 26:245–257.

KARP-BOSS, L., BOSS, E., and JUMARS, P. A. 1996. Nutrient fluxes to planktonic osmotrophs in the presence of fluid motion. *Oceanogr. Mar. Biol. Annu. Rev.* 34:71–107.

KAUPP, U. B., HILDEBRAND, E., and WEYAND, I. 2006. Sperm chemotaxis in marine invertebrates – molecules and mechanisms. *J. Cell Physiol.* 208:487–496.

KELLER, L., and SURETTE, M. G. 2006. Communication in bacteria: An ecological and evolutionary perspective. *Nature Rev. Microbiol.* 4:249–258.

KICKLIGHTER, C. E., GERMANN, M., KAMIO, M., and DERBY, C. D. 2007. Molecular identification of alarm cues in the defensive secretions of the sea hare *Aplysia californica*. *Anim. Behav.* 74:1481–1492.

KRUG, P. J., and MANZI, A. E. 1999. Waterborne and surface-associated carbohydrates as settlement cues for larvae of the specialist marine herbivore, *Alderia modesta*. *Biol. Bull.* 197: 94–103.

KUNERT, G., OTTO, S., ROSE, U. S. R., GERSHENZON, J., and WEISSER, W. W. 2005. Alarm pheromone mediates production of winged dispersal morphs in aphids. *Ecol. Lett.* 8:596–603.

KVITEK, R. G. 1991. Paralytic shellfish toxins sequestered by bivalves as a defense against siphon-nipping fish. *Mar. Biol.* 111:369–374.

KVITEK, R., and BRETZ, C. 2004. Harmful algal bloom toxins protect bivalve populations from sea otter predation. *Mar. Ecol. Prog. Ser.* 271:233–243.

KVITEK, R., and BRETZ, C. 2005. Shorebird foraging behavior, diet, and abundance vary with harmful algal bloom toxin concentration in invertebrate prey. *Mar. Ecol. Prog. Ser.* 293: 303–309.

LOREAU, M., NAEEM, S., INCHAUSTI, P., BENGTSSON, J., GRIME, J. P., HECTOR, A., HOOPER, D. U., HUSTON, M. A., RAFFAELLI, D., SCHMID, B., TILMAN, D., and WARDIE, D. A. 2001. Biodiversity and ecosystem functioning: Current knowledge and future challenges. *Science* 294:804–808.

LOUGHRIN, J. H., POTTER, D. A., and HAMILTON-KEMP, T. R. 1995. Volatile compounds induced by herbivory act as aggregation kairomones for the Japanese beetle (*Popillia japonica* Newman). *J. Chem. Ecol.* 21:1457–1467.

MAFRA-NETO, A., and CARDÉ, R. T. 1994a. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MAFRA-NETO, A., and CARDÉ, R. T. 1994b. Rate of realized interception of pheromone pulses in different wind speeds modulates almond moth orientation. *J. Comp. Physiol. A.* 182:563–572.

MARDEN, J. H. 1984. Remote perception of floral nectar by bumblebees. *Oecologia* 64:232–240.

MATRAI, P., and KELLER, M. D. 1994. Total organic sulfur and dimethylsulfoniopropionate in marine phytoplankton: Intracellular variations. *Mar. Biol.* 119:61–68.

MATSUMURA, K. 1995. Tetrodotoxin as a pheromone. *Nature* 378:563–564.

MAYER, M. S., and MCLAUGHLIN, J. R., eds. 1991. Handbook of Insect Pheromones and Sex Attractants. CRC Press, Boca Raton, FL. 992 p.

MCCLINTOCK, J. B., and BAKER, B. J., eds. 2001. Marine Chemical Ecology. CRC Press, Boca Raton, FL. 624 p.

MEAD, K. S. 2002. From odor molecules to plume tracking: An interdisciplinary, multilevel approach to olfaction in stomatopods. *Integ. Comp. Biol.* 42:258–264.

MILLER, D. C., JUMARS, P. A., and NOWELL, A. R. M. 1984. Effects of sediment transport on deposit feeding: Scaling arguments. *Limnol. Oceanogr.* 29:1202–1217.

MILLER, R. L. 1985. Sperm chemo-orientation in the metazoa, pp. 275–337, in C. B. Metz, and A. Monroy (eds.). The Biology of Fertilization, Vol. 2. Academic Press, New York, N.Y.

MOORE, P. A., and CRIMALDI, J. P. 2004. Odor landscapes and animal behavior: Tracking odor plumes in different physical worlds. *J. Mar. Systems* 49:55–64.

MULLER, C. H., MULLER, W. H., and HAINES, B. L. 1964. Volatile growth inhibitors produced by aromatic shrubs. *Science* 143:471–473.

MURDOCH, W. W. 1969. Switching in general predators: Experiments on predator specificity and stability of prey populations. *Ecol. Monogr.* 39:335–354.

MURLIS, J., ELKINTON, J. S., and CARDÉ, R. T. 1992. Odor plumes and how insects use them. *Annu. Rev. Entomol.* 37:505–532.

MURLIS, J., and JONES, C. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

MURLIS, J., WILLIS, M. A., and CARDÉ, R. T. 2000. Spatial and temporal structures of pheromone plumes in fields and forests. *Physiol. Entomol.* 25:211–222.

MYERS, R. A., and WORM, B. 2003. Rapid depletion of predatory fish communities. *Nature* 423:280–283.

MYLNE, K. R., DAVIDSON, M. J., and THOMSON, D. J. 1996. Concentration fluctuation measurements in tracer plumes using high and low frequency response detectors. *Boundary-Layer Meteorol.* 79:225–242.

NEVITT, G. A. 2000. Olfactory foraging by Antarctic Procelliform seabirds: Life at high Reynolds numbers. *Biol. Bull.* 198:245–253.

NEVITT, G. A., VEIT, R. R., and KARIEVA, P. M. 1995. Dimethyl sulfide as a foraging cue for Antarctic Procelliform seabirds. *Nature* 376:680–682.

NOWELL, A. R. M., and JUMARS, P. A. 1984. Flow environments of aquatic benthos. *Annu. Rev. Ecol. Syst.* 15:303–328.

NOWELL, A. R. M., and JUMARS, P. A. 1987. Flumes: Theoretical and experimental considerations for simulation of benthic environments. *Oceanogr. Mar. Biol. Annu. Rev.* 25:91–112.

NYLUND, G. M., GRIBBEN, P. E., DE NYS, R., STEINBERG, P. D., and PAVIA, H. 2007. Surface chemistry versus whole-cell extracts: Antifouling tests with seaweed metabolites. *Mar. Ecol. Prog. Ser.* 329:73–84.

PAINE, R. T. 1966. Food web complexity and species diversity. *Am. Nat.* 100:65–75.

PAINTER, S. D., CLOUGH, B., GARDEN, R. W., SWEEDLER, J. V., and NAGLE, G. T. 1998. Characterization of *Aplysia* attractin, the first waterborne peptide pheromone in invertebrates. *Biol. Bull.* 194:120–131.

PAWLIK, J. R. 1992. Chemical ecology of the settlement of benthic marine invertebrates. *Oceanogr. Mar. Biol. Annu. Rev.* 30:273–335.

PAWLIK, J. R., and BUTMAN, C. A. 1993. Settlement of a marine tube worm as a function of current velocity: Interacting effects of hydrodynamics and behavior. *Limnol. Oceanogr.* 38:1730–1740.

PEACOR, S. D., and WERNER, E. E. 2001. The contribution of trait-mediated indirect effects to the net effects of a predator. *Proc. Natl. Acad. Sci., USA* 98:3904–3908.

PERRET, X., STAEHELIN, C., and BROUGHTON, W. J. 2000. Molecular basis of symbiotic promiscuity. *Microbiol. Mol. Biol. Rev.* 64:180–201.

PETERSON, J. E., and HASTINGS, A. 2001. Dimensional approaches to scaling experimental ecosystems: Designing mousetraps to catch elephants. *Am. Nat.* 157:324–333.

PHILIPS, D. A., FERRIS, H., COOK, D. R., and STRONG, D. R. 2003. Molecular control points in rhizosphere food webs. *Ecology* 84:816–826.

POHNERT, G., STEINKE, M., and TOLLRIAN, R. 2007. Chemical cues, defence metabolites and the shaping of pelagic interspecific interactions. *Trends Ecol. Evol.* 22:198–204.

PREISSER, E. L., BOLNICK, D. I., and BERNARD, M. F. 2005. Scared to death? The effects of intimidation and consumption in predator-prey interactions. *Ecology* 56:501–509.

REDFIELD, R. J. 2002. Is quorum sensing a side effect of diffusion sensing? *Trends Microbiol.* 10:365–370.

RIFFELL, J. A., KRUG, P. J., and ZIMMER, R. K. 2002. Fertilization in the sea: The chemical identify of an abalone sperm attractant. *J. Exp. Biol.* 205:1439–1450.

RIFFELL, J. A., and ZIMMER, R. K. 2007. Sex and flow: The consequences of fluid shear for sperm-egg interactions. *J. Exp. Biol.* 210:3644–3660.

ROELOFS, W. L. 1995. Chemistry of sex attraction. *Proc. Natl. Acad. Sci., USA* 92:44–49.

ROELOFS, W. L., LIU, W. T., HAO, G. X., JIAO, H. M., ROONEY, A. P., and LINN, C. E. 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci., USA* 99:13621–13626.

ROELOFS, W. L., and ROONEY, A. P. 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Natl. Acad. Sci., USA* 100:9179–9184.

SAKATA, K., TSUGE, M., and INA, K. 1986. Chemical studies on phagostimulants for marine gastropods: A simple bioassay for feeding stimulants for the young sea hare, *Aplysia juliana*. *Mar. Biol.* 91:509–511.

SHULZ, S., FRANCKE, W., BOPPRÉ, M., EISNER, T., and MEINWALD, J. 1993. Defense mechanisms of arthropods: Stereochemical pathway of hydroxydanaidal production from alkaloid precursors in *Creatonotos transiens* (Lepidoptera, Arctiidae). *Proc. Natl. Acad. Sci., USA* 90:6834–6838.

SMEE, D. L., FERNER, M. C., and WEISSBURG, M. J. 2008. Alteration of sensory abilities regulates the spatial scale of nonlethal predator effects. *Oecologia*: in press.

SMEE, D. L., and WEISSBURG, M. J. 2006. Clamming up: Environmental forces diminish the perceptive ability of bivalve prey. *Ecology* 87:1587–1598.

STACHOWICZ, J. J. 2001. Mutualism, facilitation, and the structure of ecological communities. *Bioscience* 51:235–246.

STEINKE, M., STEFELS, J., and STAMHUIS, E. 2006. Dimethyl sulfide triggers search behavior in copepods. *Limnol. Oceanogr.* 51:1925–1930.

STRACK, D., FESTER, T., HAUSE, B., SCHLIEMANN, W., and WALTER, M. H. 2003. Arbuscular mycorrhiza: biological, chemical, and molecular aspects. *J. Chem. Ecol.* 29:1955–1979.

STUART, A. E., BORYCZ, J., and MEINERTZHAGEN, I. A. 2007. The dynamics of signaling at the histamergic photoreceptor synapse of arthropods. *Prog. Neurobiol.* 82:202–227.

SUGATA, Y., OKANO, M., FUJIWARA, T., MATSUMOTO, R., HATTORI, H., YAMAMOTO, M., NISHIBORI, M., and NISHIZAKI, K. 2007. Histamine H4 receptor agonists have more activities than H4 agonism in antigen-specific human T-cell responses. *Immunol.* 121:266–275.

SWANSON, R. L., DE NYS, R., HUGGETT, M. J., GREEN, J. K., and STEINBERG, P. D. 2006. In situ quantification of a natural settlement cue and recruitment of the Australian sea urchin *Holopneustes purpurascens*. *Mar. Ecol. Prog. Ser.* 314:1–14.

SWANSON, R. L., WILLIAMSON, J. E., DE NYS, R., KUMAR, N., BUCKNELL, M. P., and STEINBERG, P. D. 2004. Induction of settlement of larvae of the sea urchin *Holopneustes purpurascens* by histamine from a host alga. *Biol. Bull.* 206:161–172.

TILMAN, D. 1999. The ecological consequences of changes in biodiversity: A search for general principles. *Ecology* 80:1455–1467.

TOONEN, R. J., and PAWLIK, J. R. 1996. Settlement of the tube worm *Hydroides dianthus* (Polychaeta: Serpulidae): Cues for gregarious settlement. *Mar. Biol.* 126:725–733.

TORTO, B., BOUCIAS, D. G., ARBOGAST, R. T., TUMLIN, J. H., and TEAL, P. E. 2007. Multitrophic interaction facilitates parasite–host relationship between an invasive beetle and the honey bee. *Proc. Natl. Acad. Sci., USA* 104:8374–8378.

TRIGO, J. R., BROWN, K. S. Jr., WITTE, L., HARTMANN, T., LUDGER, L., and BARATA, L. E. S. 1996. Pyrrolizidine alkaloids: Different acquisition and use patterns in Apocynaceae and Solanaceae feeding ithomiine butterflies (Lepidoptera: Nymphalidae). *Bull. J. Linn. Soc.* 58:99–123.

TROTT, T. J., and ROBERTSON, J. R. 1984. Chemical stimulants of cheliped flexion behavior by the western Atlantic ghost crab, *Ocypode quadrata* (Fabricius). *J. Exp. Mar. Biol. Ecol.* 78: 237–252.

TRUSSELL, G. C., EWANCHUK, P. J., and BERTNESS, M. D. 2002. Field evidence of trait-mediated indirect interactions in a rocky intertidal food web. *Ecol. Lett.* 5:241–245.

TRUSSELL, G. C., EWANCHUK, P. J., and MATASSA, C. M. 2006. Habitat effects on the relative importance of trait- and density-mediated indirect interactions. *Ecol. Lett.* 9:1245–1252.

VELEZ, Z., HUBBARD, P. C., HARDEGE, J., BARATA, E. N., and CANARO, A. V. M. 2007. The contribution of amino acids to the odour of a prey species in the Senegalese sole (*Solea senegalensis*). *Aquaculture* 265:336–342.

VICKERS, N. J., and BAKER, T. C. 1992. Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone (Lepidoptera, Noctuidae). *J. Insect Behav.* 5:669–687.

VICKERS, N. J., and BAKER, T. C. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Natl. Acad. Sci., USA* 91: 5756–5760.

VICKERS, N. J., CHRISTENSEN, T. A., BAKER, T. C., and HILDEBRAND, J. G. 2001. Odour-plume dynamics influence the brain's olfactory code. *Nature* 410:466–470.

VISSER, A. W., and JACKSON, G. A. 2004. Characteristics of the chemical plume behind a sinking particle in a turbulent water column. *Mar. Ecol. Prog. Ser.* 283:55–71.

VON FRISCH, K. 1941. Über einen Schreckstoff der Fischhaut and seine biologische Bedeutung. *Z. vergl. Physiol.* 29:46–145.

WARD, G. E., BROKAW, C. J., GARBERS, D. L., and VACQUIER, V. D. 1985. Chemotaxis of *Arbacia punctulata* spermatozoa to resact, peptide from the egg jelly layer. *J. Cell Biol.* 101:2324–2329.

WEBSTER, D. R., RAHMAN, S., and DASI, L. P. 2003. Laser-induced fluorescence measurements of a turbulent plume. *J. Engineer. Mechan.-ASCE* 129:1130–1137.

WEISSBURG, M. J. 2000. The fluid dynamical context of chemosensory behavior. *Biol. Bull.* 198:188–202.

WEISSBURG, M. J., and ZIMMER-FAUST, R. K. 1993. Life and death in moving fluids: Hydrodynamic effects on chemosensory-mediated predation. *Ecology* 74:1428–1443.

WEISSBURG, M. J., and ZIMMER-FAUST, R. K. 1994. Odour plumes and how blue crabs use them in finding prey. *J. Exp.Biol.* 197:349–375.

WELLER, S. J., JACOBSON, N. L., and CONNER, W. E. 1999. The evolution of chemical defenses and mating systems in tiger moths (Lepidoptera: Arctiidae). *Biol. J. Linn. Soc.* 68:557–578.

WERNER, E. E., and PEACOR, S. D. 2003. A review of trait-mediated indirect interactions in ecological communities. *Ecology* 84:1083–1100.

WINDER, M., SPAAK, P., and MOOIJ, W. M. 2004. Trade-offs in *Daphnia* habitat selection. *Ecology* 85:2027–2036.

WOLFE, G. V. 2000. The chemical defense ecology of marine unicellular plankton: Constraints, mechanisms, and impacts. *Biol. Bull.* 198:225–244.

WOLFE, G. V., STEINKE, M., and KIRST, G. O. 1997. Grazing-activated chemical defense in a unicellular alga. *Nature* 387:894–897.

YOSHIDA, M., MURATA, M., INABA, K., and MORISAWA, M. 2002. A chemoattractant for ascidian spermatozoa is a sulfonated steroid. *Proc. Natl. Acad. Sci., USA* 99:14831–14836.

ZIMMER, R. K., and BUTMAN, C. A. 2000. Chemical signaling processes in the marine environment. *Biol. Bull.* 198:168–187.

ZIMMER, R. K., COMMINS, J. E., and BROWNE, K. A. 1999. Regulatory effects of environmental chemical signals on search behavior and foraging success. *Ecology* 80:1432–1446.

ZIMMER, R. K., and FERRER, R. P. 2007. Neuroecology, chemical defense, and the keystone species concept. *Biol. Bull.* 213:208–225.

ZIMMER-FAUST, R. K., DE SOUZA, M. P., and YOCH, D. C. 1996. Bacterial chemotaxis and its potential role in marine dimethylsulfide production and biogeochemical sulfur cycling. *Limnol. Oceanogr.* 41:1330–1334.

ZIMMER-FAUST, R. K., FINELLI, C. M., PENTCHEFF, N. D., and WETHEY, D. S. 1995. Odor plumes and animal navigation in turbulent water flow: A field study. *Biol. Bull.* 188:111–116.

ZIMMER-FAUST, R. K., STANFILL, J. M., and COLLARD, S. B. III. 1988. A fast, multi-channel fluorometer for investigating aquatic chemoreception and odor trails. *Limnol. Oceanogr.* 33:1586–1594.

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REVIEW ARTICLE

Physical Processes and Real-Time Chemical Measurement of the Insect Olfactory Environment

Jeffrey A. Riffell · Leif Abrell · John G. Hildebrand

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Abstract Odor-mediated insect navigation in airborne chemical plumes is vital to many ecological interactions, including mate finding, flower nectaring, and host locating (where disease transmission or herbivory may begin). After emission, volatile chemicals become rapidly mixed and diluted through physical processes that create a dynamic olfactory environment. This review examines those physical processes and some of the analytical technologies available to characterize those behavior-inducing chemical signals at temporal scales equivalent to the olfactory processing in insects. In particular, we focus on two areas of research that together may further our understanding of olfactory signal dynamics and its processing and perception by insects. First, measurement of physical atmospheric processes in the field can provide insight into the spatiotemporal dynamics of the odor signal available to insects. Field measurements in turn permit aspects of the physical environment to be simulated in the laboratory, thereby allowing careful investigation into the links between odor signal dynamics and insect behavior. Second, emerging analytical technologies with high recording frequencies and field-friendly inlet systems may offer new opportunities to characterize natural odors at spatiotemporal scales relevant to insect perception and behavior. Characterization of the chemical signal environment allows the determination of when and where olfactory-mediated behaviors may control ecological interactions. Finally, we argue that coupling of these two research areas will foster increased understanding of the physicochemical environment and enable researchers to determine how olfactory environments shape insect behaviors and sensory systems.

Keywords Odor plume · Insect behavior · Odor-plume tracking · PTRMS · Mass spectrometry · Gas chromatography · Odor landscape

Notation for Physical Processes and Analytical Technologies, respectively, is summarized in Appendices 1 and 2.

J. A. Riffell · J. G. Hildebrand
ARL Division of Neurobiology, University of Arizona, Tucson, AZ 857210-0077, USA

L. Abrell
Department of Chemistry, University of Arizona, Tucson, AZ 85721, USA

J. A. Riffell (✉) · L. Abrell · J. G. Hildebrand
Center for Insect Science, University of Arizona, Tucson, AZ 85721, USA
e-mail: jeffr@neurobio.arizona.edu

Introduction

Since animals crawled onto land approximately 400 mya, they have had to contend in a terrestrial, chemosensory environment where airborne signals are chemically different and fluctuate faster than under water. Airborne chemicals must have coevolved with multicellular organisms, especially with the large Angiosperm and insect diversification about 100 mya (Hildebrand 1995; Zimmer and Butman 2000; Bargmann 2006). In water or in air, the extraction of necessary chemical information from the environment has always depended upon discriminating signal from noise and resolving distance and orientation to the source.

In the last 25 years, efforts aimed at understanding how chemical signals are distributed in space and time have gained traction in a research discipline traditionally focused on identifying the behaviorally important chemical signals. A realization has occurred that the physical environment determines (a) whether or not and how much chemical signal is available to the animal and (b) the behavioral response and navigational strategies used by the recipient

organism (Weissburg and Zimmer-Faust 1993; Mafra-Neto and Carde 1994; Vickers and Baker 1994). A number of studies, pioneered by Murlis and Jones (1981), have documented that chemical signals are dynamic in both space and time (Murlis et al. 2000a; Justus et al. 2002b) due to physical processes of the fluid environment (Murlis and Jones 1981; Elkinton et al. 1984; Moore and Atema 1991; Murlis et al. 2000b; Webster and Weissburg 2001; Justus et al. 2002b). Seminal work by Baker and Cardé, in turn, has demonstrated that the physical environment dictates the navigational behavior employed by an organism through its effects on controlling the information provided by the chemical signal (Willis and Baker 1988; Mafra-Neto and Carde 1994; Vickers and Baker 1994; Kuenen and Carde 1994; Willis et al. 1994; Zimmer-Faust et al. 1995; Vickers and Baker 1996). The aerodynamic environment can dictate important ecological interactions and be a selective force on the evolution of olfactory systems, as it determines the chemical information available to an organism (Hildebrand 1995; Zimmer and Butman 2000).

The olfactory systems of terrestrial animals sample the environment and process chemical stimuli faster than once per second, but most chemical analysis methods for behaviorally relevant volatiles require extended time periods for sample collection, preparation, and separation (Tholl and Röse 2006). Thus, the temporal resolution of the olfactory information traditionally has been lost to scientists. New analytical technologies involving fast (<1 s or <1 min) sampling times and quantitative resolution are becoming available for chemical ecologists, thereby providing a means to sample the odor environment at time scales approaching that of the insect olfactory system.

In this review, we examine how the physical environment constrains olfactory-guided behavior by considering five main ideas. First, because the fluid environment controls the chemical information available to an organism, a review of some simple, but critical, physical processes that can provide linkages between the physical environment and odor-mediated behavior are considered. This information hopefully will provide researchers a set of tools to aid in characterizing the physical environment. Second, a qualitative description of the different characteristics of a turbulent odor plume is reviewed. Third, we consider the different physicochemical environments that insects navigate in. Fourth, we examine the effects of body size on the odor landscape experienced by a navigating insect. Whether an animal is <1 mm or >10 cm will potentially influence the physicochemical landscape in which it exists and the sensory strategies that it utilizes. Last, we examine the current analytical technologies available to chemical ecologists and behavioralists to examine spatial and temporal distribution of chemicals that control behavior. Taken together, the aim of these sections is to provide a broad understanding of the physical factors that control odor information and behavior and the rapidly evolving technologies that may increase our understanding of these processes.

Physics of the Odor Environment

Once odorant molecules evaporate from a source, they will instantly become subject to the physical forces of the environment and transported by ambient motions of the air. There are two physical processes that influence the transport of odor at different spatial scales: molecular diffusion and turbulence and advection. Molecular diffusion transports odorants by Brownian motion and occurs under small spatial scales (<1 mm) and long time periods (ca. 80 min for an odorant molecule to travel 1 m). In contrast, ambient motion by air can transport odorant molecules $>10^3$ times quicker than diffusion over equivalent distances and is thus the principal physical process that controls odor transport at distances >1 cm.

At these much larger spatial scales, the odor environment is dynamic, both spatially and temporally. There are two physical processes that cause this variability: advective and turbulent transport. Advection is the bulk transport of the odorants by the mean direction of the flow, whereas turbulent transport comprises the chaotic, three-dimensional mixing of odor with clean air. By using Gaussian distribution models for pollutant transport, researchers initially modeled chemical plumes as a diffusive, time-averaged process. However, the sensory systems of animals flying or walking in the terrestrial environment rarely, if at all, use such time-averaged odor information. Instead, animals use near-instantaneous concentrations of the odor plume to navigate to a source. It is, therefore, important to understand the near-instantaneous resolution of odor information to determine how animals can locate odor sources. To understand this process, it is necessary first to understand the physical forces that control odor information at these spatial and temporal scales. Excellent reviews of the instantaneous structure of plumes are found in Murlis et al. (1992), Weissburg (2000), and Koehl (2006). In this review, we examine physical processes and parameters useful to characterize the odor dynamical processes and the rapidly evolving analytical technology that may be used to characterize the terrestrial chemical environment.

Aerodynamic Parameters

Odor temporal dynamics and dispersion are governed by ambient air currents. An insect living in a calm, low wind environment will experience different chemical stimulus dynamics from one inhabiting a high-energy, windy environment. A measure of the fluid dynamic regime experienced by a navigating insect can be approximated by

the body Reynolds number (*Re*), which compares the inertial forces with viscous forces. Inertial forces represent the large momentum-containing scales that maintain the turbulent motion. In contrast, the viscous forces signify the "stickiness" of the fluid that dampens the momentum, thereby bringing it to a halt. The inertial term takes the form of ρu^2 [where ρ is the density of air (kg/m^3), and u is the wind velocity or translational velocity of the organism (m/s)], and the viscous term takes the form of $\mu u/S$ [where μ is the air viscosity (Ns/m^2), and S is the spatial scale of interest (m)]. The ratio of these two forces thus takes shape in the equation as

$$Re = \frac{\rho u^2}{\mu u/S} = \frac{\rho S u}{\mu}, \quad (1)$$

thereby producing a non-dimensional number that indicates the nature of the air flow. When $Re >> 1$, the air momentum will overwhelm viscous damping, and air motion will be turbulent. As *Re* increases, the air flow becomes more turbulent and causes the spatial scale of the turbulence to increase (e.g., the size of the turbulent eddies) and the time scales of the turbulent fluctuations to increase. As *Re* decreases and approaches unity, the spatiotemporal dynamics of the turbulent flow decrease until the flow becomes laminar (without three-dimensional structure).

The Reynolds number is useful for examining the fluid environment an organism might experience, but to accurately replicate the aerodynamic conditions of the field within the laboratory, it is necessary to go beyond the mean advective bulk flow and instead examine the fluctuations (turbulence) of air flow. Fluid (liquid or gas) near any surface will stick to the surface and not slide along that substrate. Due to this "no-slip" condition, the fluid in contact with the substrate will impose a frictional force on the fluid layers directly above it causing the fluid to shear or change in velocity with increasing distance from the substrate (Fig. 1A). Within this velocity gradient region—also called the "log layer" because of the logarithmic change in velocity with vertical distance from the substrate—momentum is transferred from the large turbulent structures (called 'eddies') to progressively smaller and smaller eddies until energy is dissipated into heat by the frictional force of viscosity near the surface (Fig. 1A). Thus, turbulence can be much greater near the surface than in the region of the free-stream velocity. The turbulence and momentum transfer to the surface can be described through shear stress (τ_R) (also called Reynolds shear stress) and is a the function of

$$\tau_R = -\rho\overline{u'w'}, \quad (2)$$

where the term $\overline{u'w'}$ is the mean of the cross-product of fluctuating velocities in the horizontal (u) and vertical (w) dimensions, and the negative term in front of the air density (ρ) denotes the downward flux of momentum. The

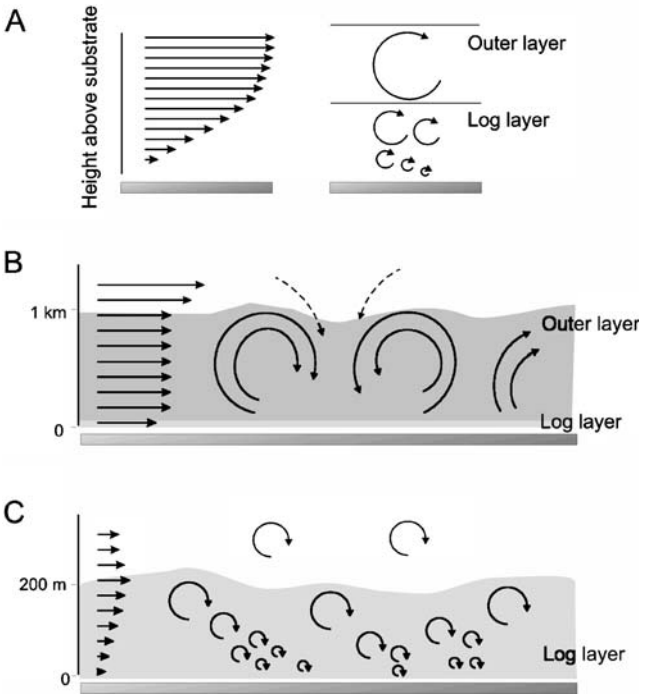


Fig. 1 The effect of flow velocity on the structure within a turbulent boundary layer and the change in diurnal conditions. **A** The boundary layer is defined by the region where the flow velocity reaches 95% of the free stream velocity (*left*). A schematic of the turbulent velocity fluctuations is shown on the right where turbulent kinetic energy is transferred from the large energy-containing eddies in the outer region of the boundary layer to progressively smaller and smaller eddies in the log layer. **B** A schematic of the daytime boundary layer conditions where surface-layer heating causes the large eddies in the outer layer to reach all the way to the substratum. **C** A schematic of the nighttime boundary layer conditions. Substrate cooling causes a stable boundary layer to develop and the kinetic turbulent energy levels to drop in comparison to daytime turbulent conditions

Reynolds shear stress provides a measure of the turbulence of a system. Another term for describing boundary layer turbulence is the friction velocity (m/s), u_*, and is related to the shear stress by $(\tau_R/\rho)^{-1/2}$. This term has the units of a velocity (m/s) but is more accurately described as the scalar of momentum in a boundary layer or the velocity at which momentum is transferred. As u_* increases, turbulent fluctuations in velocity also increase; therefore, u_* is an important scaling parameter in boundary layer and turbulent wind studies. The turbulent conditions of a boundary layer (e.g., a large grassy meadow or a desert plain) can be parameterized through the roughness Reynolds number (Re_*), which is analogous to the Reynolds number described above but provides a means to describe the turbulent conditions of a boundary layer. This term is described by the equation

$$Re_* = \frac{\rho u_* D}{\mu}, \quad (3)$$

where D is the size (m) of the elements on the substrate (e.g., the rocks and vegetation on the ground), and u_* is the friction velocity described above. For Re_* values of 3.5–6, turbulent

eddies begin to penetrate the upper region of the boundary layer, and if $Re_*>75$, then turbulence extends all the way to the substratum (Schlichting 1987; Stull 1988). An insect navigating to an odor source within a boundary layer of $Re_*>75$ will experience a dynamic, filamentous odor stimulus, whereas an insect navigating at $Re_*<6$ will experience a smooth continuous odor gradient near the substratum (where diffusional and inertial processes are near unity) and a filamentous odor stimulus farther from the substratum.

The roughness Reynolds number provides a useful parameter to describe certain environments (further detailed below), but caution must be used as it may not accurately describe the dominant physical processes for a given habitat. For instance, the velocity fluctuations of a meadow sat the foot of a 1,000-m-tall mountain will reflect the influence of the mountain on the atmospheric conditions rather than the grass and shrubs found within the meadow (D=1 m), thereby causing underestimation between realized and expected Reynolds roughness numbers. Therefore, the length scale, D, used in calculating Re_* should be chosen carefully. Nevertheless, measurement of τ_R and u_* provides valuable information regarding the level of turbulence in a given habitat and also allows these physical conditions to be simulated in the laboratory for dynamically scaled odor-plume tracking studies (Zimmer and Zimmer 2008).

In the field, an important function of the stability, or lack thereof, of the boundary layer is the atmospheric conditions (temperature and air turbulence). In the absence of energetic atmospheric events that mix the atmosphere, like frontal passages, daytime temperature-induced convection occurs from heating of the surface, causing large eddies to form and thoroughly mix the air from the surface to >1 km height (Figs. 1B and 2A,B; Kaimal and Finnigan 1994; Mole and Jones 1994). In contrast, at sunset, the temperature-driven convection weakens, and a stable boundary layer develops near the surface (0–200 m; Fig. 1C and 2A,B; Mylne 1992; Yee et al. 1993; Mole and Jones 1994). The stability of the boundary layer can be determined through the Richardson number (R_i), which is a dimensionless parameter that represents the relative importance between temperature- and wind-driven turbulence and has the form

$$R_i = \frac{\left(g/\overline{\theta}\right)\left(\partial\overline{\theta}/\partial z\right)}{\left(\partial\overline{u}/\partial z\right)^2}, \tag{4}$$

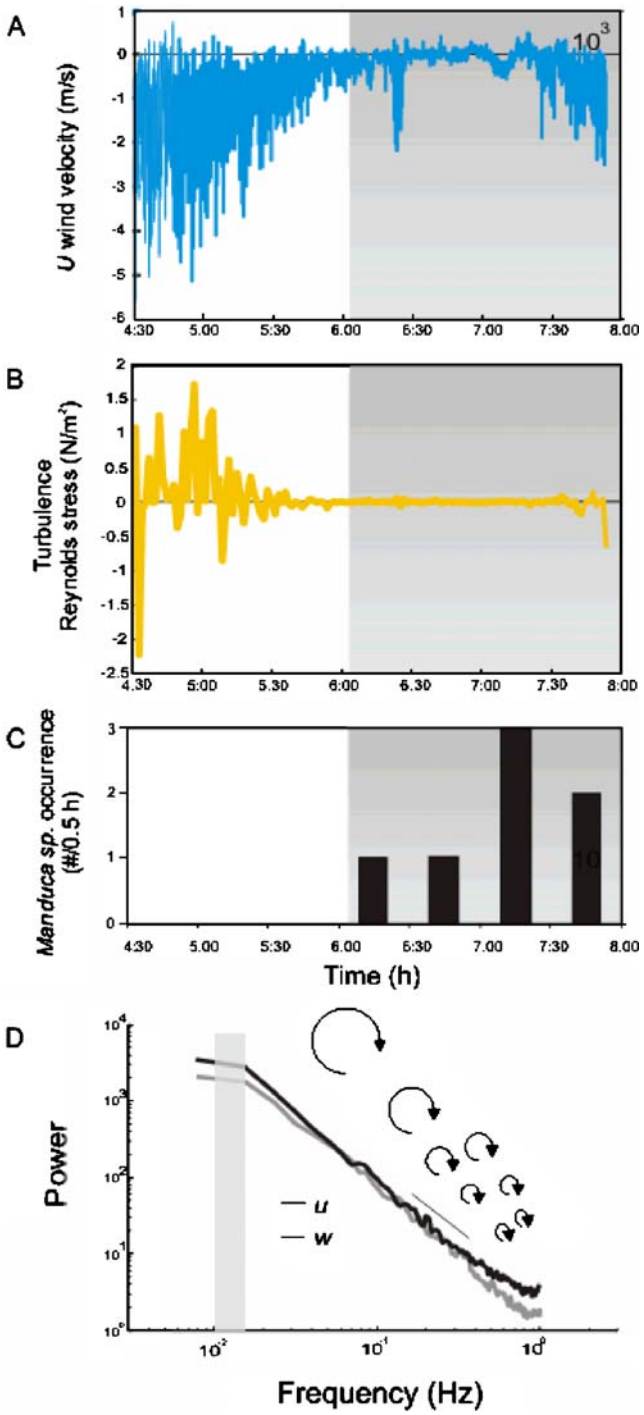
where g is the acceleration due to gravity (9.81 m/s^2), $\overline{\theta}$ is the mean temperatures, z is the height above the substrate, and $\overline{u}$ denotes the mean wind. When R_i is positive, the boundary layer is stable (Fig. 1C). R_i is negative for an unstable boundary layer (Fig. 1B) and zero for a neutral boundary layer. For example, Mole and Jones (1994) found that plume intermittency, filament concentration, and average concentration were higher for stable than for unstable conditions. Thus, when atmospheric conditions are relatively calm and uncloudy, the odor environment at night might be conducive for odor plume transport and the maintenance of plume structure and hence insect navigation (e.g., Fig. 2C).

The Odor Landscape and Turbulent Odor Plumes

Examination of olfactory tracking behavior of insects, mammals, and birds (all inhabitants of turbulent flows) has demonstrated clearly that animals do not use time-averaged properties of the odor (Elkinton et al. 1984) and instead use near-instantaneous (< 1 s) information present in the plume (Mafra-Neto and Carde 1994; Vickers and Baker 1994).

Temporal Features of the Plume At time and spatial scales <1 s and >1 mm, respectively, turbulent odor plumes are patchy (Fig. 3B) and are composed of odor spikes or 'filaments' of intense concentration interspersed with periods and regions of low or undetectable concentrations. The dynamic occurrence of the chemical signal is termed its intermittency, which is a proportional value ranging from 0 (odor continuously present) to 1 (odor absent; Cardé and Willis 2008). The spatial and temporal scales in which the odor filaments occur are directly related to the temporal and spatial scales of the turbulence (Fig. 3B; Zimmer-Faust et al. 1988).

A number of different factors can influence plume intermittency. A higher wind speed or turbulence can cause the odor filaments to become thin (Yee et al. 1993) and increase in frequency (more filaments per unit time; Fackrell and Robins 1982). However, an increase in turbulence has a corresponding effect of lowering the odor-laden filament concentration (Finelli et al. 2000; Moore and Crimaldi 2004). Moreover, the size and position above the substrate of an odor source also affects the plume dynamics. Odor sources high off the substratum will be subject to higher advective flows than those nearer the ground (Fig. 1C). For instance, flowers of the agave, *Agave palmeri*, which produce strong night time odor emissions, are 4–6 m from the ground and are subject to high advective wind velocities (2.2–7.1 m/s). Whereas flowers from the jimsonweed plant, *Datura wrightii*, are close to the ground (~0.5 m) and experience lower wind velocities (0.1–2.3 m/s). Odor source size will also affect the plume structure close to the source. For example, plume intermittency is higher for larger sources than smaller, but if a small source is in close proximity to a large structure (e.g., a female moth calling from a tree), then the plume will behave as if coming from the large structure. A rough approximation of the effects on odor source size and air velocity on plume intermittency may be determined by the



◀ **Fig. 2** A time series of the turbulent conditions near a *Datura wrightii* flower patch measured with a 3D sonic anemometer (Young, USA) sampling at 32 Hz. Regions in gray represent the time from when the sun began to set. In the daytime, wind velocities (**A**) and turbulent shear stresses (**B**) are nearly two-orders of magnitude higher then those that occur in the evening (shown in *gray*). **C** The drop in turbulent intensities corresponds with the nighttime foraging activity by *Manduca sexta* hawkmoths which feed on nectar from *D. wrightii* flowers. The drop in turbulent intensities may enable hawkmoths to effectively navigate to the strong volatile emissions of *D. wrightii* flowers. **D** Daytime energy spectra of wind velocities in the vertical (w) and longitudinal (u) dimensions. The largest energy-bearing eddies (in *shaded region*) occur at a time period of 100 s (frequencies ~10^{-2} Hz), and corresponds to n, where n is the frequency of peak intensity of the power spectrum. The large eddies progressively cascade smaller and smaller eddies until the turbulent kinetic energy is dissipated at a frequency of 0.8 Hz. Spectra of the velocity fluctuations contain information on the temporal and spatial features of the turbulence and relate directly to dispersion and structure of the plume

Strouhal number (St), which relates to the frequency in which eddies are shed by structures in flow. For cylindrical objects, the eddies are shed with a predictable rate,

$$St = \frac{fD_{\text{object}}}{u}, \tag{5}$$

where f is the frequency of eddy shedding (per second), and D_{object} is the diameter of the object (m). Eddies shed by the object will entrain the odor and impose a dominant frequency on the olfactory information transmitted in the object's wake. For smooth cylinders in the Re range of 10^2–10^5, St is a constant of 0.2, thereby allowing rearrangement of the equation for the dominant eddy frequency (and hence, plume intermittency) to be $f=0.2u/D_{\text{object}}$. We caution use of St in the field, however, under conditions of strong, three-dimensional winds, which will change the projected diameter of the obstacles (D_{objects}) thereby affecting the plume filament frequencies. Another aspect that can influence the temporal features of the odor plume is "active" behavior by the animal (or source) releasing the odor. For instance, female moths can periodically pump pheromone into the air (Conner et al. 1980) and flowers in the wind oscillate back and forth (Sprayberry and Daniel 2007). The intermittent release and odor source movement will influence the temporal structure of the plume signal, thereby creating a biologically mediated intermittency coupled to that of the turbulent flow.

Spatial Structure of the Plume The plume structure will change as a function of the distance from the odor source (Fig. 3). Research by atmospheric scientists who study pollutant transport for the past 30 years have scoured the countryside searching for large (~1 km), flat (~10 cm vegetation height) sites in which to examine the effects of boundary layer turbulence on plume structure. Experiments on the fields in UK, Canada, and USA provide a framework for understanding the effects of turbulence on odor plume structure (Mylne and Mason 1991; Yee et al. 1993; Mole and Jones 1994). For plumes on a relatively flat surface, the plume increases in width and height and decreases in filament intensity and intermittency (the proportion of time odor is present) as distance from the source increases. For instance, Dinar et al. (1988) found that chemical signal concentration in a plume was high and present 75% of the time at a position <4 m downwind from the source, but

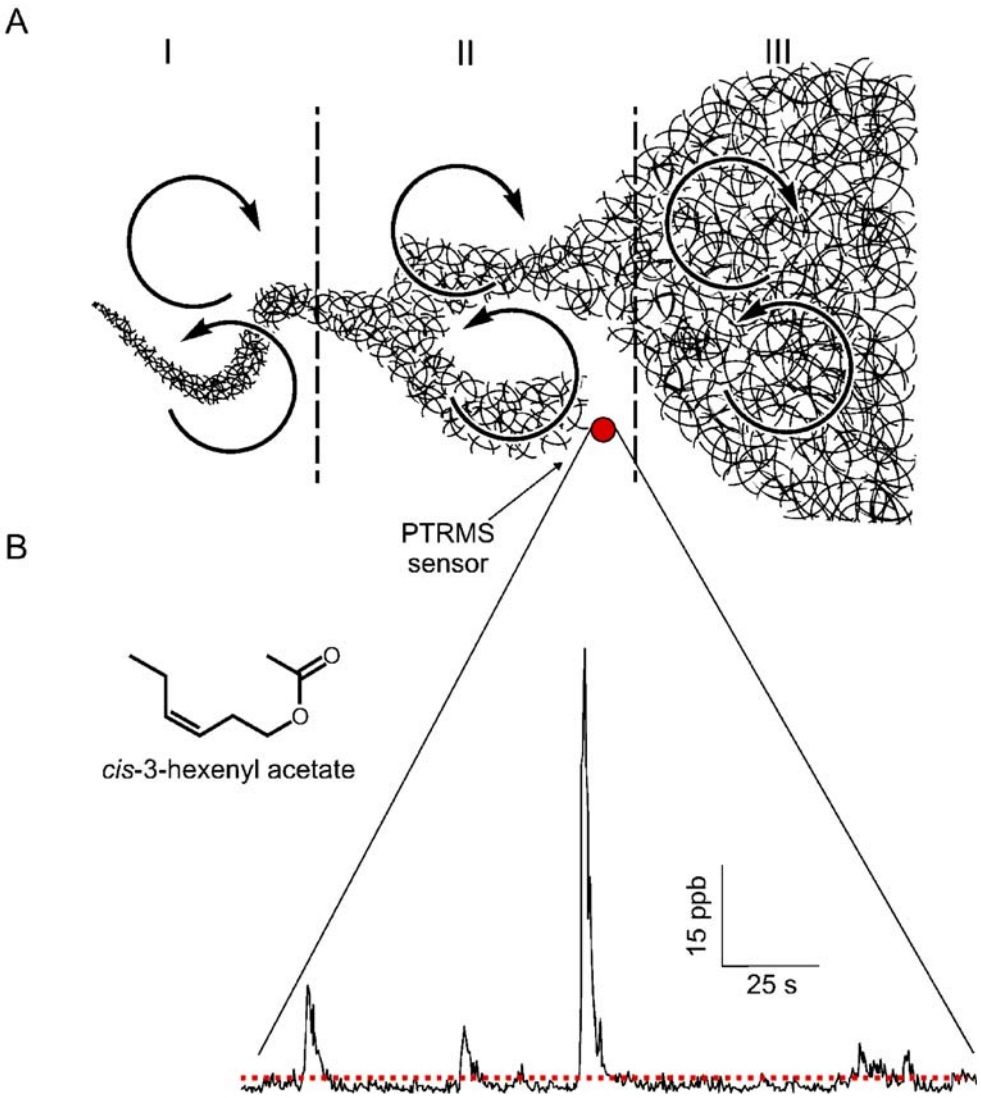


Fig. 3 Odor plumes are spatio-temporally dynamic. **A** A schematic of a conceptual model for plume dispersion modified from Mylne (1992). When the plume width is smaller than the dominant crosswind eddy size the plume will begin meandering. Once the plume width approaches the eddy size the plume becomes mixed with clean air. The plume will continue to develop and increase in size until its width exceeds the eddy scale (Environmental Effects on the Odor Landscape). The plume structure as a whole will depend upon the dominant eddy scale and the variation in concentration fluctuations of the plume. **B** A mass chromatogram of a *cis*-3-hexenyl acetate ($C_8H_{14}O_2$) plume as measured by proton transfer reaction mass spectrometry (*PTRMS*). Measurements occurred 4 m downwind from the source at an average wind velocity of 40 cm/s, dominant eddy size of 2.1 m and frequencies of 10 s. Based on conditional sampling of the plume measurements, odor bursts occurred every 11.1 s which approximately matched those predicted by Mylne (1992). The PTRMS sample rate was 4 Hz

concentrations dropped to 20% and occurred only 20% of the time at 40 m downwind (Dinar et al. 1988). Similarly, Murlis (1997) found that the chemical signal in a plume was present 40% of the time at a position 2.5 m downwind from the source but only present 10% of the time at 20 m downwind (Murlis 1997).

A conceptual model of the effects of turbulence on the large-scale plume structure can be based on the size of the turbulent eddies mixing the plume. For instance, Mylne (1992) described the formation of a plume based on its width relative to eddy size present in the turbulent flow. When the plume width is narrower than the dominant eddies, then the plume will meander (move side-to-side), thereby creating large patches of odor-laden air interspersed with odor-free air (Fig. 3A; Yee et al. 1993; Mylne et al. 1996; Finelli et al. 1999). As the plume width approaches or exceeds the dominant eddy length scale, vigorous mixing occurs with non-odor laden air transported into the interior of the plume (Fig. 3A). The distance that plumes meander can be estimated by u/n, where n is the frequency of the peak intensity of the velocity power spectrum (e.g., the dominant energy-containing eddies) and u is the wind velocity (Yee et al. 1993). Taking the range of velocities (0.2–2.0 m/s) and eddy frequencies (0.01–1.0/s) from Yee et al. (1993), Mylne and Mason (1991), and Murlis (1997), eddy lengths responsible for plume meandering range from 0.2 to >200 m. While this analysis may only approximate plume meandering under stable atmospheric conditions, it demonstrates the extreme odor patchiness that an insect must navigate toward.

While plume meandering will be critical for the large-scale structure of the plume, the edges and interior of the plume are dynamic and consist of fine-scale features important for navigating animals. For instance, the concentration of chemical signals in filamentous plumes is lower farther from the source in the along-wind direction; although some odor filaments of high concentration do

occur, they are encountered much less frequently (Murlis et al. 1992, 2000a; Murlis 1997). The intensity of concentration fluctuations also decreases with distance from the source (Murlis et al. 1992; Zollner et al. 2004). Moreover, with increasing distance from the center of the filamentous plume to the edges in the crosswind direction, odor intensity and intermittency also decrease (Mylne and Mason 1991; Yee et al. 1993; Murlis 1997). Although odor filaments occur less frequently on the edges of the plume than near the centerline, the concentration of the filaments can be as high as those in the middle.

The Dynamics of a Plume in a Laboratory Wind Tunnel Until now, we have focused on the dynamics of an odor plume in fairly idealized conditions: large area (~1 km), short vegetation (~10 cm), and steady turbulent wind conditions. Under these conditions, the plume will grow and meander. Can all of these conditions be replicated in the laboratory wind tunnel? Unfortunately, the size of a typical wind tunnel (~0.5–1 m^2 height and width and 2–5 m length; Visser 1976; Miller and Roelofs 1978) is much smaller than the dominant turbulent length scales in the field (1–200 m); therefore, a plume in a wind tunnel will not exhibit the same characteristics. However, physical turbulent conditions in the field, including turbulent fluctuations (root-mean-square velocity divided by the mean velocity) and Reynolds stresses, can be simulated in the laboratory to approximate those forces in the field (Cermak and Arya 1970; Fackrell and Robins 1982; Justus et al. 2002). Such scaling parameters are paramount, as they are necessary to replicate a natural aerodynamic regime and will dictate the plume spatiotemporal characteristics. Turbulent conditions in the field involve both the advective (mean velocity and direction) and turbulent (fluctuating velocities) components of the wind, as well as a number of other parameters that involve wind gusting (acceleration reaction) and change in direction. However, researchers that use wind tunnels have tended to simulate complex wind conditions by using single velocity measurements or, at best, single Reynolds numbers. Before beginning a behaviorally oriented, odor plume study, it is essential to determine the number and nature of the critical wind parameters (Fig. 2). Otherwise, reproducibility (i.e., dynamic similarity) is difficult to achieve (Zimmer and Zimmer 2008).

Environmental Effects on the Odor Landscape

Turbulent motion and molecular diffusion will spread a chemical plume in three dimensions to create what may be viewed as an odor landscape (Atema 1996; Murlis 1997; Zimmer and Butman 2000) in which an animal must navigate. The degree to which these motions dominate odor transport depend largely upon the physical environment. For habitats that are relatively flat (e.g., a large, grassy meadow, or a sandy plain), a useful rule of thumb for establishing the turbulence of the physical environment is by the roughness Reynolds number (Re_*). Re_* depends upon the shear velocity (u_*) and the roughness element (D) (Schlichting 1987). Therefore, the three-dimensional environment where odors are released will determine the odor intensity and spatiotemporal dynamics.

Odor Dynamics on a Desert Plain

A large, sandy plain, for example, near Maharès, Tunisia, provides an excellent example for effects of the roughness element on boundary layer turbulence. The degree to which turbulent eddies from the atmosphere come into contact with the substrate depends in large part on the size of the roughness element and wind velocity. For a shear velocity of 0.4 m/s (Wolf and Wehner 2000) and average sand grain size of 0.00025 m, the Reynolds roughness number is 6.5. At this number, turbulence begins to reach the outer regions of the boundary layer (Schlichting 1987), and the odor plume will be dominated by advection rather than large turbulent eddies thereby causing the odor plume to become a sheared continuous gradient (Weissburg 2000; Wolf and Wehner 2000). In contrast, if the sand size increases to 0.0025 m and the u_* remains 0.4 m/s, the Re_* is 65.8, which indicates that turbulent eddies are close to reaching all the way to the substrate, and hence, the boundary layer (and the odor plume) is fully turbulent. This raises an important point: The presence of roughness on the substratum decreases the velocity, which causes the boundary to become fully turbulent, and hence turbulence changes as a function of roughness element size. Observations of smoke used to simulate a plume for odor-tracking ants in this desert environment suggests that the Re_* values may be ca. 6, with the smoke remaining close to the substrate and becoming "smeared" by the low wind velocity.

Odor Dynamics in a Field

In more heterogeneous habitats, substrate differences and wind velocity begin to play a larger role in controlling the odor. For instance, by using the same shear velocity from the example above in the desert (0.4 m/s; Yee et al. 1993; Mole and Jones 1994) but using vegetation height as our roughness element (0.1–1.0 m), we find that the roughness Reynolds number ranges from 2,630 to 26,300. Because turbulence will fully dominate this habitat under a variety of spatial and temporal conditions, the odor landscape will also be dynamic reflecting the three-dimensional fluctuations in velocity. For instance, Yee et al. (1993) found that because of the large eddy sizes associated with the field

environment (u_*=0.25–7.0 m/s; D=0.5 m; Re_*=3,290–13,200), plume meandering only occurred close to the plume source, and at larger downwind distances (>100 m), the plume became well mixed, lacking a large-scale meandering structure but still being highly intermittent (Yee et al. 1993). Hence, a foraging insect far from a food odor source may experience a wide plume that has fine-scale structure and odor intermittency, but as it approaches the source—due to plume meandering and the plume width decreasing—the odor signal will become more spatiotemporally complex with longer periods of odorless air.

Odor Dynamics within a Forest

In contrast to the relatively flat surfaces of a grassy field or desert plain, the physical environment within a forest is dynamic and depends upon the vertical location between the canopy and the floor. Whereas Re_* provides a good rule of thumb for boundary layer turbulence in field and desert environments, forest habitats are so spatially complex that a developed boundary layer does not exist. This is because the upper canopy and large diameter tree trunks constrain large eddies from entering the understory regions of the forest, and thus, turbulence, radiation, thermal, physiological, and structural properties of a forest vary most in the vertical dimension (Rauner 1976; Hutchison and Baldocchi 1989), where turbulence levels are maximal near the upper canopy and decline rapidly in the understory toward the shrub layer and forest floor (Baldocchi 1989). Thus, in the understory, turbulence levels are low (u_*=0–0.2 m/s), the length of the turbulent eddies are small (1–3 m), and the bulk advective motion of the wind is minimized (0.1–1.0 m/s) relative to the turbulent eddies which cause the wind direction to shift chaotically (Kaimal and Finnigan 1994).

Studies that examine the transport of the tracer sulfur hexafluoride (SF_6) within a forest have shown that plume concentrations exhibit a logarithmic decrease in concentration with increasing distance from the source. For example, LeClerc et al. (2003) and Thistle et al. (2004) showed that mean tracer concentrations drop from 20% within 5 m of the source to 0.1% 60 m downwind. Moreover, individual filament concentrations can be tenfold higher than the average concentrations 10 m from the source, with the plume being intermittent and time between odor bursts long (40–300 s) in duration. An interesting phenomenon that affects plumes is the lack of a strong advective wind velocity, which causes the odor plume to swing back and forth extending in two directions 180° opposed (Leclerc et al. 2003; Thistle et al. 2004). Thus, a flying insect moving from a meadow into the forest will experience a totally different physical environment that may require different sensory strategies for navigation.

Effects of Scale

An important aspect that influences the odor environment experienced by animals is body size and translational speed —or wind velocity—of the animal itself. Returning to the Reynolds number, which indicates the ratio of inertial to viscous forces, and is the product of the size and speed, we can begin to examine the odor landscape experienced by insects of different sizes.

Small insects will experience a completely different physicochemical environment from larger, faster insects. The leaf-cutter ant, *Atta vollenweideri*, provides an interesting example of the effects of small size and the environment for the type of forces experienced by the ant. For instance, leaf-cutter ants are small (ca. 0.005 m) and, when in their nest, experience air flows of 0.005–0.02 m/s (Kleineidam and Roces 2000; Kleineidam et al. 2001) with corresponding Reynolds numbers of 1.6–6.5. Within this environment, ants experience a fluid dynamic regime where viscous forces nearly balance inertial forces. This will have an important effect on the odor landscape by smoothing out three-dimensional structures of the flow and hence causing the odor plumes to become sheared gradients. In contrast, when these ants leave their nest, they experience a grassy field environment with wind velocities of 1–6 m/s and the corresponding Re>300. In this environment, the odor will be fully turbulent and dynamic. For much larger or faster insects (S≥0.02 m, u≥0.5 m/s), for example, the flying hawkmoth *Manduca sexta*, Reynolds numbers are typically 1,000–10,000. These insects, then, exist in a fully turbulent environment where the odor will be intermittent and three dimensional.

Analytical Equipment

Role of Technology

In the previous sections, we detailed how atmospheric and landscape conditions may distribute chemical odors spatiotemporally. Valid measurements of something as amorphous as an odor plume may seem currently inaccessible. Nonetheless, to be able to measure chemical signals in real time as they are emitted and perceived by the organism is critical for determining how chemical signaling processes determine ecological and evolutionary interactions. Therefore, a continuous goal is the acquisition of improved technologies that will provide rapid and sensitive chemical data, which can then be merged with behavioral or electrophysiological observations and integrated into a mechanistic description of olfactory ecology.

A grand challenge to improve the sensitivity of our analytical measurements was unintentionally laid before us

nearly 40 years ago when Kaissling and Priesner (1970) discovered that, by using olfactory receptor cells on sensilla structures lining their antennae, male moths can detect a single pheromone molecule. Although fluorescence detection and other techniques have pushed limits of chemical detection into the zeptomole (10^{-21}) range, today, we still appreciate the animal nose (or insect antenna) as a type of ultimate detector.

Animal olfactory receptors remain supreme not only for their molecular sensitivity but also for their respectable sample collection speed. *M. sexta* moths, a favorite model organism, optimally collect odor samples in the range of one to five times per second (1–5 Hz; Heinbockel et al. 1999), but *Spodoptera exigua* and *Cadra cautella* moths have been shown to exhibit electroantennographic responses to stimulus intermittencies given at up to 33 Hz (Bau et al. 2002). Modern analytical techniques can also collect samples at this rate, and faster, but in most cases, these instruments are tuned to a single chemical identity. For the best information about chemical identification, gas chromatography mass spectrometry (GCMS) has remained the most reliable and popular technique for many years, but sample collection for this method is traditionally slow. Recently, GCMS sensitivity has become quite high (10^{-12} g; Song et al. 2006). Analytical equipment available to chemical and olfactory ecologists will be reviewed in the context of analyzing odor dynamics with maximal sensitivity, speed, and accurate chemical identification, where unfortunately one feature is often sacrificed for improvement of the others. Examples will range from commercially available devices to emerging technologies that tend to appear first as applications in atmospheric chemistry or in monitoring industrial environments.

Valuable reviews have addressed aspects of modern instrumentation and sampling methods useful for temporally analyzing biogenic volatile organic chemicals (BVOCs). Tholl et al. (2006) specifically address challenges and advances in monitoring environmentally dependent changes in plant volatile emissions by including in-depth descriptions of instruments, sampling techniques, and respective biases and limitations. D'Alessandro and Turlings (2006) pay special attention to above- and below-ground sampling techniques for herbivore-induced plant volatiles that mediate interactions among plants and insects. Ten emerging sensor technologies from the US Sandia National Laboratories for volatile organics monitoring are reviewed by Ho et al. (2007), and a recent review on electronic noses by Röck et al. (2008) discusses the integration of multiple sensor techniques. In this review, we focus on chemical detection technologies (and other supportive techniques) that feature low limits of detection, and rapid temporal measurement capability as a strength for maximum resolution of odor dynamics, and portability is emphasized.

Gas Chromatography

Terrestrial animal olfactory systems sample the environment and process chemical stimuli faster than once per second. In contrast, popular methodologies for analysis of behaviorally relevant volatiles involve significant time periods for collection, preparation (e.g., concentration or cryofocusing), and separation. The introduction of these samples into analytical instruments for structural characterization and quantification (e.g., by GCMS) usually results in significant loss of temporal resolution of the chemical signal. However, new, fast (ca. 200–400 s run times) GC systems are becoming available for chemical ecologists, thereby providing a means to sample the odor environment at time scales more relevant to moving, probing insects. Matisola and Dömötörová (2003) review multiple design strategies employable for streamlining GC analysis and provide tabulated examples of different approaches, including a shortlist of field-portable GC systems in use. Many of the these strategies were advanced in the laboratory of the late Richard Sacks, including at-column heating to enhance selectivity, use of atmospheric air as a carrier gas to facilitate portable GC, development of pre-concentrator systems, microelectro-mechanical system silicon columns (etched into a ca. 10 cm^2 chip), and alternative inlet devices and detectors needed to support miniaturization (Reidy et al. 2007; Sanchez and Sacks 2007).

Kunert et al. (2002) demonstrated rapid GC for improved temporal characterization of plant volatile emissions by employing the zNose™ miniature GC with a surface acoustic wave quartz microbalance detector (SAW; Electronic Sensor Technology, Newbury Park, CA, USA; or see Lu and Zellers 2002). They achieved a 3-min time resolution of lima bean volatile terpenoids in response to leaf wounding. By using standard charcoal pre-concentration, this rapid analysis is a drastic temporal improvement over classic GC runs that last 10–60 min. Even without the capability to define odor fluctuations that occur from second to second, this portable technology is useful in olfactory dynamics for making confident chemical identifications in the field just minutes after sample collection or for creating a temporal odor profile with ca. 3 min temporal resolution (e.g., if left to run continuously and automatically). Other fast GC systems with automated pre-concentration have been custom built for atmospheric monitoring from aircraft and employ helium ionization detection (HID) and electron capture detection (ECD) for selective detection down to parts per trillion level of non-methane hydrocarbons (C_2–C_5) every 5 min (Whalley et al. 2004).

Mass Spectrometry

Chemical Ionization Techniques Atmospheric pressure chemical ionization (APCI; soft ionization) mass spectrometry

techniques for environmental air analysis have attracted much interest (Charles et al. 2001). APCI mass spectrometers performing proton transfer reaction mass spectrometry (PTRMS) and selected ion flow tube mass spectrometry (SIFTMS) are both commercially available. PTRMS has been applied numerous times for measuring BVOCs (De Gouw and Warneke 2007) and has been adapted for measuring BVOC fluxes by using eddy covariance approaches (Karl et al. 2000).

PTRMS uses H_3O^+ as a reagent gas, so that H_3O^+ primary ions travel into a drift tube and collide with gaseous organic volatiles (*M*) by the proton transfer reaction

$$H_3O^+ + M \rightarrow MH^+ + H_2O$$

if energetically allowed (if *M* has a proton affinity exceeding that of water, 166.5 kcal/mol, a condition fulfilled for essentially all VOCs). Quadrupole unit mass detection limits of 5–30 ppt have been reached, and measurement times can be as low as 2 ms per observed ion, thus allowing real-time monitoring of ambient air in environmental situations that include aircraft flight missions and extended environmental sampling campaigns (Lindinger et al. 1998a, b). At 130 kg, the PTRMS is portable on wheels or can be lifted by several people for short distances. PTRMS is also available with high resolution time of flight (HRTOF) mass detection and has been adapted with a GC inlet and ion trap (IT) detection. SIFTMS also employs a quadrupole for detection limits of a few parts per billion and measurement times of a few seconds (Freeman and McEwan 2002). An additional upstream quadrupole filter is used to select reagent gas ions of H_3O^+, O_2^+, NO^+, or others. The instrument is similar in size and cost with quadrupole PTRMS. APCI inlets on benchtop liquid chromatography (LC)ITMS instruments have also been successfully modified to accept gas samples with rapid measurement times of 20–200 ms per observed ion (Jublot et al. 2005).

PTRMS has been used in a variety of different applications that involve emission of volatile compounds. Karl and others have used PTRMS for examining dynamics of green leaf volatiles, pollutants, and herbivory-induced changes in volatile emissions from plants (Karl et al. 2003; von Dahl et al. 2006; Filella et al. 2006). Recently, we have used PTRMS to measure synthetic floral odor plumes simultaneously with insect electroantennography measurements in wind tunnels. Raw and processed data both appear as a temporal evolution of mass intensities for a single, or multiple ions, over a particular time period (PTRMS measurement of a single odor species for a 190 s time period is shown in Fig. 3B).

Sample Desorption Techniques A desire to analyze samples under ambient conditions (outside of the vacuum area) has driven the development of ambient desorption techniques. Desorption ionization (DI) techniques can use a laser or charged particles (i.e., an electrospray) to dislodge and ionize ambient sample particles for introduction into a mass spectrometer (Cooks et al. 2006). The popular desorption electrospray ionization (DESI) method works well typically on solid samples. Numerous related techniques have evolved, some of which accommodate gaseous samples, like the direct analysis in real time (DART) method, which uses an excited gas (plasma) to drive the desorption process of solid, liquid, or gas samples (Cody et al. 2005; Haefliger and Jeckelmann 2007), or atmospheric pressure Penning ionization (APPeI; Iwana et al. 2006), which uses argon or helium (rare gases) activated by a negative-mode corona discharge to ionize a pulled gaseous sample before analysis by TOFMS. Laser photoionization of trace gas molecules has been developed mainly along two, parallel paths: resonance-enhanced multiphoton ionization time-of-flight mass spectrometry (REMPI-TOFMS) and single photon ionization (SPI) TOFMS (Zimmermann 2005). Real-time gas sample analyses (e.g., coffee roasting emissions at 10 Hz; Dorfner et al. 2004) have been achieved by using REMPI-TOFMS, a selective ionization method for aromatic and highly unsaturated compounds. SPI-TOFMS is a less selective ionization method that allows detection of aliphatics and aromatics. Although the DI techniques above have not yet been directly applied to volatiles from plants or insects, their high temporal resolution, direct gas sampling, and limits of detection present promising opportunities for olfactory ecology (e.g., Fig. 4). Neutral desorption of analytes by extractive electrospray ionization (EESI; Chen et al. 2007) has been used to analyze human breath and also appears to hold promise for applications in ecology.

Alternate Analytical Technologies

Ion Mobility Spectroscopy IMS is broadly applied today for detecting trace chemical substances, notably in screening air travelers' carry-on baggage for detection of chemical weapons. Eiceman et al. (1990) characterized methylsalicylate ($C_8H_8O_3$) and dimethylsulfoxide (C_2H_6OS) odor plumes in a continuous field setting with IMS in an early environmental application. IMS functions under atmospheric pressure wherein different ion species travel through a buffer gas in a small (ca. 3–15 cm length) chamber (a.k.a. drift tube; also used in PTRMS) along an electric field at different rates, depending on individual mobility, which is determined by mass, charge, and collision cross-section (Borsdorf and Eiceman 2006). IMS techniques based on this concept have evolved for over a century under different names like ion chromatography. The arrival times (i.e., time of flight (TOF); ca. 2–15 ms) of different ion species at the IMS Faraday type detector are used to distinguish them. Short ion travel times allow fast

instrument cycling and rapid sampling. Recent advances in IMS have achieved instrument miniaturization and incorporation of new ionization techniques like ESI and corona discharge. More traditional ionization techniques employ CI initiated by radioactive ^{63}Nickel, and photoionization.

Differential mobility spectroscopy (DMS) differs from IMS by the ion path in having two planar electrodes that produce an electric field that oscillates between high and low values (e.g., 500 to 1,500 V) and a sweeping compensation voltage. DMS promises excellent chemical separation for complex volatile organic mixtures in a miniature chip size. Lambertus et al. (2005) detected and separated a complex mixture of 45 organic volatiles ranging from C_2–C_9 at ca. 1–100 ppb by using a 3.0-m GC column on a microchip attached to a 15-mm microchip DMS. High-field asymmetric waveform ion mobility spectrometers (FAIMS) operate similarly to DMS (Borsdorf and Eiceman 2006).

Absorbance Spectroscopies Volatile organics strongly absorb energy at mid- to far-infrared (IR; ca. 3–1,000 μm) wavelengths, and this makes their detection possible at low concentrations. The near-IR (ca. 0.75–1.4 μm) range is also useful, but detection of VOCs based on overtones in this region is 10^2 less sensitive. Fourier transform infrared spectroscopy (FTIR) is widely used for analysis of many organic compounds, and open-path FTIR (OP-FTIR) is a direct extension of this method for open spaces (from meter to kilometer). Industrial, environmental, and remote sensing applications of OP-FTIR demonstrate its utility. Active OP-FTIR sensing (includes an instrument light source) is more sensitive than passive sensing (uses ambient light; Vogt 2006). With advanced chemometric techniques, the application of multiple or sweeping OP-FTIR spectrometers and reconstructed tomographic maps has allowed temporal visualization of some vapor plumes (Todd 2000). A direct comparison between OP-FTIR (measurement cycle time 1.2 Hz), PTRMS (ca. 100 Hz), and GC analysis of canister grab samples revealed good agreement with methods that measure parts per billion amounts of volatile organics (Christian et al. 2004).

Several portable laser absorption spectroscopic (LAS) techniques useful for trace gas detection reflect the recent

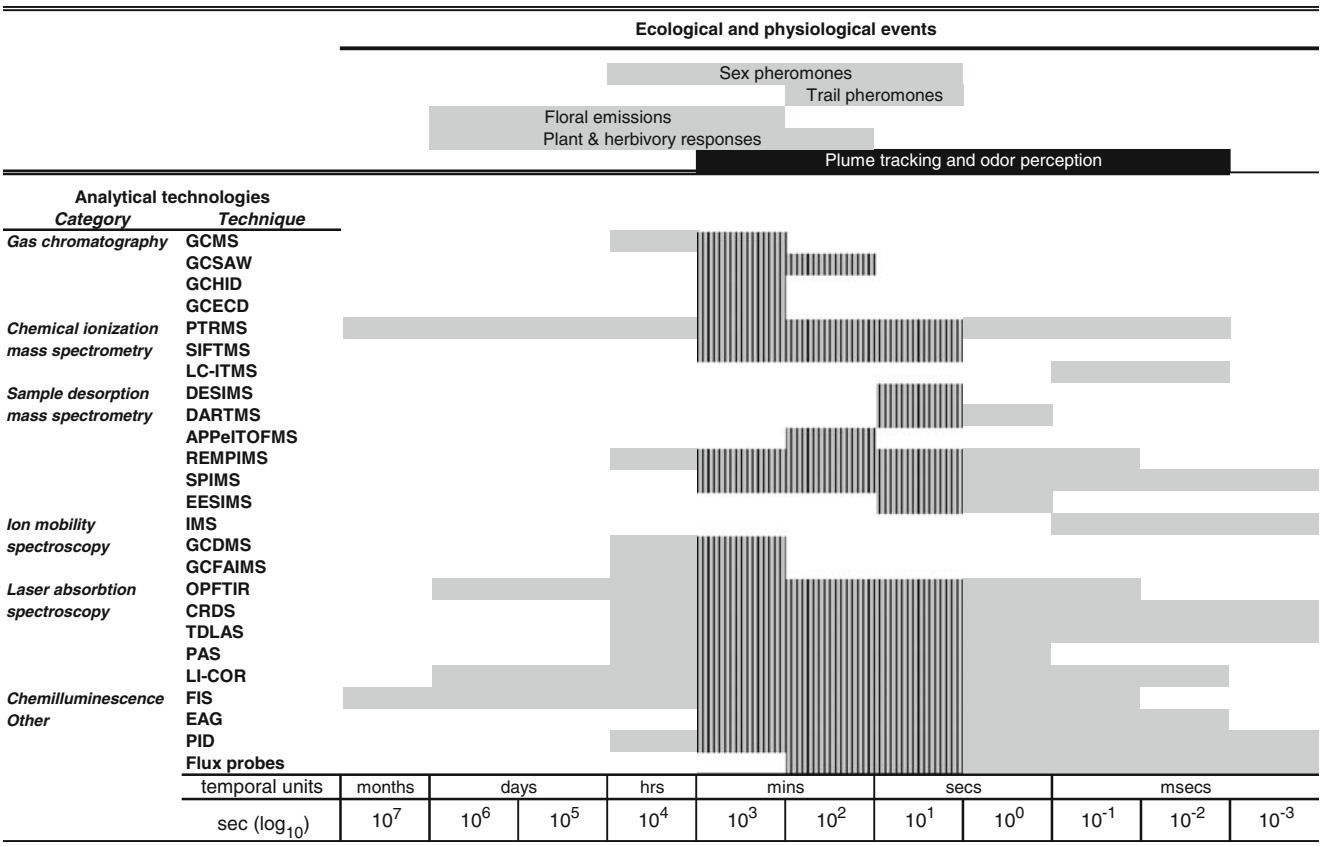


Fig. 4 Ecological and odor producing physiological activities (*upper five rows*) are compared with available analytical technologies having potential application in olfactory ecology. Ecological events are portrayed as two types: odor emissions (*gray*) lasting from seconds to hours, and odor tracking and perception (*black*) lasting from milliseconds to minutes. Temporal resolution ranges (in seconds; *lower rows*; log scale) obtainable from measurements with analytical technologies listed by category (acronyms defined in "Analytical Equipment", see also Appendix 2) are shown in *gray*. The temporal range where chemical signal emission and signal reception (olfaction) overlap is indicated by *hashed marks*

evolution of optical spectroscopy. Small and robust diode lasers with high optical intensity have mostly replaced lamp and filament light sources to provide greater sensitivity and portability for trace gas detections. However, small spectral coverage from these lasers has limited new diode laser dependant devices to the detection of a single gas species per laser. LAS applications in atmospheric science, environmental monitoring, semiconductor production, and breath analysis have demonstrated parts per million–parts per trillion detections of organics of phyto-relevance such as acetone (C_3H_6O), isoprene (C_5H_8), and methanol (CH_4O) among others (Khunemann et al. 2002; Cias et al. 2007; Ngai et al. 2007; Wang and Mbi 2007). Recently, broadly tunable diode lasers have become commercially available in the 1,528–1,608 nm (near-IR) range that are suitable for measuring gases like CO_2 and NH_3, but not appropriate for semiochemical-type VOCs (Kachanov et al. 2006). The lack of availability of lasers in the VOC-appropriate mid-infrared 1,610–1,710 nm range reflects industry needs rather than technological barriers, so commercially available mid-IR laser devices may become more common as the VOC sensor industry matures. Three primary approaches of LAS technology have led to instrumentation that is now available and in use for trace gas detection: cavity ringdown spectroscopy (CRDS), photo acoustic spectroscopy (PAS), and tunable diode laser absorption spectroscopy (TDLAS; Kachanov et al. 2006).

Infrared Gas Analyzer for CO_2 Measurement Many insects possess olfactory receptor cells that are specifically sensitive to ambient CO_2 levels. Detection of CO_2 concentration changes is important for many insect species (Stange 1996; Guerenstein and Hildebrand 2008). Expression of CO_2 sensory organs among invertebrates appears to be strongest in herbivorous Lepidoptera (Bogner 1990; Stange 1997; Guerenstein et al. 2004). Sexual dimorphism of the CO_2 sensory labial palp organs (LPOs) and sensilla therein is variable among insects. The LI-7500 non-dispersive infrared CO_2/H_2O gas analyzer (LI-COR Biosciences, Lincoln, NE, USA) is the standard tool used by ecophysiologists for measuring precise CO_2 fluctuations from ca. 0–3,000 ppm with response times from 5–20 Hz. The battery operated 0.75 kg instrument is 30×6.5 cm and mountable in any orientation. The LI-7500 has been used to measure dynamic plumes of artificially high CO_2 above host plants inside free air carbon dioxide enrichment (FACE) rings for evaluating the effects of fluctuating, elevated CO_2 on moth oviposition preference (Abrell et al. 2005).

Chemiluminescence For real-time (10 Hz measurement cycle time) detection (to 400 ppb) of isoprene, Hills and Zimmerman (1990) developed the fluorescent isoprene sensor (FIS) based on detection of chemiluminescence coming from decay of the excited formaldehyde intermediate in the reaction of isoprene (a terminal alkene) with ozone. Other existing chemiluminescence-based trace gas detectors are specific for non-semiochemical type gases.

Insect Antennae as Biosensors Use of the insect antenna for determination of biologically important volatiles has emerged as an important type of biosensor for specific VOCs, especially pheromones (Roelofs 1984; Park et al. 2002). First, electroantennography (EAG), and later, single-sensillar recording, have emerged as sensitive tools for elucidating behaviorally important volatiles (Schneider 1969; Mustaparta 1975; Guerenstein and Guerin 2001; Stranden et al. 2003). Single-sensillar recording, the electrophysiological recording of a receptor neuron located on the insect antennae, has been used only in the laboratory due to the delicate arrangement of electrophysiological recording equipment. In contrast, EAGs are robust and have been used in the field. While not as sensitive as single-sensillar responses, EAGs can provide a qualitative measure of volatile semiochemicals in the atmosphere. Established by Dietrich Schneider (1957) by using the *Bombyx mori* silkworm moth antenna, an EAG response is described as recording the sum of receptor potentials in many sensory neurons by chemical stimuli presented to the antenna (Schneider 1969). The electrical signal from the antenna is collected from electrodes placed at the base and tip of the antenna, amplified, and recorded. EAG response amplitude is dependent on the stimulus concentration and chemical structure. Both EAGs and single-sensillum recordings have been used in tandem with GCMS for determination of biologically relevant volatiles, and EAGs have been used in the field for detection of simulated and natural plumes (Murlis et al. 2000).

Chemical Tracers Unavailability of fast, sensitive, and affordable chemical detectors for volatile semiochemicals has led to the development of odor plume experiments that rely on surrogate molecules that, optimistically, behave like odor molecules in a plume. Initially, Murlis and Jones (1981) used an ion collector method downwind from an ion generator acting on air at a coastal field to generate a 15 m outdoor plume. Downwind detection of intermittent ion bursts revealed fine scale plume structure, but ion-ion repulsions may have affected plume structure. More recently, gypsy moth pheromone [(+)-disparlure, $C_{19}H_{38}O$] was released synchronously with generated ions upwind and measured by male moth antennae downwind by using EAG. This creative validation approach revealed that pheromone and ion arrival at vertical Langmuir flux probes (ca. 1.2 m^2) was concurrent but quantitatively uncorrelated (Murlis et al. 2000). A more modern surrogate

plume approach utilizes fast photoionization detection (PID; up to 330 Hz). Justus et al. (2002, 2005) controlled release of a surrogate propene gas in a 3-m wind tunnel and measured plume structure by using a miniPID from Aurora Scientific (Aurora Scientific Inc., Ontario, Canada). PID continues to offer high temporal resolution of plume structure, but the inherent shortcoming is a lack of discrimination for individual VOCs. Odor concentrations (50–3,000 ppm) measured by PID reflect the sum of all odor mixture components ionized by radiation (8.5-11.7 eV will ionize most organic volatiles $\leq C_{10}$ depending on their respective ionization potentials) from a UV lamp in a detection chamber (Palassis 1997). Development of improved odor stimuli delivery techniques requires high frequency odor detection, which PID can provide (French and Meisner 2007).When coupled with a sonic anemometer, PIDs provide a powerful means to characterize the physical odor environment.

For an insect, the behavioral (odor perception and subsequent navigation) response to an odor is generally immediate (millisecond to minute). The analytical technologies reviewed above and listed in Fig. 4 span a wide range of response times and conceptually fundamental detection methods. As GC is a separation process and not a detection process, GC speed is mainly limited by separation time rather than detection speed. In mass spectrometry, only ions are detected, so the ionization process is fundamental in determining molecule detection efficiency and sample inlet speed. Similar to GC, ion mobility spectroscopy is also a separation process, but ~10^3 times faster than GC (Fig. 4). Laser absorption spectroscopies show promise for trace gas detections in diverse environments, including remote sensing and tomographic applications. The fastest technology reviewed in this paper is photoionization detection (up to 330 Hz; Fig. 4) which Cardé and coworkers have already exploited for several years now (Justus et al. 2002). Other fast techniques shown in Fig. 4 include PTRMS, OPFTIR, LI-COR, EAG, and FIS. Among these, PTRMS, OPFTIR, and EAG have the widest ability to measure diverse chemicals in a single measurement cycle, and may be the most useful techniques going forward in olfactory ecology today.

Summary and Future Directions

The olfactory environment in which insects navigate is dynamic, with physical processes ultimately controlling the chemical signal available to the organism. In this review, we described features of the physical environment that control the dynamics of the chemical signal and a menu of technological innovations that may allow measurement of the fluctuating chemical signal. A multi-disciplinary research approach is thus necessary to effectively investigate and understand the olfactory environment that insects inhabit. Recent advances in analytical technologies allow unprecedented opportunities for chemical ecologists and behavioralists to characterize the physicochemical environment and determine when and where olfactory-mediated behaviors control ecological interactions.

Different physical environments, such as a forest or a desert plain, over land or in water, and the size of an animal body, will affect the chemical stimuli available and may operate as a selective pressure on the sensory system. It follows that different "odor landscapes" will set constraints on insect olfactory systems and odor-tracking navigational behavior. Small insects (<1 mm) inhabit a fluid regime with filamentous odors, but also smooth, sheared odor gradients will exist during periods of limited air movement (Zimmer and Zimmer 2008). In contrast, larger, faster insects inhabit a fluid regime with filamentous and three-dimensional odor. Do insects living at these different scales utilize different behavioral strategies to locate odor sources? Do animals that exist in different environments but inhabit dynamically similar fluid regimes (e.g., a moth and a salmon) use equivalent strategies (see DeBose and Nevitt 2008; Cardé and Willis 2008; Zimmer and Zimmer 2008)? Moreover, much of our understanding of odor plume dynamics comes from sampling at a fixed point. Navigating insects, on the other hand, will be translating in the same space as the filamentous plume and, thus, will be experiencing the plume from a moving reference frame (Lagrangian versus Eulerian frame). How does this influence the chemical signal variation and fine-scale features experienced by the insect? On an evolutionary scale, we might predict that the physicochemical environment has shaped the sensory system and behaviors of these organisms. A comparative, phylogenetic approach presents an exciting opportunity toward an examination of how the physical environment may have influenced organismal sensory systems, behaviors, and morphologies. The improvement of analytical technologies will foster increased understanding of the physicochemical environment and will enable researchers to determine how olfactory environments shape insect behaviors and sensory systems.

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Appendix 1

Table 1 Notation for physical processes

Symbol	Definition	Units
D	Roughness element height (Eq. 3)	m
D_{object}	Diameter of object used in Strouhal number calculation (Eq. 5)	m
f	Eddy shedding frequency (Eq. 5)	1/s
G	Gravitational acceleration.	m/s^2
μ	Air viscosity (Eq. 1)	Ns/m^2
p	Air density (Eq. 1)	kg/m^3
N	Frequency of the peak intensity of velocity spectra	1/s
Re	Reynolds number (Eq. 1). The ratio between inertial and viscous forces, $Re = \frac{\rho S u}{\mu}$	dimensionless
Re_*	Reynolds roughness number (Eq. 3). Describes boundary layer, $Re_* = \frac{\rho u_* D}{\mu}$	Dimensionless
R_i	Richardson number (Eq. 4). Describes boundary layer stability, $R_i = \frac{(g/\overline{\theta})(\partial\overline{\theta}/\partial z)}{(\partial\overline{u}/\partial z)^2}$	Dimensionless
S	Insect spatial scale (Eq. 1)	M
S_t	Strouhal number (Eq. 5). Eddy shedding frequency by objects in wind. $St = \frac{fD_{\text{object}}}{u}$	Dimensionless
u	Velocity in the horizontal dimension (Eq. 1)	m/s
$\overline{u}$	Mean horizontal velocity (Eq. 1)	m/s
u'	Fluctuating horizontal velocity (Eq. 2)	m/s
u_*	Friction velocity (Eq. 3). Describes intensity of the turbulence	m/s
w	Velocity in the vertical dimension (Eq. 2)	m/s
w'	Fluctuating vertical velocity (Eq. 2)	m/s
τ_R	Reynolds shear stress (Eq. 2), $\tau_R = -\rho\overline{u'w'}$.	N/m^2
z	Height above the substrate (Eq. 4)	m
$\overline{\theta}$	Mean temperature (Eq. 4)	Kelvin

Appendix 2

Table 2 Notation for analytical technologies

Category	Technique abbreviation	Technique
Gas chromatography	GCMS	Gas chromatography mass spectrometry
	GCSAW	Gas chromatography surface acoustic wave quartz microbalance detection
	GCHID	Gas chromatography helium ionization detection
	GCECD	Gas chromatography electron capture detection
Chemical ionization mass spectrometry	PTRMS	Proton transfer reaction mass spectrometry
	SIFTMS	Selected ion flow tube mass spectrometry
	LCITMS	Liquid chromatography ion trap mass spectrometry
	DESIMS	Desorption electrospray ionization mass spectrometry
	DARTMS	Direct analysis in real time mass spectrometry
	APPeITOFMS	Atmospheric pressure Penning ionization time of flight mass spectrometry
	REMPIMS	Resonance-enhanced multiphoton ionization mass spectrometry
	SPIMS	Single photon ionization mass spectrometry
	EESIMS	Extractive electrospray ionization mass spectrometry
Ion mobility spectroscopy	IMS	Ion mobility spectroscopy
	GCDMS	Gas chromatography differential mobility spectroscopy
	GCFAIMS	Gas chromatography high-field asymmetric waveform ion mobility spectrometry

Table 2 (continued)

Category	Technique abbreviation	Technique
Absorption spectroscopy	OPFTIR	Open-path Fourier transform infrared spectroscopy
	CRDS	Cavity ringdown spectroscopy
	TDLAS	Tunable diode laser absorption spectroscopy
	PAS	Photo acoustic spectroscopy
	IRGA	Infrared gas analyzer
	LICOR	Refers to the LI-7500 non-dispersive infrared CO_2/H_2O gas analyzer from LI-COR Biosciences
Chemiluminescence	FIS	Fluorescent isoprene sensor
Other	EAG	Electroantennography
	PID	Photoionization detection

References

ABRELL, L., GUERESNSTEIN, P. G., MECHABER, W. L., STANGE, G., CHRISTENSEN, T. A., NAKANISHI, K., and HILDEBRAND, J. G. 2005. Effect of elevated atmospheric CO_2 on oviposition behavior in *Manduca sexta* moths. *Global Change Biology* 11:1272–1282.

ATEMA, J. 1996. Eddy chemotaxis and odor landscapes: Exploration of nature with animal sensors. *Biol. Bull.* 191:129–138.

BALDOCCHI, D. D. 1989. Turbulent transfer in a deciduous forest. *Tree Physiol.* 5:357–377.

BARGMANN, C. I. 2006. Comparative chemosensation from receptors to ecology. *Nature* 444:295–301.

BAU, J., JUSTUS, K. A., and CARDÉ, R. T. 2002. Antennal resolution of pulsed pheromone plumes in three moth species. *J. Insect Physiol.* 48:433–442.

BOGNER, F. 1990. Sensory physiological investigation of the carbon dioxide receptors in Lepidoptera. *J. Insect Physiol.* 36:951–957.

BORSDORF, H., and EICEMAN, G. A. 2006. Ion mobility spectrometry: Principles and applications. *Appl. Spectrosc. Rev.* 41:323–375.

CARDÉ, R. T., and WILLIS, M. A. 2008. Navigation strategies used by insects to find distant, wind-borne sources of odor. *J. Chem. Ecol.* (this volume).

CERMAK, J. E., and ARYA, S. P. S. 1970. Problems of atmospheric shear flows and their laboratory simulation. *Boundary-Layer Meteorol.* 1:40–60.

CHARLES, L., RITER, L. S., and COOKS, R. G. 2001. Direct analysis of semivolatile organic compounds in air by atmospheric pressure chemical ionization mass spectrometry. *Anal. Chem.* 73:5061–5065.

CHEN, H., WORTMANN, A., ZHANG, W., and ZENOBI, R. 2007. Rapid in vivo fingerprinting of nonvolatile compounds in breath by extractive electrospray ionization quadrupole time-of-flight mass spectrometry. *Angew. Chem. Int. Ed.* 46:580–583.

CHRISTIAN, T. J., KLEISS, B., YOKELSON, R. J., HOLZINGER, R., CRUTZEN, P. J., HAO, W. M., SHITAI, T., and BLAKE, D. R. 2004. Comprehensive laboratory measurements of biomass-burning emissions: 2. First intercomparison of open-path FTIR, PTR-MS, and GC-MS//FID//ECD. *J. Geophys. Res.* 109:1–12.

CIAS, P., WANG, C., and DIBBLE, T. S. 2007. Absorption cross-sections of the C-H overtone of volatile organic compounds: 2 methyl-1,3-butadiene (isoprene), 1,3-butadiene, and 2,3-dimethyl-1,3-butadiene. *Appl. Spectrosc.* 61:230–236.

CODY, R. B., LARAME'E, J. A., and DURST H. D. 2005. Versatile new ion source for the analysis of materials in open air under ambient conditions. *Anal. Chem.* 77:2297–2302.

CONNER, W. E., EISNER, T., MEER, R. K., GUERRERO, A., GHIRINGELLI, D., and MEINWALD, J. 1980. Sex attractant of an arctiid moth (*Utetheisa ornatrix*): A pulsed chemical signal. *Behav. Ecol. Sociobiol.* 7:55–63.

COOKS, R. G., OUYANG, Z., TAKATS, Z., and WISEMAN, J. M. 2006. Ambient mass spectrometry. *Science* 311:1566–1570.

D'ALESSANDRO, M., and TURLINGS, T. C. J. 2006. Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. *Analyst* 131:24–32.

DE GOUW, J., and WARNEKE, C. 2007. Measurements of volatile organic compounds in the Earth's atmosphere using proton-transfer-reaction mass spectrometry. *Mass Spectrom. Rev.* 26:223–257.

DEBOSE, J. L., and NEVITT, G. A. 2008. Behavioral responses and navigational strategies to natural olfactory stimuli II: fish and birds. *J. Chem. Ecol.* (this volume).

DINAR, N., KAPLAN, H., and KLEIMAN, M. 1988. Characterization of concentration fluctuations of a surface plume in a neutral boundary layer. *Boundary-Layer Metereol.* 45:157–175.

DORFNER, R., FERGE, T., YERETZIAN, C., KETTRUP, A., and ZIMMERMANN, R. 2004. Laser mass spectrometry as on-line sensor for industrial process analysis: Process control of coffee roasting. *Anal. Chem.* 76:1386–1402.

EICEMAN, G. A., SNYDER, A. P., and BLYTH, D. A. 1990. Monitoring of airborne organic vapors using ion mobility spectrometry. I. *J. Env. Anal. Chem.* 38:415–425.

ELKINTON, J. S., CARDÉ, R. T., and MASON, C. J. 1984. Evaluation of time-average dispersion models for estimating pheromone concentration in a deciduous forest. *J. Chem. Ecol.* 10:1081–1108.

FACKRELL, J. E., and ROBINS, A. G. 1982. The effects of source size on concentration fluctuations in plumes. *Boundary-Layer Meteorol.* 22:335–350.

FILELLA, I., PENUELAS, J., and LLUSIA, J. 2006. Dynamics of the enhanced emissions of monoterpenes and methyl salicylate, and decreased uptake of formaldehyde, by *Quercus ilex* leaves after application of jasmonic acid. *New Phytologist* 169:135–144.

FINELLI, C. M., PENTCHEFF, N. D., ZIMMER-FAUST, R. K., and WETHEY, D. S. 1999. Odor Transport in Turbulent Flows: Constraints on Animal Navigation. *Limnol. Oceanogr.* 44:1056–1071.

FINELLI, C. M., PENTCHEFF, N., ZIMMER, R. K., and WETHEY, D. S. 2000. Physical constraints on ecological processes: A field test of odor-mediated foraging. *Ecology* 81:784–797.

FREEMAN, C. G., and MCEWAN, M. J. 2002. Rapid analysis of trace gases in complex mixtures using selected ion flow tube-mass spectrometry. *Australian J. Chem.* 55:491–494.

FRENCH, A. S., and MEISNER, S. 2007. A new method for wide frequency range dynamic olfactory stimulation and characterization. *Chem. Senses* 32:681–688.

GUERENSTEIN, P., and GUERIN, P. 2001. Olfactory and behavioural responses of the blood-sucking bug *Triatoma infestans* to odours of vertebrate hosts. *J. Exp. Biol.* 204:585–597.

GUERENSTEIN, P., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2004. Sensory processing of ambient CO_2 information in the brain of the moth *Manduca sexta*. *J. Comp. Physiol. A* 190:707–725.

GUERENSTEIN, P., and HILDEBRAND, J. G. 2008. Roles and effects of environmental carbon dioxide in insect life. *Annu. Rev. Entomol*. 53:161–178.

HAEFLIGER, O. P., and JECKELMANN, N. 2007. Direct mass spectrometric analysis of flavors and fragrances in real applications using DART. *Rapid Commun. Mass Spectrom.* 21:1361–1366.

HEINBOCKEL, T., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 1999. Temporal tuning of odor responses in pheromone-responsive projection neurons in the brain of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* 409:1–12.

HILDEBRAND, J. G. 1995. Analysis of chemical signals by nervous systems. *Proc. Natl. Acad. Sci. USA* 92:67–74.

HILLS, A. J., and ZIMMERMAN, P. R. 1990. Isoprene measurement by ozone-induced chemiluminescence. *Anal. Chem.* 62:1055–1060.

HO, C. K., ROBINSON, A., MILLER, D. R., and DAVIS, M. J. 2007. Overview of sensors and needs for environmental monitoring. *Sensors* 5:4–37.

IWANA, T., MITSUTAKA, H., ISAO, Y., OKADA, H., and HIRAOKA, K. 2006. Development of sniffing atmospheric pressure Penning ionization. *J. Mass Spectrom. Soc. Japan* 54:227–233.

JUBLOT, L., LINFORTH, R. S. T., and TAYLOR, A. J. 2005. Direct atmospheric pressure chemical ionisation ion trap mass spectrometry for aroma analysis: Speed, sensitivity and resolution of isobaric compounds. *I. J. Mass Spectrom.* 243:269–277.

JUSTUS, K. A., MURLIS, J., JONES, C. D., and CARDÉ, R. T. 2002. Measurement of odor-plume structure in a wind tunnel using photoionization detector and a tracer gas. *Env. Fluid Mech.* 2:115–142.

JUSTUS, K. A., CARDÉ, R. T., and FRENCH, A. S. 2005. Dynamic properties of antennal responses to pheromone in two moth species. *J. Neurophysiol.* 93:2233–2239.

KACHANOV, A. A., CROSSON, E. R., and PALDUS, B. A. 2006. Tunable diode lasers: Expanding the horizon for laser absorption spectroscopy. *Optics Photonics News* 16:44–50.

KAIMAL, J., and FINNIGAN, J. 1994. Atmospheric Boundary Layer Flows, 1 edn. Oxford University Press, Oxford.

KAISSLING, K. E., and PRIESNER, E. 1970. Die Riechschwelle des Seidenspinners. *Naturwissenschaften* 57:23–28.

KARL, T., GUENTHER, A., JORDAN, A., FALL, R., and LINDINGER, W. 2000. Eddy covariance measurement of biogenic oxygenated VOC emissions from harvesting. *Atmos. Env.* 35:491–495.

KARL, T., JOBSON, T., KUSTER, W. C., WILLIAMS, E., STUTZ, J., SHETTER, R., HALL, S. R., GOLDAN, P., FEHSENFELD, F., and LINDINGER, W. 2003. Use of proton-transfer-reaction mass spectrometry to characterize volatile organic compound sources at the La Porte super site during the Texas Air Quality Study 2000. *J. Geophys. Res.* 108:ACH13–1–ACH13/15.

KHUNEMANN, F., WOLFERTZ, M., ARNOLD, S., LAGEMANN, M., POPP, A., SCHULER, U., JUX, A., and BOLAND, W. 2002. Simultaneous online detection of isoprene and isoprene-d_2 using infrared photoacoustic spectroscopy. *App. Physics B* 75:397–403.

KLEINEIDAM, C., and ROCES, F. 2000. Carbon dioxide concentrations and nest ventilation in nests of the leaf-cutting ant *Atta vollenweideri*. *Insectes Sociaux* 47:241–248.

KLEINEIDAM, C., ERNST, R., and ROCES, F. 2001. Wind-induced ventilation of the giant nests of the leaf-cutting ant *Atta vollenweideri*. *Naturwissenschaften* 88:301–305.

KOEHL, M. A. R. 2006. The fluid mechanics of arthropod sniffing in turbulent odor plumes. *Chem. Senses* 31:93–105.

KUENEN, L., and CARDE, R. T. 1994. Strategies for recontacting a lost pheromone plume: Casting and upwind flight in the male gypsy moth. *Physiol. Entomol.* 19:15–29.

KUNERT, M., BIEDERMANN, A., KOCH, T., and BOLAND, W. 2002. Ultrafast sampling and analysis of plant volatiles by a hand-held miniaturised GC with pre-concentration unit: Kinetic and quantitative aspects of plant volatile production. *J. Sep. Sci.* 25:677–684.

LAMBERTUS, G. R., FIX, C. S., REIDY, S. M., MILLER, R. A., WHEELER, D., NAZAROV, E., and SACKS, R. 2005. Silicon microfabricated column with microfabricated differential mobility spectrometer for GC analysis of volatile organic compounds. *Anal. Chem.* 77:7563–7571.

LECLERC, M. Y., MESKHIDZE, N., and FINN, D. 2003. Comparison between measured tracer fluxes and footprint model predictions over a homogeneous canopy of intermediate roughness. *Agric. For. Meteorol.* 117:145–158.

LINDINGER, W., HANSEL, A., and JORDAN, A. 1998a. Proton-transfer-reaction mass spectrometry (PTR-MS): on-line monitoring of volatile organic compounds at pptv levels. *Chem. Soc. Rev.* 27:347–354.

LINDINGER, W., HANSEL, A., and JORDAN, A. 1998b. On-line monitoring of volatile organic compounds at pptv levels by means of proton-transfer-reaction mass spectrometry (PTR-MS). Medical applications, food control, and environmental research. *I. J. Mass Spectrom.* 173:191–241.

LU, C. J., and ZELLERS, E. T. 2002. Multi-adsorbent preconcentration/focusing module for portable-GC/microsensor-array analysis of complex vapor mixtures. *Analyst* 127:1061–1068.

MAFRA-NETO, A., and CARDE, R. T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MATISOLA, E., and DÖMÖTÖROVÁ, M. 2003. Fast gas chromatography and its use in trace analysis. *J. Chromatogr. A* 1000:199–221.

MILLER, J. R., and ROELOFS, W. L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

MOLE, N., and JONES, C. D. 1994. Concentration fluctuation data from dispersion experiments carried out in stable and unstable conditions. *Boundary-Layer Meteorol.* 67:41–74.

MOORE, P. A., and ATEMA, J. 1991. Spatial information in the three-dimensional fine structure of an aquatic odor plume. *Biol. Bull.* 181:408–418.

MOORE, P., and CRIMALDI, J. 2004. Odor landscapes and animal behavior: tracking odor plumes in different physical worlds. *J. Mar. Sys.* 49:55–64.

MURLIS, J. 1997. Odor plumes and the signal they provide, pp. 221–231, in R. T. Carde, and A. K. Minks (eds.). Insect Pheromone Research: New Directions. Chapman and Hall, New York.

MURLIS, J., and JONES, C. 1981. Fine-scale structure of odour plumes in relation to insect orientation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

MURLIS, J., ELKINTON, J. S., and CARDE, R. T. 1992. Odor plumes and how insects use them. *Annu. Rev. Entomol.* 37:505–532.

MURLIS, J., WILLIS, M. A., and CARDE, R. T. 2000. Spatial and temporal structures of pheromone plumes in fields and forests. *Physiol. Entomol.* 25:211–222.

MUSTAPARTA, H. 1975. Responses of single olfactory cells in the pine weevil *Hylobius abietis* L. (Col.: Curculionidae). *J. Comp. Physiol. A* 97:271–290.

MYLNE, K. R. 1992. Concentration fluctuation measurements in a plume dispersing in a stable surface layer. *Boundary-Layer Meteorol.* 60:15–48.

MYLNE, K. R., DAVIDSON, M. J., and THOMSON, D. J. 1996. Concentration fluctuation measurements in tracer plumes using

high and low frequency response detectors. *Boundary-Layer Meteorol.* 79:225–242.

MYLNE, K., and MASON, P. 1991. Concentration fluctuation measurements in a dispersing plume up to a range of 1000 m. *Quart. J. Roy. Meteorol. Soc.* 117:177–206.

NGAI, A. K. Y., PERSIJN, S. T., HARREN, F. J. M., VERBRAAK, H., and LINNARTZ, H. 2007. Selective trace gas detection of complex molecules with a continuous wave optical parametric oscillator using a planar jet expansion. *Appl. Phys. Lett.* 90:081109–1–081109-3.

PALASSIS, J. 1997. Portable photoionization instruments. *Appl. Occup. Environ. Hyg.* 12:528–531.

PARK, K. C., OCHIENG, S. A., ZHU, J., and BAKER, T. C. 2002. Odor discrimination using insect electroantennogram responses from an insect antennal array. *Chem. Senses* 27:343–352.

RAUNER, J. L. 1976. Deciduous forests, pp. 241–264, in J. L. Monteith (ed.). Vegation and the Atmosphere, Vol. II Case Studies. Academic Press, London.

REIDY, S., GEORGE, D., AGAH, M., and SACKS, R. 2007. Temperature-programmed GC using silicon microfabricated columns with integrated heaters and temperature sensors. *Anal. Chem.* 79:2911–2917.

RÖCK, F., BARSAN, N., and WEIMAR, U. 2008. Electronic nose: Current status and future trends. *Chem. Rev.* 108:705–725.

ROELOFS, W. L. 1984. Electroantennogram assays: Rapid and convenient screening procedures for pheromones, pp. 131–159, in H. E. Hummel, and T. A. Miller (eds.). Techniques in Pheromone ResearchSpringer, New York.

SANCHEZ, J. M., and SACKS, R. 2007. Performance characteristics of a new prototype for a portable GC using ambient air as carrier gas for on-site analysis. *J. Sep. Sci.* 30:1052–1060.

SCHLICHTING, H. 1987. Boundary-Layer Theory, 8th edn. McGraw-Hill, New York.

SCHNEIDER, D. 1957. Elektrophysiologishe untersuchungen von chemo-und mechanorezeptoren der antennne des Seidenspinners *Bombyx mori* L. *Z Vergi. Physiol.* 40:8–41.

SCHNEIDER, D. 1969. Insect olfaction: Deciphering system for chemical messages. *Science* 163:1031–1037.

SONG, M. S., MARRIOTR, P., RYANT, D., and WYNNE, P. 2006. Analytical limbo: How low can you go? *LCGC North America* 24:1012–1029.

SPRAYBERRY, J. D. H., and DANIEL, T. L. 2007. Flower tracking in hawkmoths: behavior and energetics. *J. Exp. Biol.* 210:37–45.

STANGE, G. 1996. Sensory and behavioural responses of terrestrial invertebrates to biogenic carbon dioxide gradients, pp. 223–253, in G. Stanhill (ed.). Advances in Bioclimatology. Springer, Heidelberg.

STANGE, G. 1997. Effects of changes in atmospheric carbon dioxide on the location of hosts by the moth, *Cactoblastis cactorum*. *Oecologia* 110:539–545.

STULL, R. B. 1988. An Introduction to Boundary Layer Meteorology. Kluwer, Dordrecht.

STRANDEN, M., LIBLIKAS, I., KÖNIG, W. A., ALMAAS, T. J., BORG-KARLSON, A.-K., and MUSTAPARTA, H. 2003. (–)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships. *J. Comp. Physiol A* 189:563–577.

THISTLE, H. H., PETERSON, H., ALLWINE, G., LAMB, B., STRAND, T., HOLSTEN, E. H., and SHEA, P. J. 2004. Surrogate Pheromone plumes in three forest trunk spaces: composite statistics and case studies. *For. Sci.* 50:610–625.

THOLL, D., BOLAND, W., HANSEL, A., LORETO, F., RÖSE, U. S. R., and SCHNITZLER, J.-P. 2006a. Practical approaches to plant volatile analysis. *Plant J.* 45:540–560.

THOLL, D., and RÖSE, U. S. R. 2006b. Detection and identification of floral scent compounds, pp. 3–25, in N. Dudavera, and E. Pichersky (eds.). Biology of Floral Scent. CRC, Boca Raton, FL.

TODD, L. 2000. Mapping the air in real-time to visualize the flow of gases and vapors: occupational and environmental applications. *Appl. Occup. Environ. Hyg.* 15:106–113.

VICKERS, N. J., and BAKER, T. C. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Natl. Acad. Sci. USA* 91:5756–5760.

VICKERS, N. J., and BAKER, T. C. 1996. Latencies of behavioral response to interception of filaments of sex pheromone and clean air influence flight track shape in *Heliothis virescens* (F.) males. *J. Comp. Physiol. A* 178:831–847.

VISSER, J. H. 1976. The design of a low-speed wind tunnel as an instrument for the study of olfactory orientation in the Colorado beetle (*Leptinotarsa decemlineata*). *Ent. Exp. Appl.* 20:275–288.

VOGT, F. 2006. Trends in remote spectroscopic sensing and imaging - experimental techniques and chemometric concepts. *Cur. Anal. Chem.* 2:107–127.

VON DAHL, C. C., HEAVECKER, M., SCHLOEGL, R., and BALDWIN, I. T. 2006. Caterpillar-elicited methanol emission: A new signal in plant-herbivore interactions? *Plant J.* 46:948–960.

WANG, C., and MBI, A. 2007. A new acetone detection device using cavity ringdown spectroscopy at 266 nm: evaluation of the instrument performance using acetone sample solutions. *Meas. Sci. Tech.* 18:2731–2741.

WEBSTER, D. R., and WEISSBURG, M. J. 2001. Chemosensory guidance cues in a turbulent chemical odor plume. *Limnol. Oceanogr.* 46:1034–1047.

WEISSBURG, M. J. 2000. The fluid dynamical context of chemosensory behavior. *Biol. Bull.* 198:188–202.

WEISSBURG, M. J., and ZIMMER-FAUST, R. K. 1993. Life and death in moving fluids: Hydrodynamic effects on chemosensory-mediated predation. *Ecology* 74:1428–1443.

WHALLEY, L. K., LEWIS, A. C., MCQUAID, J. B., PURVIS, R. M., LEE, J. D., STEMMLER, K., ZELLWEGER, C., and RIDGEOPN, P. 2004. Two high-speed, portable GC systems designed for the measurement of non-methane hydrocarbons and PAN: Results from the Jungfraujoch high altitude observatory. *J. Env. Mon.* 6:234–241.

WILLIS, M. A., DAVID, C. T., MURLIS, J., and CARDÉ, R. T. 1994. Effects of pheromone plume structure and visual stimuli on the pheromone-modulated upwind flight of male gypsy moths (*Lymantria dispar*) in a Forest (Lepidoptera: Lymantriidae). *J. Insect Behav.* 7:385–409.

WILLIS, M. A., and BAKER, T. C. 1988. Effects of varying sex pheromone component ratios on the zigzagging flight movements of the oriental fruit moth, *Grapholita molesta*. *J. Insect Behav.* 1:357–371.

WOLF, H., and WEHNER, R. 2000. Pinpointing food sources: olfactory and anemotactic orientation in desert ants, *Cataglyphis fortis*. *J. Exp. Biol.* 203:857–868.

YEE, E., KOSTENIUK, P. R., CHANDLER, G. M., BILTOFT, C. A., and BOWERS, J. F. 1993. Statistical characteristics of concentration fluctuations in dispersing plumes in the atmospheric surface layer. *Boundary-Layer Meteorol.* 65:69–109.

ZIMMER, R. K., and BUTMAN, C. A. 2000. Chemical signaling processes in the marine environment. *Biol. Bull.* 198:168–187.

ZIMMER, R. K., and ZIMMER, C. A. 2008. Dynamic scaling in chemical ecology. J. Chem. Ecol. (this volume).

ZIMMER-FAUST, R. K., STANFILL, J. M., and COLLARD, S. B. 1988. A fast, multi-channel fluorometer for investigating aquatic chemoreception and odor trails. *Limnol. Oceanogr.* 33:1586–1594.

ZIMMER-FAUST, R. K., FINELLI, C. M., PENTCHEFF, N. D., and WETHEY, D. S. 1995. Odor plumes and animal navigation in turbulent water flow: A field study. *Biol. Bull.* 188:111–116.

ZIMMERMANN, R. 2005. Laser ionisation mass spectrometry for online analysis of complex gas mixtures and combustion effluents. *Anal. Bioanal. Chem.* 381:57–60.

ZOLLNER, G. E., TORR, S. J., AMMANN, C., and MEIXNER, F. X. 2004. Dispersion of carbon dioxide plumes in African woodland: implications for host-finding by tsetse flies. *Physiol. Entomol.* 29:381–394.

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REVIEW ARTICLE

Navigational Strategies Used by Insects to Find Distant, Wind-Borne Sources of Odor

Ring T. Cardé · Mark A. Willis

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Abstract Insects locate many resources important to survival by tracking along wind-borne odor plumes to their source. It is well known that plumes are patchy distributions of high concentration packets of odor interspersed with clean air, not smooth Gaussian distributions of odor intensity. This realization has been crucial to our understanding of plume-tracking behavior, because insect locomotory movements and sensory processing typically take place in the range of tens to hundreds of milliseconds, permitting them to respond to the rapid changes in odor concentration they experience in plumes. Because odor plumes are not comprised of smooth concentration gradients, they cannot provide the directional information necessary to allow plume-tracking insects to steer toward the source. Many experiments have shown that, in the species examined, successful source location requires two sensory inputs: the presence of the attractive odor and the detection of the direction of the wind bearing that odor. All plume-tracking insects use the wind direction as the primary directional cue that enables them to steer their movements toward the odor source. Experimental manipulations of the presence and absence of the odor, and the presence, absence, or direction of the wind during plume tracking, have begun to resolve the relationship between these two sensory inputs and how they shape the maneuvers we observe. Experiments, especially those undertaken in the natural wind and odor environments of the organisms in question and those directed at understanding the neural processing that underlie plume tracking, promise to enhance our understanding of this remarkable behavior.

Keywords Orientation · Odor dispersion · Optomotor anemotaxis · Drosophila · Moth · Tsetse fly · Pheromone · Host odor

R. T. Cardé (✉)
Department of Entomology, University of California,
Riverside, CA 92521, USA
e-mail: ring.carde@ucr.edu

M. A. Willis
Department of Biology, Case Western Reserve University,
2080 Adelbert Rd.,
Cleveland, OH 44106, USA

Introduction

Many kinds of organisms use odors to locate and identify a wide variety of resources, such as potential mates, conspecifics for aggregation, food, and sites for oviposition. Finding these sources involves a number of potential selective forces—such as economy of movement and the rapidity and likelihood of source location. In some cases, the time to location may be paramount, as in the case of a female moth that is emitting pheromone. She may be mated by the first male that reaches her, and consequently, there should be a considerable selective advantage accorded to males that have an efficient strategy for finding her pheromone plume and then navigating rapidly and accurately along the plume to its source. Greenfield (1981) categorized this competition as "a race to locate females." In other cases, such as finding a fruit suitable for oviposition, the resource may be available continuously, and, therefore, the importance of rapid navigation to the odor's source may be lessened.

This review focuses on examples selected to exemplify the diversity of navigational strategies that insects employ to find a distant, wind-borne odor source by navigating along its plume. An odor is typically a mixture of chemicals that evokes a specific behavioral reaction, upwind orientation in

the context of this review. Odors that are incomplete may not evoke "natural" orientation behavior, as in the case of a partial blend of pheromone components or a complete mixture presented at an unnatural ratio (see Vickers 2006). This review is confined to cases where the odor presented is assumed to be the single natural chemical or the naturally occurring mixture. Among the issues considered are: How the odor is distributed within the plume, strategies for finding an odor plume, mechanisms to detect wind flow, direction of wind flow as a directional guide to the odor's source, and the kinds of orientation maneuvers used in navigation.

Odor Dispersion

A thorough consideration of the dynamics of odor dispersion in wind is beyond the intent of this review. However, a brief description of this process is necessary to begin to understand how the spatial distribution of odor within the plume and the direction of wind flow modulate orientation maneuvers. In this paper, we define the plume as the volume wherein odor concentration is generally above behavioral threshold; because of turbulence, the odor is unevenly distributed in the plume, with odor-containing eddies interspersed with air that is odor-free or below behavioral threshold. Important reviews on the patterns of odor dispersion in air and water as they affect animal responses to semiochemicals include those of Elkinton and Cardé (1984), Murlis et al. (1992), Weissburg (2000), Conover (2007), Willis (2008), and Riffell et al. (this issue).

Early analyses of the dispersion of a pheromone plume in wind used a time-averaged (Gaussian) model developed by Sutton (1953) to depict concentration distributions downwind of the source (Bossert and Wilson 1963). The work of Fares et al. (1983) incorporated turbulent diffusion and accounted for atmospheric stability, i.e., the scale of turbulence, but these models also generated Gaussian distributions. All such approaches produce time-averaged distributions (e.g., the Sutton equation estimates a 3-min average of concentration), whereas, as first pointed out by Wright (1958), insects typically react to very brief exposures to odors (e.g., ≈10 ms in the case of the almond moth, *Cadra cautella*, to a single filament of its pheromone, Mafra-Neto and Cardé 1996). By using the wing-fanning reaction of the male gypsy moth, *Lymantria dispar*, to pheromone, Elkinton et al. (1984) found that time-averaged dispersion models substantially underestimated the instantaneous concentration of pheromone in a forest habitat. Aylor et al. (1976) and Miksad and Kittredge (1979) proposed plume models that calculate instantaneous concentration relative to the plume's centerline, but these did not account for the lateral movement of the plume (i.e., meander), and consequently, these models can not be used to predict concentration at a fixed point downwind (Elkinton and Cardé 1984).

Dispersion of odor in wind is dominated by the forces of turbulent diffusion that stretch and stir odor filaments as they are released from the odor source, simultaneously creating gaps of odor-free air within the plume as it expands and is transported downwind (Murlis et al. 1992). Molecular diffusion, on the other hand, is so slow that it is important only at very small distances. The diffusion coefficient for ethanol is only 1.32×10^{-5} m^2 s^{-1} and that for hexadecanol (similar in size to many moth pheromones) is 2.5×10^{-6} m^2 s^{-1} (Loudon 2003). The slow rates of molecular diffusion mean that the distribution of odor in the plume is due mainly to turbulence rather than molecular diffusion, and in cases where the odor is a mixture, the ratio of compounds throughout the plume will be close to the ratio emitted at the source. Relatively undiluted packets of odor ("bursts") can thus persist many meters downwind of the plume's origin (Murlis and Jones 1981).

Insects navigating along turbulent plumes thus encounter odor in bursts interspersed with patches of clean air (Fig. 1). In analyzing the temporal features of such intermittent signals, it is helpful to use only those portions of the record when the signal is present, the so-called conditional signal. This variable presence of a signal at a fixed sampling point downwind is termed its intermittency (a proportional value that ranges from a value of 1 when the signal is *not* present to zero when it is present continuously—see Fig. 1). Intermittency is caused by turbulent diffusion and plume

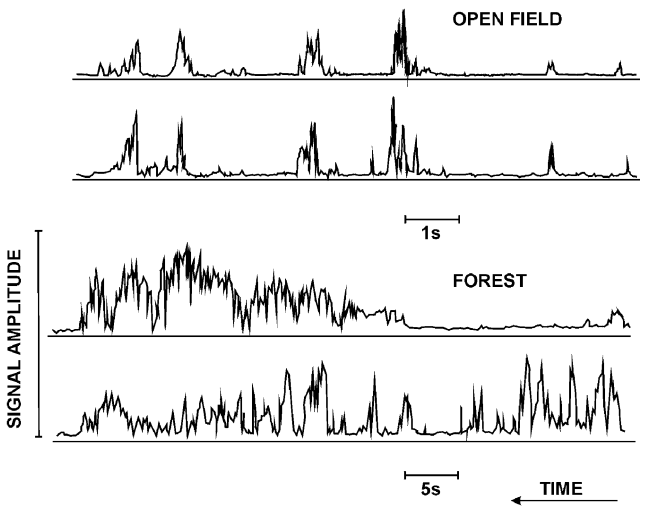

Fig. 1 Ion signals measured in daytime with a Langmuir probe in an open field and in a forest 2.5 m downwind of an ion source. The ions served as a surrogate for odor. Note the difference in the time scales for measurements in the two habitats. In the open field, there are many gaps of several seconds duration when no ions were detected, showing that the signal sampled at a fixed point in space is highly intermittent. When the signal is present (in "bursts"), it varies enormously in concentration. See Murlis et al. (2000) for details.

meander and undulation. As the plume is carried downwind, the conditional mean concentration of odor bursts within the plume and the conditional peak-to-mean ratio of odor intensity both decrease, while signal intermittency increases (Murlis et al. 2000). However, given the patchy distribution and highly variable concentration of odor filaments within the plume, the use of changes in odor concentration as a guide to the direction toward the source is not reliable unless the odor source is decimeters or so away (Murlis et al. 1992).

The effect of wind speed on small and large scale turbulence is complex. As wind velocity increases, generally the directional flow of the plume is more consistent (i.e., the plume is straighter) and thus the plume's outer envelope encompasses a reduced volume and increased concentration (Griffiths et al. 1995). Episodes of increased wind speed and consistency of directional flow seem to account for time periods when gypsy moths navigate successfully over tens of meters along pheromone plumes in a deciduous forest (Elkinton et al. 1987).

The initial size of odor sources that are important to insects varies enormously. Even moderately sized insects such as arctiid moths (e.g., 1–3 cm in length) can generate an initially small plume of pheromone, on the order of several square millimeters in cross-section, emanating from two small pores at the tip of their abdomen. Pheromone-emitting ("calling") arctiid females elevate their wings in a "V" posture, enabling air to flow freely around their body and, thereby, preserving plume size as the odor is carried away from the female (Conner and Best 1988). However, insects may more typically emit odor while perched within foliage or on tree trunks, thereby generating much larger plumes. For example, a gypsy moth (*L. dispar*) female typically calls while perched on a tree trunk. Eddies swirling in the trunk's downwind wake immediately expand her pheromone plume to approximately the tree's diameter (Charlton and Cardé 1990; see also Brady et al. 1989; Willis et al. 1991; Wyatt et al. 1993).

Whether odor emitting individuals choose calling sites specifically to enhance signal propagation is currently not well understood. In the case of the pink bollworm moth, *Pectinophora gossypiella*, Kaae and Shorey (1973) found that females select calling sites near the top of the cotton canopy when night winds are light and farther down in the canopy in windy conditions. Female-baited traps placed in both positions caught more males in the upper position on calm nights and more in the lower position on windy nights, suggesting that the male's orientation success is influenced by wind speed and position of the signal in the canopy. We do not, however, know how the interactions of position in the foliage and wind speed influence the plume's distribution or the male's ability to track it.

Odor plumes from the body of vertebrates generally would have an initial size roughly as large as the size of the body from which the odor emanates. Sizes range for example from a small bird to a large ungulate or even a herd of ungulates. The odor cues within such plumes, however, may be unevenly distributed, particularly close to the source. A female mosquito blood feeds preferentially, dependent on mosquito species, on different regions of the torso, legs, arms, and head of a seated human (de Jong and Knols 1996). Such partitioning of feeding sites may be mediated in part by odor profiles that differ by body region and may dictate orientation maneuvers. Odor from human breath, particularly CO_2, which itself is highly attractive to host-seeking female mosquitoes, forms a plume that is pulsed with each exhalation; initially, this plume would not overlap with the body odor plume except near the head. A fluctuating intensity of CO_2, due mainly to turbulent diffusion, has been suggested to be the principal odor cue used at a distance by female yellow fever mosquitoes, *Aedes aegypti*, to initiate plume following (Dekker et al. 2005).

Many other odor sources also have an initially large plume size. Mass attack of hundreds of bark (scolytid) beetles boring into a tree releases pheromone and phloem volatiles that generate a plume that initially is as wide as the tree trunk and that has a vertical expanse matching the trunk area that is infested. Volatiles released by leaves often serve as orientation cues, and the odor plume's initial size typically would be as large as the plant. These examples indicate that initial plume size varies enormously with the kind of odor source. Plumes issuing from small sources have reasonably defined borders that are readily crossed and detected by a flying insect within meters to centimeters of the source; such plume boundaries may facilitate orientation by collimating its flight path. Indeed, recent studies of male cockroaches, *Periplaneta americana*, that track plumes of different widths while walking have identified specific turns back into the plume that appear to be triggered by these animals nearing or crossing the lateral boundary of a wind-borne plume (Willis and Avondet 2005). Large plumes may have ragged or ill-defined edges, which, because of the plume's large size, are not routinely traversed or detected during orientation. Far from the source (e.g., tens of meters), plumes may be so wide that the expanded individual filaments of the plume may be treated like "plumes" by animals with the size and movement scales of a flying insect.

Structural features of the habitat also influence a plume's fine-scale distribution and meander. Landscape-level features such as trees and shrubs, when they contact the plume, can stir and dilute it (mechanical turbulence) or cause it to meander around the obstacle. Plume structure also varies with the daily patterns of wind velocity and levels of turbulence. Wind velocities on average peak from late morning to mid afternoon and then generally decline until reaching a daily low near dawn. Levels of turbulence can

be related either to atmospheric stability, which is governed by incoming solar radiation during daytime and the extent of nighttime cloud cover or to vertical temperature gradients (Fares et al. 1983). Directional variability (plume meander) is correlated inversely with wind speed (Brady et al. 1989) and, generally, is less over open ground than in woodlands (Brady et al. 1989; Murlis et al. 2000). Fares et al. (1983) have suggested that observed variation in the daily times that *Dendroctonus* bark beetles are attracted to their pine hosts is correlated with favorable periods for semiochemical dispersal—namely when stable atmospheric conditions tend to "trap" the plume under the canopy. Conover (2007) provides a thorough consideration of how structural features of the habitat such as trees and atmospheric conditions influence plume structure.

Given that weather can differ enormously from day to day and the habitat that a particular species occupies also typically varies in structure, there may be no single, optimal navigational strategy. Rather, the orientation maneuvers must be sufficiently flexible to operate across a range of wind speeds and turbulence conditions (Belanger and Willis 1996).

Wind Direction

Generally, heading upwind while in contact with the odor is the principal mechanism that flying and walking insects employ during orientation along a plume. However, shifting wind direction causes the plume to meander and, surprisingly, the instantaneous wind direction within the plume is not always aligned with the plume's long (downwind) axis. When the wind holds at a relatively steady speed but changes direction, upwind direction within such a meandering plume generally points toward the odor's source (David et al. 1982), but heading upwind also may lead the insect out of the plume (Fig. 2b). Surrogate odors (puffs of smoke) released over an open field typically travel in a straight line for at least 25 m. When the wind speed and direction both fluctuate as would be typical in nature, however, the upwind direction may lead out of the plume and be a poor directional guide to the odor's source. Such meandering can be accentuated by the presence of shrubs and trees around which plumes may flow (Elkinton et al. 1987; Brady et al. 1989). The consequences of variable directionality and wind velocity on plume following are that insects flying upwind along plumes would be expected routinely to exit the plume's boundaries. As we explain later, insects are well adapted to deal with this challenge.

The last consideration is the possible vertical undulation of the plume. A plume traveling through a forest into a patch of sunlight will be warmed and consequently will rise in thermal chimneys (e.g., Fares et al. 1983). At nighttime in the Costa Rican rain forest, pheromone plumes from cockroaches calling on tree trunks also flow upward because of a temperature gradient, and accordingly, males of a given species appear to position themselves on trunks higher than conspecific females (Schal 1982). In relatively still air, odor plumes that emanate from a warm source (e.g., odors from human skin) may have a significant vertical component because of convective flow until the plume cools to the ambient temperature. There is not a great deal of information on how much vertical distribution of plumes influences odor plumes at spatial scales relevant to insect orientation, but vertical undulations, when they do occur, must add to the navigational challenge.

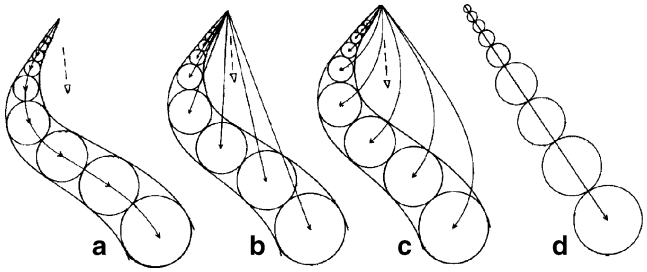

Fig. 2 Schematic of the possible effects of changing wind direction on the trajectory of meandering pheromone plumes as viewed from above. Pheromone is depicted as a series of puffs (circles), which expand with time. The trajectories of the puffs are shown by the lines leading from the source to the center of each puff with the arrow tips depicting the instantaneous wind direction within each puff. The dashed lines and arrow tips represent the mean direction of wind flow measured at the source over the time course of each plume's meandering. **a** Trajectory assumed by workers before the David et al. (1982) publication. The plume's centerline and the instantaneous wind direction at any point are assumed to be aligned. Therefore, heading upwind would lead to the odor source. **b** Conforms with the experiments of David et al. (1982) in an open field with a shifting wind direction but a relatively constant wind speed. Heading upwind in the downwind sector of the plume routinely leads the responder out of the plume. **c** Matches observations of Elkinton et al. (1987) beneath a forest canopy and Brady et al. (1989) in an open savannah; this effect is correlated with changes in wind direction and velocity measured at the source. **d** Shows a steady wind direction—during such "favorable" and typically brief alignments of wind direction and the plume's centerline, insects can make rapid progress toward the source. Reprinted with permission of Wiley–Blackwell from Elkinton et al. (1987), p. 400.

Finding an Odor Plume

Although this review emphasizes how the distribution of odors influences the navigation of insects to an odor's source, also of importance are the strategies employed to contact an odor plume. These have been termed "searching," "ranging," "questing," "wandering," and "appetitive" behaviors, but their key properties are a straightened-out locomotory path and a lowered threshold of responsiveness to resource-linked stimuli. The optimal strategies to locate a wind-borne odor plume often have been considered as a

separate theoretical issue (e.g., Sabelis and Schipper 1984; Dusenbery 1989, 1990). Generally, implicit in such models is an ability of the organism to estimate and average wind direction and its variability over some time interval, with the resultant optimal course for contacting a wind-borne plume being aimed on average crosswind, upwind, or downwind, depending on the model's assumptions. For example, if the wind is shifting direction more than 60°, it could be more advantageous to head either upwind or downwind than to head crosswind, because the crosswind expanse of the plume will exceed the distance of its downwind projection (Sabelis and Schipper 1984). There are, however, unstated assumptions implicit in this model. The distribution of odor across a plume that shifts more than 60° is patchy because of turbulence, and the probability of meeting odor may differ in traversing such a plume's via its downwind versus its crosswind axis (even if a plume is not continuous along its windline axis, the likelihood of contacting it while heading crosswind ought to be higher than approaching the plume from upwind or downwind directions). Other assumptions are that the searching organism must able to "calculate" the mean wind direction (Zanen et al. 1994), must be able to determine over the relevant time interval that the wind is shifting by more than 60°, and, most importantly, be able to assign these wind vectors to an absolute cardinal heading, such as fluctuating ≈60° around due north. This requires the organism to have a directional "map" of the immediate area and a memory of the wind's fluctuations.

Behavioral evidence to support any of these optimality models is scanty. Field observations of gypsy moths (*L. dispar*) have documented that female-seeking males flying within a meter or so of ground level take random courses with respect to the instantaneous wind direction (Elkinton and Cardé 1983). Although many gypsy moth females call from heights within 2 m of ground level, some call throughout the height of the tree canopy. Males observed in dense populations also search for calling females by flying vertically along tree trunks, apparently without reference to wind direction (Cardé and Hagaman 1984). Field observations of initial flight direction upon take-off of female onion flies, *Delia antiqua*, in absence of host plant odor appear random with respect to wind direction (Judd and Borden 1988, 1989). Wind-tunnel observations of the malaria mosquito, *Anopheles gambiae s.s.*, in an unscented air flow of steady direction determined that many females in the host-seeking stage generally flew upwind (Takken et al. 1997; Costantini et al. 2001). In field observations of orientation to human "baits" with netting barriers deployed in differing directions of wind flow, mosquitoes were documented to fly downwind before host encounter, in accord with a model of optimal energy expenditure to find a plume (Gillies and Wilkes 1974). However, in subsequent field work Gillies and Wilkes (1978) discounted their previous interpretation of a downwind host-seeking strategy, because they determined that screened fences had a negligible effect on mosquito movement. However, field observations of tsetse flies in absence of host odor or visual cues indicated that such "ranging" flights have a downwind bias (Gibson et al. 1991). Manipulations of hungry *Drosophila* fruit flies in a small wind tunnel in steady and shifting wind directions, however, suggest that they can shuttle between two strategies (Zanen et al. 1994). In a steady wind direction, they flew generally crosswind, in agreement with the predictions for an optimal path to encounter a plume, whereas in shifting winds they flew upwind in conformity with Dusenbery's (1990) analysis.

Provided the organism could use only currently or very recently sensed wind direction as a guide, a crosswind searching strategy would be optimal if the search organisms detected little variability in wind direction. Conversely, if the organism experienced continual changes in wind direction, then heading upwind or downwind by using the then-sensed wind direction would be optimal. These simple rules would not necessitate knowing the amount of wind variability (is it >60°?) nor its cardinal direction, but the general effects on searching success should be similar to using the implicit assumptions of the Sabelis and Schippers model. This suggests that strategies for finding the plume by assuming a particular heading with respect to *current* wind flow would be most effective: upwind or downwind when the wind direction varies, and crosswind when the wind direction holds steady. Additional issues that add to the complexity to calculating an optimal strategy are the relative speed of the wind versus the speed of the flying insect (see Dusenbery 1990).

An interesting "special case" has recently been discovered (Wolf and Wehner 2006). The desert ant *Cataglyphis* sp. uses visual cues from polarized light and inertial cues from the movement of its own body to steer a return course to its nest or good foraging sites (Wehner 1996). However, when returning to distant feeding sites, they apparently approach from downwind and intentionally steer off the wind line to intercept the plume of odor issuing from the food, and thus assure location of its source (Wolf and Wehner 2006). In this case, memory of the location of a good food source is combined with wind information to allow the ant to steer its walking course into a quadrant of the environment where it should encounter a reliable cue to locate important resources.

Finding a "Lost" Odor Plume

An insect flying upwind along the odor plume can lose contact with the plume in three ways: (1) Gaps within the plume caused by turbulence may be of sufficient size to

cause a cessation in upwind displacement; (2) insects may exit the plume's boundaries because the direction upwind may not always lead along the plume's path (Fig. 2b,c); and (3) the moth's own plume-tracking maneuvers may carry it outside the time-averaged plume boundaries. A strategy used by moths to re-contact a lost plume is called "casting," in which upwind displacement ceases and the moth flies to and fro in increasingly wider lateral zigzags across the windline (e.g., David et al. 1983; Kennedy 1983; Kuenen and Cardé 1994). The vertical component of flight also expands upon loss of the pheromone plume (von Keyserlingk 1984; Vickers and Baker 1996). Casting moths have been observed to hold station near the point of plume loss or drift downwind as the width of their tracks increases (Baker and Haynes 1987). This flight pattern may allow the moth to regain contact with the plume by reentering it or, if casting persists for a sufficient time, be in a location where the plume's meander itself causes re-contact. Casting moths continue to orient their flight with respect to the wind direction (David et al. 1983; Baker and Haynes 1987), and in some cases, this may enable them to re-contact the plume closer to the source then where it was lost (David et al. 1983). Another strategy for re-contacting the plume is to loop downwind, as observed in a parasitoid wasp (Kaiser et al. 1994) and the oriental fruit moth (Baker and Haynes 1996). A third option is simply station keeping until the plume swings back to the insect's location. Once the plume is re-contacted, upwind flight can resume. If contact is not re-established within a set time (a "giving-up time"), the insect should return to plume searching (Kuenen and Cardé 1994).

Odor-Induced Orientation to Wind

Important recent reviews of the mechanisms of insect flight to odor sources include those of Gibson and Torr (1999), Vickers (2000, 2006), and Hardie et al. (2001). Kennedy (1983) provided a comprehensive review and analysis of the early literature. Reviews of plume following in water are also of interest, as the issues of turbulent diffusion of an odor in water, information processing, and orientation are parallel to those when the plume is airborne (Atema 1996; Weissburg 2000).

Optomotor Anemotaxis The principal navigational mechanism used by a flying insect in location of an odor source is to head upwind while in contact with the odor. Detection of the direction upwind once an insect is airborne is accomplished by the optomotor response. An insect flying along the windline perceives an image flow viewed below as moving front to rear. When the insect's heading is not due upwind, the image flow has a transverse component because of wind-induced drift. This navigation system was first verified by Kennedy (1939) by using female *Aedes aegypti* mosquitoes stimulated to fly upwind by human breath introduced into a wind tunnel. Mosquitoes reacted to their apparent visual displacement which was provided by a pattern projected onto the tunnel's floor. Mosquitoes headed in the visually perceived upwind direction when the floor pattern was moved to simulate the effect of downwind displacement. This steering system is termed "optomotor anemotaxis."

Male Moth Orientation to Pheromone Much of what has been learned about odor-induced in-flight orientation has come from studies of male moth navigation along plumes of female-emitted pheromone. Most of these studies have used wind tunnels that permit a moderate degree of manipulation of the pheromone plume's structure, wind speed, and visual environment. The moth's maneuvers generally have been recorded in planar view, and so, the analyses of flight tracks have been two dimensional. A few studies have recorded flight tracks of moths flying freely in the field, often using a visual marker such as soap bubbles to indicate the path the odor plume has taken (Murlis et al. 1982; David et al. 1983; Willis et al. 1994; Vickers and Baker 1997).

Upwind flight of moths along a pheromone plume has typically been described as having a zigzag form, with regular left and right reversals across the windline. These turns in the gypsy moth are remarkably metronymic, occurring ≈3.5 to 4 reversals s^{-1} in a variety of conditions of plume concentration, plume structure, ambient temperature, and light level (Charlton et al. 1993; Cardé and Knols 2000), all of which change other flight parameters such as flight speed. A robustness of turning frequency during plume tracking is characteristic of all moth species in which it has been measured (Willis and Arbas 1991—*Manduca sexta*; Vickers and Baker 1992—*Heliothis virescens*; Willis and Baker 1987—*Grapholita molesta*). Often the only way to experimentally alter the turning frequency is to remove the odor plume altogether (Willis and Arbas 1991). Kennedy (1983) viewed zigzag turns in plume following and in casting as modulated by the same motor program and termed them "counterturns." The possibility that counterturns are caused by exiting the plume's boundary and then turning back toward where it was last sensed was once widely assumed (e.g., Farkas and Shorey 1972, 1974; Kennedy and Marsh 1974; Marsh et al. 1978; Cardé and Hagaman 1979) and had been described as "feeling for the edges." However, as turns can be executed well inside a plume (Kuenen and Baker 1982) or successively in clean air (i.e., casting flight; Kennedy and Marsh 1974), loss of odor contact is not the initiating stimulus for counterturning.

The establishment of the direct effects of a plume's internal structure and boundary position on orientation maneuvers has been at times contentious. Wright (1958)

proposed that flight along the plume was governed by encountering successive filaments of odor within the turbulent plume, with the pattern of contact shaping the form of the flight track. His analysis, however, assumed that the directional cues used in orientation were entirely chemical, in the plume's internal fenestration and outer boundaries, a view he later abandoned (Daykin et al. 1965). Simply detecting wind flow and then heading upwind while in contact with the odor was not considered a relevant navigational mechanism.

Farkas and Shorey (1972) proposed that moths sensed the overall shape of the pheromone plume while flying a zigzag path that carried them in and out of the plume's boundaries. The fact that they referred to this behavior as "aerial trail following" suggests that they imagined that plume-tracking male moths used chemo-orientation mechanisms similar to those used by walking ants to follow pheromone trails deposited on the ground (Hangartner 1967). Successive comparisons of the plume's position would enable flight along the plume's long axis toward the pheromone's source. In wind-tunnel experiments with the pink bollworm, *P. gossypiella*, a plume was first drawn out in wind, a male was then released into the plume, and the wind was immediately stopped (Farkas and Shorey 1972). The males' tracks were monitored after wind stop, during which time directional orientation could not be attributed to wind cues. According to Farkas and Shorey (1972), the only possible explanation for the successful plume tracking observed was longitudinal klinotactic chemo-orientation, that is, orientation directed by comparing concentration changes along the plume's length. Farkas and Shorey (1972) attempted to demonstrate an optomotor reaction that could support optomotor anemotaxis by moving a treadmill floor pattern beneath the tunnel, but no consistent response was observed. Therefore, they concluded that pink bollworm moth males did not use optomotor anemotaxis to direct their flight orientation in their wind tunnel, rather that moths steered by orienting directly to the odor concentrations in the plume.

This mechanism was challenged by Kennedy and Marsh (1974) who showed in their wind-tunnel trials that Indian meal moth, *Plodia interpunctella*, males steered their flight toward the source by reacting to changes in feedback from their visual flow field as predicted by optomotor anemotaxis. However, the principal observation of Farkas and Shorey (1972) that moths that have initiated flight along a plume in wind indeed can continue successful orientation along the plume in still air has been substantiated in two additional moths, *G. molesta* (Baker and Kuenen 1982; Kuenen and Baker 1982) and *L. dispar* (Willis and Cardé 1990). Given the variability of wind in nature, the ability to follow plumes in still air or wind velocities too low to resolve wind-induced drift by using optomotor feedback (Cardé and Knols 2000) should be a great advantage in mate finding. It has been demonstrated (Kennedy and Marsh 1974; Baker et al. 1984; Cardé 1984), and is now widely accepted, that plume-tracking moths use optomotor anemotaxis to direct their flight upwind toward the source. However, the mechanisms used to track plumes during lulls in wind are not well understood and are still open to study.

The first experiments to manipulate plume structure systematically sought to determine if signal intermittency was important to orientation. Kennedy et al. (1981) found with the summerfruit tortrix moth, *Adoxophyes orana*, that initial contact with pheromone initiated regular zigzag turning, but immediately, subsequent emersion in a homogeneous miasma of pheromone inhibited upwind flight; the males initiated casting flight as if they had lost contact with odor. Conversely, flight in a cloud of pheromone with a fluctuating intensity promoted upwind flight similar to that observed during plume tracking (Baker et al. 1985). Cessation of upwind movement in a homogeneous pheromone environment has been observed now in two other moth species, the oriental fruit moth, *G. molesta* (Willis and Baker 1984), and the pink bollworm, *P. gossypiella* (Justus and Cardé 2002). The almond moth, *C. cautella*, however, is capable of flying upwind in a homogeneous cloud of pheromone, at least over short distances, although the heading was aimed at an angle averaging ≈15° off of due upwind (Justus and Cardé 2002).

A precise role for contact with pheromone filaments in setting an upwind course was proposed by Baker (1990). According to this idea, two parallel control systems are activated upon contact with pheromone. The first generates the regular counterturning thought to underlie the zigzagging flight tracks we observe (either upwind or casting). The second suppresses this turning and steers the moth more directly upwind, resulting in an upwind surge toward the source. Subsequent experiments with *Heliothis virescens* showed that puffs of pheromone generated at the source at a rate of 4 Hz induced sustained upwind flight and source location and in some individuals, almost completely suppressed casting maneuvers, resulting in prolonged bouts of upwind flight with few detectable turns (Vickers and Baker 1992).

Working with the almond moth in a laminar flow wind tunnel, Mafra-Neto and Cardé (1994, 1995) monitored upwind surges after either interception of a filament of pheromone in a ribbon plume or a single puff of pheromone. A single encounter with a filament or puff induced an upwind surge within ≈200 ms, followed by initiation of crosswind casting. The duration of pheromone contact positively influenced the distance (and duration) of the surge, and, provided that contacts with filaments occurred at a sufficiently high rate, the surges could be linked into an essentially due-upwind heading. The rate of filament

generation at the pheromone source, ensuring a relatively due upwind course, appeared to be 10 Hz and above (Mafra-Neto and Cardé 1994, 1995; Justus et al. 2002b). Given the moth's airspeed, the in-flight rate of filament encounter would be higher than the rate of production at the source (Baker and Vickers 1994; Mafra-Neto and Cardé 1995).

Vickers and Baker (1994, 1996), working contemporaneously with *H. virescens*, reached the same conclusion: Contact with a sequence of pheromone filaments sets a relatively due-upwind course. The cast—filament contact—upwind surge model seems to explain both the zigzag flight track commonly noted in moth orientation to pheromone (and likely female orientation to host plant and flower volatiles, see Mechaber et al. 2002) and occasional segments of straight upwind flight. Recent studies of freely flying *D. melanogaster* fruit flies show that they behave in a manner similar to the almond moth (Budick and Dickinson 2006). When presented with a ribbon plume of banana odor, *D. melanogaster* individuals generate flight trajectories that are essentially indistinguishable from those of male moths tracking pheromone plumes (Fig. 5 in Budick and Dickinson 2006). Like freely flying moths, *D. melanogaster* responses vary, from tracks almost straight upwind, to moth-like zigzagging. However, unlike most moths studied so far, *D. melanogaster* flies straight upwind when presented with a homogeneous fog of attractive odor. This behavior is consistent with that observed from the almond moth (Justus and Cardé 2002) and with previous anecdotal reports of *D. melanogaster* flight behavior (Kellogg et al. 1962). The common house fly, *Musca domestica*, also generates moth-like flight trajectories when tracking plumes of attractive odors (Cossé and Baker 1996). No experimental manipulation of odor stimuli has been performed with this species, and we can only speculate that the mechanisms underlying its behavior are similar to those that govern the orientation of male moths to pheromone.

Mosquito orientation to host odors Many cues are implicated in the attraction of female mosquitoes to a vertebrate host for a blood meal, including expired CO_2, other body odors, heat, humidity, and visual features (reviewed by Clements 1999). Long-distance orientation (tens of meters downwind of the host and beyond visual range) could be governed by expired CO_2, body odors, or a combination of both. CO_2 is unusual among odors in that it is present as a constant "background" odor (≈0.04% ambient concentration), whereas it is expired by vertebrates at ≈4%. In the case of CO_2 released from a point source at the same rate as breathe from an ox into a riparian woodland in Africa, bursts of CO_2 above ambient were detected by using a high-resolution analyzer up to 64 m downwind (Zöllner et al. 2004). The importance of CO_2 relative to other host odors in mosquito attraction to a host is debated, and the value of this cue in inducing upwind flight may be in part contingent on the breadth of the host range of a given mosquito species (Gillies 1980; Takken and Knols 1999; Dekker et al. 2001), with generalist species being more likely to rely on CO_2 alone as a reliable indicator of a possible upwind host and with other body odors (along with humidity, heat, and visual cues) mediating orientation at close range.

Grant and O'Connell (1996) found that receptors on the maxillary palps of female mosquitoes are exquisitely sensitive to changes in concentration of CO_2, with fluctuations of as little as 40 ppm reflected by changes in receptor firing rates. Thus, well downwind of the host, the plume of CO_2 should be detectable by mosquitoes as a fluctuating concentration above ambient. Geier et al. (1998) demonstrated in a small wind tunnel that *A. aegypti* females readily flew upwind in turbulent and filamentous plumes of CO_2, but a homogeneous plume of CO_2 was far less likely to evoke upwind orientation. Conversely, plumes of human skin odor had the opposite effect, with homogeneous plumes being more apt to elicit upwind flights than turbulent and filamentous plumes. Dekker et al. (2001) found with the malaria mosquito, *A. gambiae s.s.*, that a turbulent flow of CO_2 improved orientation (measured in a large assay chamber with a choice between two entrance ports) when CO_2 was mixed with skin odor. A homogeneous flow of CO_2 mixed with skin odor, however, depressed the catch over skin odor alone. Although CO_2 alone presented as either a turbulent or homogeneous flow evoked less trap-entering behavior than clean air, CO_2 in either a turbulent or homogeneous flow improved orientation when flight nearby the port entrance was assayed via contact with an electric net. These comparisons establish that a turbulent flow of CO_2 is important to upwind orientation of malaria and yellow fever mosquitoes and suggest that the cast—filament encounter—upwind surge model that dictates moth flight to pheromone is applicable to mosquito response to CO_2 plumes.

Dekker et al. (2001) also assessed the responses of *A. aegypti* to the same combinations of CO_2 plumes and skin odor. In both species, homogeneous CO_2 plumes reduced trap entry compared to a clean air control, whereas homogeneous skin odor plumes increased trap entry over clean air. These studies suggest that processing of CO_2 and complex odor mixtures such as skin odor may have differing time requirements, with the latter requiring longer, continuous exposure.

Further wind tunnel studies with *A. aegypti* have shown that a single, transient encounter with a filament of CO_2 instantly lowers the threshold concentration evoking upwind flight to human skin odor (Dekker et al. 2005). This suggests that CO_2 alone in *A. aegypti* could be the long-

distance orientation cue, with body odors being utilized when the mosquito is close to a potential host. In highly anthropophilic species such as the yellow fever and malaria mosquitoes, females may enter or rest outside human dwellings before feeding. There they would be exposed to residual human odors. The encounter of a fluctuating concentration of CO_2, however, signifies the nearby presence of a potential host whose chemical signature can be verified by sensing the constituents of body odor.

Studies with two highly anthropophilic species point to a fluctuating concentration of CO_2 that induces upwind orientation (Geier et al. 1998; Dekker et al. 2001), with human body odor being most effective when its concentration remains relatively constant, as would be the case close to the host. In the yellow fever mosquito, a transient encounter with a filament of CO_2 induces a salience for skin odor, such that the concentration of skin odor needed to induce orientation is reduced (Dekker et al. 2005). Clearly, we need to know how the structure of odor plumes generally modulates host finding in additional species, especially non-anthropophilic species, with broad host ranges.

Aim-Then-Shoot Orientation of Diptera A second mechanism for orientation relies on mechanoreceptor sensing of wind direction before initiating flight and then heading that direction while in contact with the plume. This navigation system is termed "aim-then-shoot" (Kennedy 1986). Flight is presumably visually guided, as in optomotor anemotaxis, by maintaining a generally front-to-rear image flow of the ground pattern as long as contact with the odor plume is maintained. If the wind direction changes while the organism is in flight, then transverse image flow is generated, which if sensed would indicate an off windline heading. In-flight adjustments to course based on the detection of wind-induced drift are not assumed for the aim and shoot navigation. Orientation to hosts over a distance of many meters presumably consists of a series of short flights interspersed by landings when contact with host odor is lost, "as a series of steps" (Bursell 1987).

Evidence in support for an aim-then-shoot mechanism is, to date, limited to a few Diptera. Some tsetse flies (*Glossina*) upon detection of host odor take off heading directly upwind, quickly achieving an airspeed of ≈5 m s^{-1}, considered too fast to permit the flies to detect crosswind drift and, therefore, to use in-flight optomotor anemotaxis (Griffiths et al. 1995). By using released tsetse flies and monitoring their arrival at an upwind source of host odor, Griffiths et al. (1995) found that the "fast cohort" of released flies (about half of the flies) arrived at odor sources 30, 50, and 75 m upwind in less than 40 s! The rest of the flies took from 1 to >20 min, presumably by orienting in a series of short flights and landings. Wind-tunnel trials, however, also support the ability of tsetse flies to use conventional optomotor anemotaxis (e.g., Colvin et al. 1989), and so, it is possible that tsetse flies toggle between these two navigational mechanisms. The meteorological observations of Brady et al. (1989) in the tsetse's natural woodland habitat demonstrated that the direction of arrival of wind-borne host-odor would be a reliable guide to the actual direction toward a host 15 m away only about a third of the time; 20% of the time it would direct the fly at least 90° away from the host. How the tsetse fly uses information on wind direction, given its tenuous correlation with direction to the host, remains unclear. Finally, as the tsetse fly closely approaches a prospective host, visual cues presented by the host may become important for orientation. The final stage of orientation may be guided visually and, in fact, may be made from the upwind direction (Gibson and Torr 1999).

Cabbage root flies (Hawkes and Coaker 1979) and onion flies (Dindonis and Miller 1980) also seem to orient toward upwind host plant odors by the aim-then-shoot mechanism, but the use of this orientation strategy by other insect groups remains to be documented. As pointed out by Griffiths et al. (1995), the aim-then-shoot mechanism as used by tsetse seems relatively crude in comparison to the precision track of a male moth locating a female. However, it may serve in locating a large and mobile host by enabling sampling of a large area where the host is many meters away. When the host is in the range of tens of meters, it may be effective in rapid host finding. However, to date, the validity of the aim-then-shoot mechanism by tsetse remains largely an argument by exclusion—tsetse flight speed is too fast to permit in-flight processing of wind drift and, therefore, use optomotor navigation—rather than a mechanism supported by definitive experiments.

Future Work

One of the major impediments to understanding the precise relationship between in-flight orientation maneuvers and the pattern of encountering either odor filaments or more evenly dispersed odor patches is that we cannot readily establish how individual maneuvers correspond to the pattern of encountering patches of odor. We can measure the general, spatial features of odor plumes in wind tunnels (e.g., Justus et al. 2002a, with propylene as an odor) and in the field (Murlis and Jones 1981; Murlis et al. 2000, with negatively charged ions as a surrogate for odor), but it is difficult to establish how the patterns of odor contact in plumes with complex spatial structures modulate orientation, because we can only correlate the average patterns of odor distribution with behavioral output at the most general level of analysis, namely the overall flight track. In wind-

tunnel trials of moth flight along a pheromone plume, moths typically slow their rate of upwind progress and have a more narrow flight track as they approach the source (e.g., Justus et al. 2002b; Willis and Baker 1984; Willis and Arbas 1991). Which of the many features of plume structure (e.g., intermittency, peak concentration, peak-to-mean concentration, etc.) that change with distance to the source (Murlis et al. 1990; Justus et al. 2002a) and dictate these changes in flight track is unknown. By manipulating the rate of odor encounter and concentration, Vickers and Baker (1992) demonstrated that both of these variables affect the structure of *H. virescens* responses. Further studies are needed to determine to what extent other parameters of the odor plume affect the structure of plume-tracking behavior.

To record what a flying insect perceives as odor input while maneuvering along the plume would seem intractable. Vickers and Baker (1994), however, mounted a third antenna on the thorax of an *H. virescens* male and simultaneously recorded, via fine wires, its electroantennogram (EAG) responses to pheromone filaments encountered while it flew along the plume in a wind tunnel. Not surprisingly, bursts of pheromone were most often contacted near the plume's centerline; bursts confirmed the expected intermittency of the signal. The amplitude of flying EAGs, however, was unexpectedly higher than those recorded for a stationary EAG rig mounted at the same positions in the plume or one artificially moved upwind. Recent results from tethered flying *M. sexta* show that air flow, from head to tail, induced by the flapping wings should increase the speed of encounters with odor filaments by the antennae (Sane and Jacobson 2006). This is a possible explanation for the unexpectedly larger EAGs observed previously (Vickers et al. 2001). Unfortunately, no obvious relationship between EAG and moth behavior (other than pheromone present during plume tracking) was observed in this study, and no follow-up study has appeared. One important caveat for studies such as this is that the antenna recorded from was not providing the information that the moth was using to control its flight.

One useful technique for matching odor contact and behavioral reactions is the use of a long, thin ribbon plume in a laminar flow in a wind tunnel and to record the immediate reaction of an insect after it contacts the odor filament (e.g., Mafra-Neto and Cardé 1994; Dekker et al. 2005; Budick and Dickinson 2006). The plume's precise position relative to the insect can be determined by adding a visual tracer to the plume or by three-dimensional video recording. Such stretched-out, narrow plumes do not occur naturally, but an argument can be made that a single encounter with the ribbon plume corresponds to contact with a single odor filament in a turbulent plume. Another approach is to use tethered insects "flying" in a virtual world with movement simulated by flow of their visual surround (e.g., Gray et al. 2002). This system would enable presentation of precise patterns of odor impingement and measurement of resultant thrust and turning maneuvers. However, tethering may restrict maneuvering, and it has been interpreted to yield misleading conclusions about what inputs control counterturning (cf. Preiss and Kramer 1986; David and Kennedy 1987).

Simulation modeling with virtual insects in a virtual environment is another approach to understanding what kinds of sensory inputs from the plume can be extracted to navigate a course to its source. Models can specify the sensory inputs and navigational rules needed to create a zigzag track along a plume in a wind tunnel (Belanger and Arbas 1998), or the full spectrum of field behavior that include plume finding, plume following, maneuvers to re-contact a lost plume, and declaration that the source has been found (Li et al. 2001) in a simulated plume in a directionally fluctuating wind field (Farrell et al. 2002). These navigational strategies have been implemented and tested on an underwater robotic vehicle that has successfully searched for and located over a range of ≈1 km the source of a visual tracer that served as a surrogate for an odor (Li et al. 2006). Simulation models enable us to explore how varying sensory inputs and maneuvering rules modulate the effectiveness of the navigational strategy, but we should recognize that such simulations and robotic implementations can be successful without employing the same inputs and rules as the insects themselves utilize.

Field observations that meld meteorological measurements with flight track records offer insights into the distances over which odors are attractive and the kinds of flight tracks that occur under natural conditions. Such observations, so far, have been limited largely to day-active insects such as tsetse flies, gypsy moths, and oriental fruit moths that can be readily observed (however, see Vickers and Baker 1997), but they provide insights into the real-world capabilities of insects searching for distant odor sources that cannot be gleaned from wind tunnel studies.

Lastly, orientation to odor sources is a common reaction among many kinds of insects, but what we know of the mechanisms that govern this process is based on a handful of model systems. Such orientation is often simply termed "attraction," which as Kennedy (1978) pointed out, is a convenient but teleological term that fails to reveal the complexity of this process and the diversity of underlying mechanisms.

References

ATEMA, J. 1996. Eddy chemotaxis and odor landscapes: exploration of nature with animal sensors. *Biol. Bull.* 191:129–138.

AYLOR, D. E., PARLANGE, J.-Y., and GRANETT, J. 1976. Turbulent dispersion of disparlure in the forest and male gypsy moth response. *Environ. Entom.* 5:1026–1032.

BAKER, T. C. 1990. Upwind flight and casting flight: complementary and tonic systems used for location of sex pheromone sources by male moths, pp. 18–25, in K. B. Døving (ed.). Proceedings of the Tenth International Symposium on Olfaction and Taste. GCS A/S, Oslo, Norway.

BAKER, T. C., and HAYNES, K. F. 1987. Manoeuvres used by flying male oriental fruit moths to relocate a sex pheromone plume in an experimentally shifted wind-field. *Physiol. Entomol.* 12:263–279.

BAKER, T. C., and HAYNES, K. F. 1996. Pheromone-mediated optomotor anemotaxis and altitude control exhibited by male oriental fruit moths in the field. *Physiol. Entomol.* 21:20–32.

BAKER, T. C., and KUENEN, L. P. S. 1982. Pheromone source location by flying moths: a supplementary non-anemotactic mechanism. *Science* 216:424–427.

BAKER, T. C., and VICKERS, N. J. 1994. Behavioral reaction times of male moths to pheromone filaments and visual stimuli: determinants of flight track shape and direction, pp. 838–841, in K. Kurihura, N. Suzuki, and H. Ogawa (eds.). Olfaction and Taste IXSpringer, Tokyo, Japan.

BAKER, T. C., WILLIS, M. A., and PHELAN, P. L. 1984. Optomotor anemotaxis polarizes self-steered zigzagging in flying moths. *Physiol. Entomol.* 9:365–376.

BAKER, T. C., WILLIS, M. A., HAYNES, K. F., and PHELAN, P. L. 1985. A pulsed cloud of sex pheromone elicits upwind flight in male moths. *Physiol. Entomol.* 10:257–265.

BELANGER, J. H., and ARBAS, E. 1998. Behavioral strategies underlying pheromone-modulated flight in moths: lesions from simulation studies. *J. Comp. Physiol. A.* 183:345–360.

BELANGER, J. H., and WILLIS, M. A. 1996. Adaptive control of odor-guided locomotion: behavioral flexibility as an antidote to environmental unpredictability. *Adapt. Behav.* 4:217–253.

BOSSERT, W. H., and WILSON, E. O. 1963. The analysis of olfactory communication in animals. *J. Theor. Biol.* 5:443–469.

BRADY, J., GIBSON, G., and PACKER, M. J. 1989. Odour movement, wind direction, and the problem of host-finding by tsetse flies. *Physiol. Entomol.* 14:369–380.

BUDICK, S. A., and DICKINSON, M. H. 2006. Free-flight responses of *Drosophila melanogaster* to attractive odors. *J. Exp. Biol.* 209:3001–3017.

BURSELL, E. 1987. The effect of wind-borne odours on the direction of flight in tsetse flies. *Physiol. Entomol.* 12:149–156.

CARDÉ, R. T. 1984. Chemo-orientation in flying insects, pp. 111–124, in W. J. Bell, and R.T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall Ltd., London.

CARDÉ, R. T., and HAGAMAN, T. E. 1979. Behavioral responses of the gypsy moth in a wind tunnel to air-borne enantiomers of disparlure. *Environ. Entomol.* 8:475–484.

CARDÉ, R. T., and HAGAMAN, T. E. 1984. Mate location strategies of gypsy moths in dense populations. *J. Chem. Ecol.* 10:25–31.

CARDÉ, R. T., and KNOLS, B. G. J. 2000. Effects of light levels and plume structure on the orientation manoeuvres of male gypsy moths flying along pheromone plumes. *Physiol. Entomol.* 25:141–150.

CHARLTON, R. E., and CARDÉ, R. T. 1990. Orientation of male gypsy moths, *Lymantria dispar* (L.), to pheromone sources: the role of olfactory and visual cues. *J. Insect Behav.* 3:443–469.

CHARLTON, R. E., KANNO, H., COLLINS, R. D., and CARDÉ, R. T. 1993. Influence of pheromone concentration and ambient temperature on flight of the gypsy moth, *Lymantria dispar* (L.), in a sustained-flight wind tunnel. *Physiol. Entomol.* 18:349–362.

CLEMENTS, A. N. 1999. The Biology of Mosquitoes. vol. 2. Sensory Reception and Behaviour. CABI Publishing, Wallingford, Oxon, UK.

COLVIN, J., BRADY, J., and GIBSON, G. 1989. Visually-guided, upwind turning behaviour of free-flying tsetse flies in odour-laden wind: a wind-tunnel study. *Physiol. Entomol.* 14:31–39.

CONNER, W. E., and BEST, B. A. 1988. Biomechanics of release of sex pheromone in moths: effects of body posture on local airflow. *Physiol. Entomol.* 13:15–20.

CONOVER, M. R. 2007. Predator–Prey Dynamics. The Role of Olfaction. CRC Press, Boca Raton, FL.

COSSÉ, A. A., and BAKER, T. C. 1996. House flies and pig manure volatiles: Wind tunnel behavioral studies and electrophysiological evaluations. *J. Agric. Entomol.* 13:301–317.

COSTANTINI, C., BIRKETT, M. A., GIBSON, G., ZIESMANN, J., SAGNON, N' F., MOHAMMED, H. A., COLUZZI, M., and PICKETT, J. A. 2001. Electroantennogram and behavioural responses of the malaria vector *Anopheles gambiae* to human-specific sweat components. *Med. Vet. Entomol.* 15:259–266.

DAVID, C. T., and KENNEDY, J. S. 1987. The steering of zigzagging flight by male gypsy moths. *Naturwissenschaften* 74:194–196.

DAVID, C. T., KENNEDY, J. S., LUDLOW, A. R., PERRY, J. N., and WALL, C. 1982. A reappraisal of insect flight towards a distant point source of wind-borne odor. *J. Chem. Ecol.* 8:1207–1215.

DAVID, C. T., KENNEDY, J. S., and LUDLOW, A. R. 1983. Finding of a sex pheromone source by gypsy moths released in the field. *Nature* 303:804–806.

DAYKIN, P. N., KELLOGG, F. E., and WRIGHT, R. H. 1965. Host finding and repulsion of *Aedes aegypti*. *Can. Entomol.* 97:239–263.

DE JONG, R., and KNOLS, B. G. J. 1996. Selection of biting sites by mosquitoes, pp. 89–103, in G. R. Bock, and G. Cardew (eds.). Olfaction in Mosquito–Host Interactions, CIBA Found. Symp. 200Wiley, Chichester, UK.

DEKKER, T., TAKKEN, W., and CARDÉ, R. T. 2001. Structure of host-odour plumes influences catch of *Anopheles gambiae s.s.* and *Aedes aegypti* in a dual-choice olfactometer. *Physiol. Entomol.* 26:124–134.

DEKKER, T., GEIER, M., and CARDÉ, R. T. 2005. Carbon dioxide instantly sensitizes female yellow fever mosquitoes to human skin odors. *J. Exp. Biol.* 208:2963–2972.

DINDONIS, L. L., and MILLER, J. R. 1980. Host finding behavior of onion flies, *Hylemia antiqua*. *Environ. Entomol.* 9:769–772.

DUSENBERY, D. B. 1989. Optimal search direction for an animal flying or swimming in a wind or current. *J. Chem. Ecol.* 15:2511–2519.

DUSENBERY, D. B. 1990. Upwind searching for an odor plume is sometimes optimal. *J. Chem. Ecol.* 16:1971–1976.

ELKINTON, J. S., and CARDÉ, R. T. 1983. Appetitive flight behavior of male gypsy moths (Lepidoptera: Lymantriidae). *Environ. Entomol.* 12:1702–1707.

ELKINTON, J. S., and CARDÉ, R. T. 1984. Odor dispersion, pp. 73–91, in W. J. Bell, and R. T. Cardé (eds.). Chemical Ecology of Insects. Chapman and Hall, London.

ELKINTON, J. S., CARDÉ, R. T., and MASON, C. J. 1984. Evaluation of time-average dispersion models for estimating pheromone concentration in a deciduous forest. *J. Chem. Ecol.* 10:1081–1108.

ELKINTON, J. S., SCHAL, C., ONO, T., and CARDÉ, R. T. 1987. Pheromone puff trajectory and upwind flight of male gypsy moths in a forest. *Physiol. Entomol.* 12:399–406.

FARES, Y., SHARPE, P. J. H., and MAGNUSON, C. E. 1983. Pheromone dispersion in forests. *J. Theor. Biol.* 84:355–359.

FARKAS, S. R., and SHOREY, H. H. 1972. Chemical trail-following by flying insects: a mechanism for orientation to a distant odor source. *Science* 178:67–68.

FARRELL, J. A., MURLIS, J., LONG, X., LI, W., and CARDÉ, R. T. 2002. Filament-based atmospheric dispersion model to achieve short time-scale structure of odor plumes. *Environ. Fluid Mech.* 2:143–169.

GEIER, M., BOSCH, O. J., and BOECKH, J. 1998. Influence of host odour plume on upwind flight of mosquitoes towards hosts. *J. Exp. Biol.* 202:1939–1648.

GIBSON, G., and TORR, S. J. 1999. Visual and olfactory responses of haematophagous Diptera to host stimuli. *Med. Vet. Entomol.* 13:2–23.

GIBSON, G., PACKER, M. J., STEULLET, P., and BRADY, J. 1991. Orientation of tsetse flies to wind, within and outside host odour plumes in the field. *Physiol. Entomol.* 16:47–56.

GILLIES, M. T. 1980. The role of carbon dioxide in host-finding by mosquitoes (Diptera: Culcidae): a review. *Bull. Entomol. Res.* 80:525–532.

GILLIES, M. T., and WILKES, T. J. 1974. Evidence for downwind flight by host-seeking mosquitoes. *Nature* 252:388–389.

GILLIES, M. T., and WILKES, T. J. 1978. The effect of high fences on the dispersal of some West African mosquitoes (Diptera: Culicidae). *Bull. Entomol. Res.* 68:401–408.

GRANT, A. J., and O'CONNELL, R. J. 1996. Electrophysiological responses from receptor neurons in mosquito maxillary palp sensilla, pp. 233–253, in G.R. Bock, and G. Cardew (eds.). Olfaction in Mosquito–Host Interactions, CIBA Found. Symp. 200. Wiley, Chichester, UK.

GRAY, J. R., PAWLOWSKI, V., and WILLIS, M. A. 2002. A method for recording behavior and multineuronal CNS activity from tethered insects flying in virtual space. *J. Neurosci. Methods* 120:211–223.

GREENFIELD, M. D. 1981. Moth sex pheromones: an evolutionary perspective. *Fla. Entomol.* 64:4–17.

GRIFFITHS, N., PAYNTER, Q., and BRADY, J. 1995. Rates of progress up odour plumes by tsetse flies: a mark-release video study of the timing of odour source location by *Glossina pallidipes*. *Physiol. Entomol.* 20:100–108.

HANGARTNER, W. 1967. Spezifität und Inaktivierung des Spurpheromons von *Lasius fuliginosus* Latr. und Orientierung der Arbeiterinnen im Duftfeld. *Zeit. Vergl. Physiol.* 57:103–136.

HARDIE, J., GIBSON, G., and WYATT, T. D. 2001. Insect behaviours associated with resource finding, pp. 87–109, in I. P. Woiwod, D. R. Reynolds, and C. D. Thomas (eds.). Insect Movement: Mechanisms and Consequences. CAB International, Wallingford, Oxon, UK.

HAWKES, C., and CROAKER, T. H. 1979. Factors affecting the behavioural responses of the adult cabbage root fly, *Delia brassicae*, to host plant odour. *Entomol. Exp. Appl.* 25:45–58.

JUDD, G. J. R., and BORDEN, J. H. 1988. Long-range host-finding behaviour of the onion fly *Delia antiqua* (Diptera: Anthomyiidae): ecological and physiological constraints.. *J. Appl. Ecol.* 25:829–845.

JUDD, G. J. R., and BORDEN, J. H. 1989. Distant olfacotory response of the onion fly, *Delia antiqua*, to host-plant odour in the field. *Physiol. Entomol.* 14:429–441.

JUSTUS, K. A., and CARDÉ, R. T. 2002. Flight behaviour of males of two moths, *Cadra cautella* and *Pectinophora gossypiella*, in homogenous clouds of pheromone. *Physiol. Entomol.* 27:67–75.

JUSTUS, K. A., MURLIS, J., JONES, C., and CARDÉ, R. T. 2002a. Measurement of odor-plume structure in a wind tunnel using a photoionization detector and a tracer gas. *Environ. Fluid Mech.* 2:115–142.

JUSTUS, K. A., SCHOFIELD, S. W., MURLIS, J., and CARDÉ, R. T. 2002b. Flight behaviour of *Cadra cautella* males in rapidly pulsed pheromone plumes. *Physiol. Entomol.* 27:58–66.

KAAE, R. S., and SHOREY, H. H. 1973. Sex pheromones of Lepidoptera. 44. Influence of environmental conditions on pheromone communication and mating in *Pectinophora gossypiella*. *Environ. Entomol.* 2:1081–1084.

KAISER, L., WILLIS, M. A., and CARDÉ, R. T. 1994. Flight manoeuvers used by a parasitic wasp to locate host-infested plant. *Entomol. Exp. Appl.* 70:285–294.

KELLOGG, F. E., FRIZEL, D. E., and WRIGHT, R. H. 1962. The olfactory guidance of flying insects. IV. *Drosophila*. *Can Entomol.* 94:884–888.

KENNEDY, J. S. 1939. The visual responses of flying mosquitoes. *Proc. Zool. Soc. London* 109:221–242.

KENNEDY, J. S. 1978. The concepts of olfactory 'arrestment' and 'attraction.'. *Physiol. Entomol.* 3:91–98.

KENNEDY, J. S. 1983. Zigzagging and casting as a programmed response to wind-borne odour: a review. *Physiol. Entomol.* 8:109–120.

KENNEDY, J. S. 1986. Some current issues in orientation to odour sources, pp. 11–25, in T. L. Payne, M. C. Birch, and C. J. E. Kennedy (eds.). Mechanisms in Insect OlfactionOxford University Press, Oxford, England.

KENNEDY, J. S., and MARSH, D. 1974. Pheromone-regulated anemotaxis in flying moths. *Science* 184:999–1001.

KENNEDY, J. S., LUDLOW, A. R., and SAUNDERS, C. J. 1981. Guidance of flying male moths by wind-borne sex pheromone. *Physiol. Entomol*. 6:395–412.

KUENEN, L. P. S., and BAKER, T. C. 1982. A non-anemotactic mechanism used in pheromone source location by flying moths. *Physiol. Entomol.* 7:277–289.

KUENEN, L. P. S., and CARDÉ, R. T. 1994. Strategies for recontacting a lost pheromone plume: casting and upwind flight in the male gypsy moth. *Physiol. Entomol.* 19:15–29.

LI, W., FARRELL, J. A., and CARDÉ, R. T. 2001. Tracking of fluid-advected odor plumes: strategies inspired by insect orientation to pheromone. *Adapt. Behav.* 9:143–167.

LI, W., FARRELL, J. A., PANG, S., and ARRIETA, R. M. 2006. Moth-inspired chemical plume tracing on an autonomous underwater vehicle. *IEEE Trans. Robotics* 22:292–307.

LOUDON, C. 2003. The biomechanical design of an insect antenna as an odor capture device, pp. 609–630, in G. J. Blomquist, and R. G. Vogt (eds.). Insect Pheromone Biochemistry and Molecular BiologyElsevier Academic, Amsterdam, The Netherlands.

MAFRA-NETO, A., and CARDÉ, R. T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MAFRA-NETO, A., and CARDÉ, R. T. 1995. Influence of plume structure and pheromone concentration on upwind flight of *Cadra cautella* males. *Physiol. Entomol.* 20:117–133.

MAFRA-NETO, A., and CARDÉ, R. T. 1996. Dissection of the pheromone-modulated flight of moths using single-pulse response as a template. *Experientia* 52:373–379.

MARSH, D., KENNEDY, J. S., and LUDLOW, A. R. 1978. An analysis of anemotactic zigzagging flight in male moths stimulated by pheromone. *Physiol. Entomol.* 3:221–240.

MECHABER, W. L., CAPALDO, C. T., and HILDEBRAND, J. G. 2002. Behavioral responses of adult female tobacco hornworms, *Manduca sexta*, to hostplant volatiles change with age and mating status. 8 pp. *J. Insect Sci.* 2.5. Available online: insectscience.org/2.5.

MIKSAD, R. W., and KITTREDGE, J. 1979. Pheromone aerial dispersal: a filament model. *14th Conf. Agric. For. Met., Am. Met. Soc.* pp. 236–243.

MURLIS, J., and JONES, C. D. 1981. Fine-scale structure of odour plumes in relation to distant pheromone and other attractant sources. *Physiol. Entomol.* 6:71–86.

MURLIS, J., BETTANY, B. W., KELLEY, J., and MARTIN, L. 1982. The analysis of flight paths of male Egyptian cotton leafworm moths, *Spodoptora littoralis*, to a sex pheromone source in the field. *Physiol. Entomol.* 7:435–441.

MURLIS, J., WILLIS, M. A., and CARDÉ, R. T. 1990. Odour signals: patterns in time and space, pp. 6–17, in K. Døving (ed.). Proceedings of the Tenth International Symposium on Olfaction and Taste. GCS A/S, Oslo, Norway.

MURLIS, J., ELKINTON, J. S., and CARDÉ, R. T. 1992. Odor plumes and how insects use them. *Annu. Rev. Entomol.* 37:505–532.

MURLIS, J., WILLIS, M. A., and CARDÉ, R. T. 2000. Spatial and temporal structures of pheromone plumes in fields and forests. *Physiol. Entomol.* 25:211–222.

PREISS, R., and KRAMER, E. 1986. Mechanism of pheromone orientation in flying moths. *Naturwissenschaften* 73:555–557.

SABELIS, M. W., and SCHIPPER, P. 1984. Variable wind directions and anemotactic strategies of searching for an odour plume. *Oecologia* 63:225–228.

SANE, S. P., and JACSOBSON, N. P. 2006. Induced airflow in flying insects II. Measurement of induced flow. *J. Exp. Biol.* 209:43–56.

SCHAL, C. 1982. Intraspecific vertical stratification as a mate finding mechanism in tropical cockroaches. *Science* 215:1405–1407.

SUTTON, O. G. 1953. Micrometerology. McGraw-Hill, New York.

TAKKEN, W., and KNOLS, B. G. J. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol*. 44:131–157.

TAKKEN, W., DEKKER, T., and WIJNHOLDS, Y. G. 1997. Odor-mediated flight behavior of *Anopheles gambiae* Giles *sensu stricto* and *An. stephensi* Liston in response to CO_2, acetone, and 1-octen-3-ol (Diptera: Culicidae). *J. Insect Behav.* 3:395–407.

VICKERS, N. J. 2000. Mechanisms of animal navigation in odor plumes. *Biol. Bull.* 198:203–212.

VICKERS, N. J. 2006. Winging it: moth flight behavior and responses of olfactory neurons are shaped by pheromone plume dynamics. *Chem. Senses* 31:155–166.

VICKERS, N. J., and BAKER, T. C. 1992. Male *Heliothis virescens* maintain upwind flight in response to experimentally pulsed filaments of their sex pheromone (Lepidoptera: Noctuidae). *J. Insect Behav.* 5:669–687.

VICKERS, N. J., and BAKER, T. C. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Nat. Acad. Sci. U. S. A.* 91:5756–5760.

VICKERS, N. J., and BAKER, T. C. 1996. Latencies of behavioral response to interception of filaments of sex pheromone and clean air influence flight track shape in *Heliothis virescens* (F.) males. *J. Comp. Physiol. A.* 178:831–847.

VICKERS, N. J., and BAKER, T. C. 1997. Flight of *Heliothis virescens* males in the field in response to sex pheromone. *Physiol. Entomol.* 22:277–285.

VICKERS, N. J., CHRISTENSEN, T. A., BAKER, T. C., and HILDEBRAND, J. G. 2001. Odour-plume dynamics influence the brain's olfactory code. *Nature* 410:466–470.

VON KEYSERLINGK, H. 1984. Close range orientation of flying Lepidoptera to pheromone sources in a laboratory wind tunnel and the field. *Meded. Fac. Landbouwwet. Rijksuniv. Gent* 49:683–689.

WEHNER, R. 1996. Middle scale navigation: the insect case. *J. Exp. Biol.* 199:125–127.

WEISSBURG, M. 2000. The fluid dynamical context of chemosensory behavior. *Biol. Bull.* 198:188–202.

WILLIS, M. A. 2008. Odor plumes and animal orientation, in A. Basbaum et al. (eds). Volume 4, Olfaction & Taste: In, The Senses: A Comprehensive References, Elsevier, Oxford (in press).

WILLIS, M. A., and ARBAS, E. A. 1991. Odor-modulated upwind flight of the sphinx moth, *Manduca sexta*. *J. Comp. Physiol. A.* 178:699–706.

WILLIS, M. A., and AVONDET, J. L. 2005. Odor-modulated orientation in walking male cockroaches, *Periplaneta americana* (L.), and the effects of odor plumes of different structures. *J. Exp. Biol.* 208:721–735.

WILLIS, M. A., and BAKER, T. C. 1984. Effects of intermittent and continuous pheromone stimulation on the flight behaviour of the oriental fruit moth, *Grapholita molesta*. *Physiol. Entomol.* 9:341–354.

WILLIS, M. A., and BAKER, T. C. 1987. Comparison of maneuvers used by walking versus flying *Grapholita molesta* males during pheromone-mediated upwind movement. *J. Insect Physiol.* 33:875–883.

WILLIS, M. A., and CARDÉ, R. T. 1990. Pheromone-modulated optomotor response in male gypsy moths, *Lymantria dispar* L.: upwind flight in a pheromone plume in different wind speeds. *J. Comp. Physiol. A* 167:699–706.

WILLIS, M. A., MURLIS, J., and CARDÉ, R. T. 1991. Pheromone-mediated upwind flight of male gypsy moths, *Lymantria dispar*, in a forest. *Physiol. Entomol.* 16:507–521.

WILLIS, M. A., DAVID, C. T., MURLIS, J., and CARDÉ, R. T. 1994. Effects of pheromone plume structure and visual stimuli on the pheromone-modulated upwind flight of male gypsy moths (*Lymantria dispar*) in a forest (Lepidoptera: Lymantriidae). *J. Insect Behav.* 7:385–409.

WOLF, H., and WEHNER, R. 2006. Desert ants compensate for navigation uncertainty. *J. Exp. Biol.* 208:4223–4230.

WRIGHT, R. H. 1958. The olfactory guidance of flying insects. *Can. Entomol.* 90:81–89.

WYATT, T. D., PHILLIPS, A. D. G., and GRÉGOIRE, J. C. 1993. Turbulence, trees and semiochemicals: wind-tunnel orientation of the predator *Rhizophagus grandis*, to its barkbeetle prey, *Dendroctonus micans*. *Physiol. Entomol.* 18:204–210.

ZANEN, P. O., SABELIS, M. W., BUONACCORSI, J. P., and CARDÉ, R. T. 1994. Search strategies of fruit flies in steady and shifting winds in the absence of food odours. *Physiol. Entomol.* 19:335–341.

ZÖLLNER, G. E., TORR, S. J., AMMANN, C., and MEIXNER, F. X. 2004. Dispersion of carbon dioxide plumes in African woodland: implication for host finding by tsetse flies. *Physiol. Entomol.* 29:381–394.

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REVIEW ARTICLE

The use of Odors at Different Spatial Scales: Comparing Birds with Fish

Jennifer L. DeBose · Gabrielle A. Nevitt

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Abstract Salmon travel hundreds of kilometers of open ocean and meandering rivers to return to their natal stream to spawn; procellariiform seabirds soar over thousands of kilometers of the ocean's surface searching for foraging opportunities and accurately return to their nesting islands. These large-scale olfactory-guided behaviors are among the most dramatic examples of animal navigation ever described. At much closer ranges, the sense of smell can be used for behaviors as diverse as tracking prey, nest location, and mate selection. Both fish and birds face similar problems interpreting olfactory information in fluid mediums where odors are dispersed as filamentous patches. Similar to insects, which have served as model organisms for investigating olfactory related behaviors, the few fish and bird species that have been studied tend to use olfactory information in conjunction with other sensory modalities. Similar to insects, fish and birds also employ oscillatory or cross-stream movement as sampling mechanisms. This review compares and contrasts the use of odors by fish and birds over a range of spatial scales that span from thousands of kilometers to less than a meter. In so doing, we identify behavioral similarities and new questions that need to be addressed regarding the olfactory ecology of these diverse groups of organisms.

Keywords Odor tracking · Fish · Birds · Odor plume · Pigeon · Procellariiform · Vulture · Salmon · Lamprey · Eel

J. L. DeBose · G. A. Nevitt (✉)
Department of Neurobiology, Physiology and Behavior,
University of California,
One Shields Ave.,
Davis, CA 95616, USA
e-mail: ganevitt@ucdavis.edu

J. L. DeBose · G. A. Nevitt
Bodega Marine Laboratory,
2099 Westside Rd,
Bodega Bay, CA 94923, USA

Present address:
J. L. DeBose
Flower Garden Banks National Marine Sanctuary (NOAA),
4700 Ave. U, Bldg 216,
Galveston, TX 77551, USA

Introduction

Both fish and birds use olfactory information over a range of spatial scales. At large spatial scales on the order of hundreds of kilometers, olfactory-guided behaviors are among the most dramatic examples of animal navigation ever described. For instance, salmon require olfaction to relocate their natal streams, and pigeons released in unfamiliar territory are able to set a course for their home loft by using information derived from odors at the release site. At much closer ranges, the sense of smell can be used for behaviors as diverse as foraging and mate selection. Although one group swims and the other flies, both fish and birds face similar problems interpreting olfactory information in natural environments. Both groups have evolved to operate in three-dimensional space, in fluid mediums that share similar characteristics relevant to odor transport. Water and air both move in currents, and these currents distribute odors such that plumes have a filamentous, discontinuous structure (Zimmer-Faust et al. 1995; Finelli et al. 1999). As these odor filaments move away from a source, they can be widely dispersed depending on the turbulence of their environment (Zimmer-Faust et al. 1995; Moore et al. 2000); the higher the turbulence, the more irregular and patchier the distribution of odor filaments.

How organisms decipher reliable information from such discontinuity has consequently been a topic of much interest.

Not surprisingly, many advances in this field have been informed by previous work with insects, which have served as model organisms for investigating olfactory-related behaviors in freely flying organisms (see Willis 2005; Cardé and Willis 2008). As with insects, the few fish and bird species that have been studied tend to use olfactory information in conjunction with input from other sensory modalities. While insects combine odor cues with visual flow to direct upwind flight, a variety of environmental cues provide directional information to fish and birds, and these cues can vary, depending on the spatial scale of the problem. In many fish species, olfactory information is interpreted with respect to input from the lateral line system, whereas in birds (pigeons, for example), olfactory, visual, and geomagnetic information are likely used together in different ways, depending on whether the bird is homing from familiar territory near to its loft or from unfamiliar territory hundreds of kilometers away. Like insects, many species of fish and birds use up-current or up-wind movement coupled with zigzagging or casting movements to focus search activity to the source of a plume (Fig. 1), but given that most of these species cannot be studied easily in laboratory conditions, the dynamics of odor tracking are not as well characterized as they are in moths and other insects. Unlike insects, where tracking behavior can be induced by the controlled release of well-defined, species-specific pheromone blends, in most fish and bird species, potential odor cues tend to be poorly defined or characterized, and information about their distribution, production, and decay in the natural environment is typically lacking. This mini-review will compare and contrast the use of odors by fish and birds over a range of spatial scales, spanning from thousands of kilometers to less than a meter, to better define similarities in the use of scent and identify new questions that need to be addressed.

Olfactory-Mediated Behaviors in Fish

Fish use their sense of smell for behaviors that are performed over both large and small spatial scales, the dimensionality of which is relative to the species in question. For example, many species of salmon (*Oncorhynchus* spp.) use olfactory cues to home over hundreds of kilometers to a natal river (reviewed by Dittman and Quinn 1996), whereas black rockfish (*Sebastes inermis*) experimentally displaced as little as 4 km also require olfaction to return to their original,

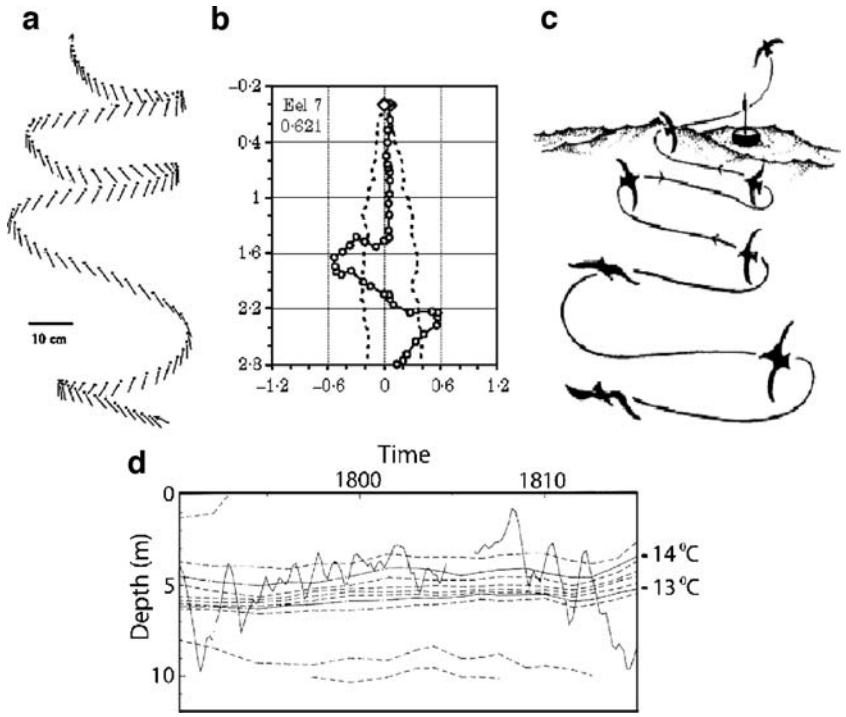

Fig. 1 A comparison of olfactory-search behaviors from moths, fish, and birds. **a** A moth exhibits cross-wind casting behavior (adapted from Belanger and Willis 1996). **b** A freshwater eel shows similar counter-turning behavior within the boundaries of an odor plume (adapted from Carton and Montgomery 2003). **c** Procellariiform seabirds show counter-turning behavior in response to prey odors (courtesy of Hutchison and Wenzel 1980). **d** Atlantic salmon show vertical oscillations across water layers of different temperatures (courtesy of Døving et al. 1985)

familiar home range (Mitamura et al. 2005). How fish use olfactory information to locate a goal over such large distances is not yet well understood despite considerable research effort. By contrast, olfactory cues mediate other types of behaviors at much closer ranges. For example, single amino acids introduced into a flow tank almost instantaneously increase foraging-related activity (e.g., surfacing and jumping) in lake charr (*Salvelinus namaycush*; Hara 2006), whereas specific alarm pheromones elicit antipredator behaviors in fathead minnows (*Pimephalus promelas*) even in the absence of a visual cue (Hartman and Abrahams 2000). At close range, odors can also aid some species in identifying potential mates. For example, living in turbid environments where visibility is limited, Lake Malawi cichlids (*Pseudotropheus emmiltos*), depend on olfaction to identify conspecifics (Plenderleith et al. 2005).

Although considerable research effort has focused on investigating large-scale, olfactory-guided migrations, with particular attention paid to commercially important fish species, the most detailed investigations of olfactory tracking to date have been conducted in laboratory or flume settings. These studies have focused on behaviors that can be elicited by olfactory cues that operate at close range. The emphasis on flume studies is, in part, due to logistic considerations of tracking animals in the field, yet no fish species has been studied in as much detail as moths at any spatial scale (see Cardé and Willis 2008). A consideration of data across insect and fish species suggests that olfactory tracking is highly adapted to and constrained by characteristics of odor dispersal linked to flow (Belanger and Willis 1996; Vickers 2000). Not surprisingly, many fish species have well-developed peripheral olfactory systems (Caprio 1988), including elaborate sniffing apparatuses (Nevitt 1991), and olfactory detection thresholds can be quite low. For instance, sea lamprey (*Petromyzon marinus*) can detect larval bile acids at 10^{-10} M (Bjerselius et al. 2000), and sablefish (*Anoplopoma fimbria*) can detect dilute squid extract made up of free amino acid concentrations between 10^{-13} and 10^{-17} M (Davis et al. 2006). In the following sections, we review recent advances that have been made in our understanding of how fish use olfactory information under natural conditions over relatively large and small spatial scales.

Large Spatial Scales

Olfactory-guided behaviors that occur over large spatial scales (which we will define as on the order of tens to hundreds of kilometers for this discussion) have been studied primarily in the context of homing (Hasler et al. 1978; Dittman and Quinn 1996; Ueda et al. 1998; Døving and Stabell 2003; Mitamura et al. 2005; Nordeng and Bratland 2006; Keefer et al. 2006). In fisheries studies, homing typically refers to the directed movement of displaced fish back to an original location or home range. A number of these investigations have used tracking methods to study how fish move through the water as they home, and certain trends emerge. For example, when navigating over these large distances to locate a goal, various species have been shown to move vertically up and down in the water column (Fig. 1d; Døving et al. 1985; Døving and Stabell 2003). These behaviors have suggested to researchers that, in order to home, fish need to sample the water column for olfactory cues released from a particular habitat or odor source even when they are kilometers or even tens of kilometers from that source. Other studies have attempted to identify these 'habitat' cues and suggest that they are produced, in part, by conspecifics or by specific populations of fishes that reside within the given habitat. For example, sea lamprey (*Petromyzon marinus*) have been shown to home to specific rivers that carry scented compounds produced by larval sea lamprey (Bjerselius et al. 2000; Vrieze and Sorensen 2001; Sorensen et al. 2003).

Many fish species also navigate across the expanse of oceans, lakes, and rivers, at times without obvious visual reference points. The mechanisms by which fish determine direction in these often visually homogeneous environments are not yet well understood. While it commonly has been assumed that odors mediate rheotaxis, more integrative hypotheses have been proposed to explain how fish track odors up-current without a fixed point of reference. These questions have been studied in salmonids, freshwater eels, and sea lamprey, which we will review in more detail in the following sections.

Salmonids Salmon (e.g., *Salmo* spp., *Oncorhynchus* spp.) are anadromous fishes. They start and end their lives in freshwater but typically spend most of their sub-adult lives foraging at sea. During sensitive periods of development linked to surges in plasma thyroid hormone, juveniles imprint to the scent of their natal stream (e.g., Dittman et al. 1996; reviewed by Dittman and Quinn 1996; Lema and Nevitt 2004; Nevitt and Dittman 2004). After undergoing a physiological metamorphosis (parr–smolt transformation) that prepares them for life in seawater, juvenile fish outmigrate to the ocean. Toward the end of their life cycle, depending on the species, salmon navigate over hundreds of kilometers to return to spawn in their natal stream. It is not known how fish return to the appropriate river system; however, numerous studies have shown that olfaction is essential in the freshwater homing migration (Hasler et al. 1978; Dittman et al. 1996; Courtenay et al. 1997; Nordeng and Bratland 2006).

Westerberg (1982) and Døving's laboratory in Norway (Døving et al. 1985; Døving and Stabell 2003) have

provided some of the most intriguing results to date to suggest how salmon track odors at large spatial scales. In Norway, Atlantic salmon (*Salmo salar*) must navigate fjords before entering the river system on the homeward migration. The fjord portion of the migration, thus, provides an excellent opportunity to track salmon before they reach their home river, as fish can be followed more easily in the confines of fjords than in the open ocean. Moreover, in fjords, as in the ocean, there are micro-structured, horizontal layers in the water column, and these layers vary in temperature, thickness, and other parameters (Westerberg 1982). By using passive sonic tracking methods to follow individually tagged fish, Døving et al. (1985) have convincingly demonstrated that salmon oscillate vertically through these layers, which the authors speculate to have distinct origins (Westerberg 1982; Døving and Stabell 2003). According to this idea, as the fish moves up and down in the water column, it comes into contact with layers of water that originate from different sources.

Westerberg (1982) reasoned that these layers provide different chemical information to fish that is presented in conjunction with directional information via the thermal fluctuations that result from current shear. In support of this idea, Døving et al. (1985) found that unmanipulated (olfactory-intact) salmon limited their oscillatory swimming behavior to specific temperature layers of the water column, typically between the surface and 30 m in depth, whereas anosmic salmon did not restrict their swimming behavior and continued to oscillate between the surface and 80 m in depth. Furthermore, Døving and co-authors showed that olfactory-intact salmon made brief, vertical dives (Fig. 1d). They hypothesized that the purpose of these explorations was to sample the fine-structure of different water layers for both olfactory cues and directional cues provided by thermal shear or other input (i.e., infrasound; see Sand and Karlsen 2000).

In conjunction with these studies, Johnsen (1982) proposed a mechanism to explain how salmon locate their home streams within the river system. He suggested that familiar home-stream waters trigger positive rheotaxis and zigzag swimming behavior, whereas the absence of home-stream odor triggers either downstream drift or negative rheotaxis. In support of this hypothesis, Nordeng and Bratland (2006) found that juvenile salmonids displaced to neighboring river systems, which each emptied into the Salangen Fjord approximately 10 km apart, returned to their natal rivers with high precision. They reasoned that these fish homed by swimming up-current in response to odorants of their own population and down-current in the absence of population odors. Døving and Stabell (2003) have since postulated that salmon will exhibit positive or negative rheotaxis in response to a set of chemical signals, depending on the physiological state of the fish. According to this idea, fish use the same chemical cues for foraging as they do to find the direction of their natal river when in a migratory state.

Salmon homing can, thus, be explained as a two-step process. As salmon enter the fjords, they sample odors by swimming in a pattern of vertical oscillations through the water column. This behavior occurs in conjunction with a combination of positive and negative rheotaxis in response to specific odors. Olfactory-mediated rheotaxis then persists as individuals enter the river system and find their way home to spawn.

Freshwater Eels Freshwater eels (*Anguilla* spp.) have a catadromous life history in that they are spawned in saltwater, migrate as juveniles to freshwater, then out-migrate to spawn in the Sargasso Sea. Yellow-phase American eels (*Anguilla rostrata*) initially migrate into the river system. During most of this stage of their life history, they reside as brackish or freshwater bottom-dwellers, moving between estuaries and river systems where they stay until they sexually mature. Similar to salmon, American eels undergo a physiological metamorphosis before out-migration that prepares them for life in seawater. These silver-phase eels migrate from freshwater and estuary habitats back to the Sargasso Sea. Both yellow and silver-phase eels use selective tidal stream transport, or STST, to travel into and out of the estuary during the appropriate migratory phase of their life cycle (Barbin 1998; Barbin et al. 1998). By using STST, eels can travel horizontally over tens of kilometers (between 12 and 32 km in these studies) with energetic efficiency, so long as they are able to choose the appropriate tide. Barbin et al. (1998) found that this ability depended on the eel having an intact sense of smell, although other sensory modalities also contributed to this behavior. Similar to the situation in fjords, in estuaries, differences in temperature and salinity lead to layering of water masses with a defined density boundary, or pycnocline. The depth of the pycnocline varies depending on hydrographic conditions but, in this study, ranged from 0.7 to 10.7 meters, with the bottom depth ranging from approximately 5 to 18 m (Barbin 1998). Barbin (1998) showed that olfactory-intact yellow-phase eels ascended to the surface and descended to the pycnocline with equal frequency as they used STST; ascents and descents were defined as a change in depth of greater than 3 m (Barbin 1998). In contrast, anosmic eels moved sporadically throughout the water column, rarely reaching the depth of the pycnocline, and ascended to the surface more frequently than olfactory-intact eels.

The results of Barbin et al. (1998) showed that anosmic, silver-phase (out-migrating) eels were less able to use STST and failed to choose the correct tidal currents. Only two of the eight anosmic silver-phase eels migrated out of the estuary within 7 days of release, and the other six had not left after 9 days (Barbin et al. 1998). In contrast, olfactory-

intact silver-phase eels timed their vertical movements with the tidal cycle and were able to move out of the estuary (over 32 km) in 4 days, on average, which is only slightly longer than the time it takes for water to move through the estuary on ebb tides (three nights on average). Behavioral differences between anosmic and olfactory-intact eels were similar to what Døving et al. (1985) observed with Atlantic salmon in fjords.

Sea Lamprey Like salmon, sea lamprey (*Petromyzon marinus*) are anadromous fish that begin life in the river as suspension feeding larvae and then make their way to lakes and oceans as fish parasites. Years later, they use olfactory cues that guide them back to an appropriate river to spawn. In contrast to salmon, this river is not necessarily their natal river. Instead, adult sea lamprey use pheromones released by larval lamprey to find suitable spawning streams (Bjerselius et al. 2000; Sorensen et al. 2003). A major advance that facilitates working with this system is that the pheromone blends have been characterized (Vrieze and Sorenson 2001). Consequently, the dynamics of odor search can be explored with respect to a known cue that can be identified as present or absent in natural water systems.

With this goal in mind, Sorensen et al. (2003) have studied movement patterns of sea lamprey in the process of searching for appropriate spawning rivers. As part of this study, the researchers tracked both anosmic and sham-treated lamprey in the Great Lakes by using acoustic telemetry (see Vrieze and Sorensen 2001; Sorensen et al. 2003). Similar to what Døving et al. (1985) reported for salmon, they found that lamprey actively swam on constant bearings while vertically migrating or oscillating through the water column of the lake. Once a lamprey encountered a river plume, it would begin circling before swimming upstream into the river. Though movement patterns were not reported for anosmic lamprey, stream capture rates revealed that only 10% of anosmic lamprey located streams, whereas nearly half of the sham-treated lamprey succeeded (see Vrieze and Sorensen 2001). Laboratory trials that used two-choice mazes have also shown that lamprey prefer waters from rivers and streams where larval pheromones are present (see Bjerselius et al. 2000; Vrieze and Sorensen 2001; Sorensen et al. 2003). Taken together, results from both sea lamprey and eels provide more general support for Døving's and Westerberg's hypotheses originally proposed in salmon, suggesting that vertical oscillations aid fish in determining the direction of an odor source when navigating over distances of kilometers.

Small Spatial Scales

By far, the majority of investigations have been carried out at much smaller spatial scales (typically less than 10 m from the odor source). Most studies suggest that fish are capable of tracking odor plumes to the source within this more-limited range and that this behavior requires other sensory modalities, including the lateral line system. The use of olfaction in tandem with the lateral line system to direct movement up-current contrasts with insects that tend to use optical feedback to progress upwind to an odor source (Willis 2005). Our understanding of small-scale olfactory-mediated behavior comes from a combination of field manipulations and flume experiments, which we review below.

Field Experiments Relatively little work has been conducted under natural, field conditions. However, the research that has been done indicates that current olfactory information is needed for fish to locate odor sources. For example, Montgomery et al. (1999) examined olfactory search tracks in Antarctic fish (*Trematomus bernacchii*), which are adapted to forage in low light conditions under Antarctic ice sheets. Working in the field, they made video observations of benthic Antarctic fish tracking an experimental odor plume from 5 m to the source. The odor plume was created by passing a solution of minced fish through a hose. By using computer simulations, they determined what search strategies fish used to find the source of this odor plume. The matching of field observations to computer simulations suggested that fish could not locate the source unless a combination of chemosensory and current information was provided.

Elliott et al. (1995) conducted an elegant test of olfactory-guided host-selection in anemonefish (*Amphiprion* spp.). Anemonefish lay their eggs next to the trunk of their host anemone where the adults tend to them until they hatch into pelagic larvae. Most species of anemonefish are obligate symbionts to a species-specific host anemone. This means that the pelagic larvae must settle and re-locate a host anemone after their pelagic life-stage concludes. Elliott et al. (1995) found that when they released juvenile anemonefishes greater than 1 m down-current from a host anemone, they would swim in zigzag patterns or counterturns in the direction of the anemone, turning less frequently as they got closer. If the anemonefish were instead released within 1 m down-current of its anemone, it would swim in a straight path toward it. If an anemonefish were released either perpendicular or up-current with respect to the anemone, it frequently was unable to locate its host. Interestingly, in these situations, the fish swam either laterally or drifted until it was down-current of the anemone. It would then descend to the substrate and swim up-current until it reached its host. At distances of half a meter or more, anemonefish were not visually attracted to their host anemones and required down-current chemical cues to locate them. These experiments went on to illustrate how currents effectively increase the

range of detection by fishes. With no current, anemonefishes' ability to locate their host anemones was severely reduced. At current velocities greater than 2.5 cm s^{-1}, fish were attracted to host anemones from up to 8 m down-current. From these and other experiments, Elliott et al. (1995) suggested that anemonefish used "decision rules" to locate a host anemone (i.e., if downstream from a host, swim directly upstream staying within 1 to 5 cm from the substrate, or if the odor plume is lost, turn and swim laterally across and down-current in a zigzag fashion until the stimulus is encountered again).

Another elegant field system has been developed to study foraging behavior of cod (*Gadus morhua*). Løkkeborg and Fernö (1999) tagged cod with acoustic transmitters and tracked them while the fish searched for a baited line, again in the fjords of Norway. Their tracks ran for several hundred meters, with an average of 262 m between the baited line and tagged fish. Cod found the bait line through chemically mediated rheotaxis (Løkkeborg 1998). Cod increased their swimming speed when heading up-current, and these faster swimming fish also tended to succeed in locating the baited line. The faster the fish were swimming, the higher the probability that they would encounter the odor plume and head up-current to the source. As fish also found the bait much more quickly during the day, the authors concluded that visual cues were probably used in conjunction with olfaction and rheotaxis to mediate bait search.

Thus far, the picture presented from field studies is that fish use olfaction in combination with other cues, that include rheotactic and visual cues, to locate odor sources in close proximity. Next, we will review results from flume studies that have examined olfactory search mechanisms with greater precision.

Laboratory Flume Experiments Blind cave fish (*Astyanax fasciatus*) are an ideal system for studying problems related to multimodal search in the absence of vision. These fish evolved in dark environments where they have become adapted to forage by using information from water movements and chemical cues. Baker and Montgomery (1999) found that both odor and current stimuli are required for blind cave fish to locate an odor source (for similar results in nocturnal banded kokopu *Galaxias fasciatus*, see Baker et al. 2002). If an appropriate odor stimulus was presented, fish oriented or swam upstream when current velocities were as low as 0.4 cm s^{-1}. The rheotactic threshold increased to 3 cm s^{-1} if olfactory cues were absent. In no-current conditions, fish tended to position themselves randomly throughout the tank and showed little attraction to the odor source. Because they used an experimental tank with a water inflow that was physically separated from an odor injection site, they could also determine how current and odor worked together to trigger olfactory search. Interestingly, they found that, when an odor stimulus was paired with a unidirectional current, fish tended to cluster around the current inflow and not the odor injection site. This experiment showed that blind cave fish need a combination of olfactory and rheosensory input to locate an odor source.

Flume work with other species has provided evidence for horizontal casting behavior during olfactory search. For example, Carton and Montgomery (2003) looked at the behavioral reactions of freshwater eels (*Anguilla australis* and *Anguilla dieffenbachii*) in response to food odors in a semi-natural, turbulent flow raceway (5 m in length). They found evidence for both odor-mediated rheotaxis and cross-stream casting in response to experimental odor injections (Fig. 1b). At distances greater than 0.9 m from the odor source, eels swam up-current. Eels seemed to detect and track the edges of the odor plumes, as they initiated cross-stream, horizontal casting behaviors one second after moving beyond the lateral margins of the plume. These casting movements were slower than direct, up-current movements, and eels spent more time casting and searching closer to the odor source than farther away.

In summary, these flume experiments support field observations in suggesting that fish typically use a combination of olfaction and rheotaxis to locate the source of an odor plume. Although subtle differences appear in each of the studies described here, common themes are to use cross-stream casting behaviors to search for the odor source or to stay within the plume and to head up-current when the plume is detected. We now review how fish locate odor sources in no-flow and variable flow situations.

No-Flow or Variable-Flow Situations Although all of the studies we have discussed so far have concluded that currents are required for fishes to locate an odor source, species probably differ in their ability to locate odor sources depending on their specific adaptations. For example, working with brown bullhead catfish (*Ameiurus nebulosus*), Sherman and Moore (2001) showed that these fish were always successful in locating an odor source under no-flow conditions, whereas success rate diminished when current was present.

There also is evidence for area-restricted search (ARS) in response to odors in non-uniform flow situations. Bonnethead sharks (*Sphyrna tiburo*) showed behavioral responses to chemical stimuli in experimental circular tanks (1.8-m-diameter pools; Johnsen and Teeter 1985). When these fish were presented with blue crab homogenate in still water, they began turning in tight circles near the stimulus site, even in the absence of a distinct visual cue. Johnsen and Teeter (1985) speculated that this searching behavior functioned to keep the shark near its initial point of contact

with the stimulus. In contrast, when the same experiment was done with the addition of a current, sharks would reverse direction when they contacted the cue. Sharks would then swim in loops that extended progressively down-current, giving the impression that they were tracking the stimulus within the flow structure of the tank. How bonnethead sharks use odors to mediate ARS in natural situations requires further study.

Sniffing Fish are also able to sample odors through active sniffing—a behavior that resembles a cough in some species (Nevitt 1991). During a sniffing event, a spontaneous jaw protrusion increases water flow to accessory olfactory sacs. Nevitt (1991) recorded pressure fluctuations in the nasal sacs of flounders during spontaneous coughing and respiration. These experiments demonstrated that ventilation over the olfactory epithelium was mechanically linked to respiration. In controlled behavioral studies, coughing behavior was elicited as flounders sampled a defined odor plume within a flow tank. From these and other investigations, Nevitt concluded that coughing was analogous to "sniffing" in air-breathing organisms. Coughing in fishes has since been used as a measure of olfactory responsiveness in laboratory situations (e.g., Nevitt 1991; Murphy et al. 2001; Belanger et al. 2006). Ventilation rates in round gobies have been shown to correspond to voltage changes in electro-olfactograms recorded at the olfactory epithelium. Murphy et al. (2001) suggested that these changes were due to odor-induced ventilation. Further support for this connection between olfaction and ventilation rate was provided through olfactory deprivation studies in gobies (Belanger et al. 2006). They found that rendering fish anosmic, either through copper sulfate treatment or nasal occlusion with dental impression material, inhibited their reaction to pheromones as measured by gill ventilation rate. These results suggest that the increase in ventilation is another mechanism by which fish sample the aquatic environment for pertinent olfactory cues, especially in those species that are benthic or less active.

Olfactory-Mediated Behaviors in Birds

Questions related to the use of olfaction in birds have been hampered by the erroneous yet common assumption that most birds are anosmic. Consequently, compared to fish, little is known about how birds are able to track odors in natural situations (for review, see Roper 1999). Unlike insects that sense odors through olfactory receptor neurons housed on complex filamentous structures, the olfactory systems of birds are enclosed and, like fish, accessed typically through paired naral openings. In some groups such as the procellariiform seabirds, these naral openings are sometimes modified into a single, tube-like structure at the top of the bill. By contrast, in turkey vultures and ducks, naral openings laterally traverse the bill, thus allowing continuous airflow between them. From a comparative perspective, the sense of smell in birds continues to be relatively overlooked (for exceptions, see Bang 1960, 1971; Bang and Wenzel 1985; Wenzel 1987; reviewed by Roper 1999), but nearly every species that has been investigated behaviorally for olfactory abilities has been shown to have them, from highly specialized species such as ground dwelling Kiwis (Wenzel 1960) and Kokapo (Hagelin 2004) to even the most pedestrian—starlings, chickens, and ducks (reviewed by Roper 1999; Van Buskirk and Nevitt 2007). Birds may present the next advance in understanding how olfactory information is used across an enormous range of spatial scales. In this paper, we review three of the best-described systems: homing pigeons, procellariiforms, and turkey vultures.

Homing Pigeons

One of the most intensely studied problems in animal navigation is the homing ability of the domesticated rock pigeon (*Columba livia*), and nearly all olfactory work in birds has focused on this problem. Pigeons released hundreds of kilometers from their home loft will fly home in a goal-oriented way, even when released from completely unfamiliar territory (for reviews, see Papi 1990; Able 1991, 1995; Walcott 1996). This behavior requires multimodal integration of an array of environmental cues, conceptualized in the navigation literature as the 'compass' (an orientation mechanism) and 'map' (a spatial coordinate system) senses (Kramer 1952). With respect to the compass, it is now generally accepted that pigeons use redundant, more-or-less condition-dependent mechanisms for orientation. For example, displaced birds orient by using either a sun compass or earth-strength magnetic field depending on whether or not the sun is visible (for review, see Walcott 1996; Wiltschko and Wiltschko 1998). With respect to the map, the physical substrate continues to be hotly debated with researchers arguing for both magnetic and olfactory input (see Wallraff 2000; Wiltschko and Wiltschko 2000). This discussion focuses on the olfactory map, but we refer readers to numerous, more comprehensive treatments of the field (e.g., Able 1991, 1995; Roper 1999; Alerstam 2006; Papi 2006).

The idea behind the olfactory map is that pigeons learn to associate windborne olfactory information with compass direction before they leave the loft. Papi was the first to suggest that pigeons form what was later termed a 'mosaic' map of the olfactory environment that surrounds the loft, which is then extended during exploratory flights

(e.g., Papi et al. 1971; reviewed by Papi 1990). In an effort to explain the ability of pigeons to home from distant (greater than 100 km), unfamiliar sites, Wallraff further proposed that odors form large-scale, two-dimensional gradient maps (e.g., Wallraff 1989; reviewed by Wallraff 2004, 2005). According to this model, scented winds provide directional information that the bird learns before leaving the loft area. From this information, the bird can extrapolate relative positional information at an unfamiliar release site by comparing spatial variation in relative odor concentrations between the release site and the loft. In search of a physical substrate, the model assumes spatially explicit, monotonic gradients in odor profiles that run along a bi-coordinate axis surrounding the loft and extending to distant sites.

Elements of these two models are summarized as follows: (1) Pigeons are exposed to a radial spectrum of wind-borne odor blends at or in the vicinity of the loft; (2) they learn to associate the directions of the scented winds with general compass headings (either by using the sun compass or the magnetic compass); (3) upon release, pigeons sample the air and make a mental comparison of the ambient odor blend (or ratio, according to Wallraff) with the blend at the release site; and (4) they then use this information to set a course for home. According to Papi, this would be achieved by following a simple set of learned rules (i.e., in response to odor blend X at the release site, go in the opposite direction from which odor blend X was experienced at the loft). According to Wallraff, a pigeon would deduce a course home at the release site by comparing the ratio of odors at the release site with the memory of the ratio at the loft and extrapolating relative positional information to set a course for home.

In support of each of these theories, a variety of carefully conducted research documents that pigeons require an intact sense of smell to home and that homing does not involve simply tracking an odor or odor blend to its source (reviewed by Able 1995; Papi 1990; see also Gagliardo et al. 2001a). Similar to studies with salmon, rendering birds anosmic (either by olfactory nerve transection, chemical removal of cilia, or by application of a local anesthetic) interferes with homing (for example, see Bingman and Benvenuti 1996; Bingman et al. 1998). Homing ability is also impacted by changes in rearing environment in that pigeons must have access to windborne odors at or in the vicinity of the loft to later be able to home from unfamiliar areas (for example, see Gagliardo et al. 2001b). Among numerous studies, Papi has demonstrated that olfactory homing is seasonally and geographically variable, and limited to distances of 500 km in some locations, and that range can be extended if birds are allowed access to the odor environment during transport (reviewed by Papi 1990). As with salmon, a major stumbling block has been to identify and characterize the odor cues involved, which is, in the case of pigeons, the biogenic substrate for the putative map (for a more complete discussion on this topic, see Roper 1999).

To better define an odor map, Wallraff's model suggests a spatially explicit gridwork of odor gradients, which offers predictive value in estimating location relative to the loft. To pin this gridwork to a genuine, physical substrate, Wallraff and Andreae (2000) collected air samples over three consecutive years from 96 rural sites distributed within a 400-km-diameter circle around an experimental pigeon loft near Wurzburg, Germany. They then used gas-chromatography to measure a suite of largely anthropogenic hydrocarbons distributed over the landscape and analyzed their spatial distribution as a proxy for the spatial distribution of natural odor blends that pigeons might use to home. Their data show rising and falling profiles in the proportions of volatile hydrocarbons in the spatial domain that were fairly consistent from year to year and resilient to changing wind conditions (Fig. 2). They went on to document a systematic, directional relationship between wind direction at the loft and relative amounts of aromatics correlated to positional direction.

While this experiment was clearly monumental, potential limitations that the authors pointed out are that, with the exception of isoprene (Fig. 2), the study focused on aromatics that were not natural, biogenic compounds but rather long-lived, anthropogenic pollutants, and there was no demonstration that birds could smell these or other, more appropriate compounds at the concentrations measured. By necessity, air was collected along roads and much closer to

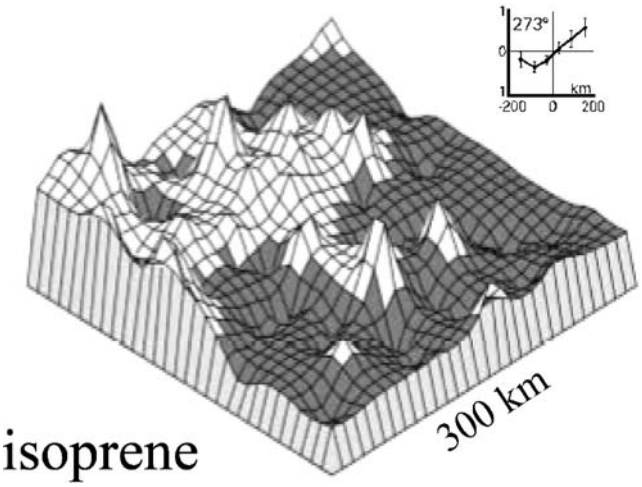

Fig. 2 Isoprene concentrations expressed as a proportion of six hydrocarbons (adapted from Wallraff and Andreae 2000). Data were collected from 96 rural locations in a 200-km radius around a pigeon loft in Wurzburg, Germany. Complete landscapes were computed by interpolation. Areas with above-average values have lighter shading, while areas with below-average values have darker shading (for further details, see Wallraff and Andreae 2000)

the substrate (4.5 m) than a pigeon normally flies. The physical association between point sources (potential emission sites such as roads, factories, or mining operations) and the ratios measured could not be considered in the analysis. Finally, samples were not taken synchronously, and sampling duration and volume (48 l of air over 2 h), as well as subsequent analysis, averaged out fine-scale structure (odor filaments), the unit the bird is likely to be sampling (Wallraff and Andreae 2000; reviewed by Wallraff 2005).

However, somewhat analogously to the work of Døving et al. (1985) with salmon, this approach is among the first to attempt a description of the spatial complexity in the odor landscape through which pigeons navigate. Further work is needed to determine flux rates of volatile substances produced by that landscape and to make clear-cut associations between what can be measured and what can be smelled. While the compounds analyzed were fairly stable, the properties of biogenic odorants are likely to vary with respect to lifetime and decay time. Thus, it will be critical to show that biogenic odor blends that are produced in a given area retain some spatial homogeneity and that pigeons can link that homogeneity to a compass direction back at the loft via experience with windborne delivery. In an alternative scenario, depending on the dynamics of fine-scale mixing and odor transport, a pigeon might get an occasional whiff of source concentration produced some distance away and still learn to associate those occurrences with compass direction. In this hypothetical example, intermittency of the pigeons' encounter rate with source concentration might also encode distance from the loft. The possibility that the fine-scale, filamentous nature of odor transport could add a different dimension to the problem ought to be considered.

The monotonic gradient model provides a starting point for humans who tend to lack an intuitive framework (and vocabulary) to describe and understand how olfactory features are related over large spatial scales on the order of hundreds of kilometers (we are reminded of the 'aroma of Tacoma', a paper mill city south of Seattle). However, this framework currently imposes unrealistic constraints on the spatial distribution of odors. Wallraff's relief maps showing standardized ratios of hydrocarbons reinforce this idea—they are as complex as mountain ranges, depending on which way an axis is drawn. However, this is not a shortcoming and only illustrates that the problem presents challenges comparable to those presented by other sensory features. For example, characteristics of visual landscapes that seem intuitively obvious to humans can be difficult to parameterize in the context of pigeon homing (e.g., Lau et al. 2006). Thus, a major strength of this study (Wallraff and Andreae 2000) is that it illustrates that identifiable, relatively stable features occur in the chemical realm, opening up new avenues for inquiry into an age-old problem.

Procellariiforms

Like pigeons, procellariiforms present an attractive model for investigating olfactory navigation over large distances. This Order, commonly referred to as the tube-nosed seabirds, is represented by the petrels, albatrosses, and shearwaters, which are in many ways like fishes of the air. These birds live highly pelagic lifestyles, coming to land only for a few months each year or every other year to breed and rear a single offspring (for comprehensive review of procellariiforms, see Warham 1990, 1996). These birds generally mate for life and routinely forage over hundreds and thousands of square kilometers of open ocean in search of patchily distributed prey. Not surprisingly, they have among the largest olfactory bulbs of any bird and, depending on the species, rely more or less upon their unusually highly developed sense of smell to find their prey. Procellariiforms use odors to forage over vast distances of open ocean, and much research has focused on understanding the mechanisms different species use to find prey (reviewed in Nevitt and Bonadonna 2005a). We have shown, for example, that procellariiforms can use olfactory cues at both large (hundreds to thousands of square kilometers in this case) and small (less than a hundred square kilometers) spatial scales for foraging. Our current understanding is that, at large spatial scales, birds use odors to identify productive areas for foraging, whereas at smaller spatial scales, they use odors in combination with other cues to track prey directly (reviewed by Nevitt 2000; Nevitt 2008).

Large Spatial Scales Results from extensive behavioral testing performed mostly at sea have led to classifications of olfactory responsiveness to prey-related odors among different species (reviewed by Nevitt 2008). These differences in behavior have provided us with a better understanding of the various ways different species use scent, both in terms of long-distance navigation and tracking prey. Toward this end, we have overcome a major stumbling block that confronts researchers working in the area of pigeon navigation by identifying a measurable, biogenic, scented compound that contributes to stable and predictable landscape features on the ocean surface. Dimethyl sulfide (DMS) is a breakdown product of dimethylsulfoniopropionate (DMSP), which is, in turn, a metabolite produced by various species of phytoplankton (most notably, *Phaeocystis* in the sub-Antarctic). Phytoplankton tend to aggregate where upwelling occurs, and, during senescence or consumption by phytoplankton grazers

(e.g., krill, fish, and squid), DMSP and DMS are released. DMS is not very soluble in seawater, and so it is quickly emitted into the atmosphere where it also can serve as a local, small-scale foraging cue for seabirds (Nevitt et al. 1995; see also Nevitt et al. 2004). Biogenic DMS has been shown to be a significant source of biogenic sulfur on a planetary scale and plays a key role in global climate regulation, so considerable research has been devoted to mapping regions noted for DMS production and flux (Kettle et al. 1999). The elucidation of such areas suggested to us that predictable odor features are superimposed upon the ocean, thus offering seabirds olfactory landmarks to identify useful foraging regions such as shelf breaks, sea mounts and upwelling zones (reviewed by Nevitt 1999; 2000). We coined the term "olfactory landscape" to describe this idea (Nevitt et al. 1995). We have since shown through physiological, behavioral, and observational tracking studies that procellariiforms respond to DMS at biogenic concentrations and will orient to it in non-foraging contexts (for example, Nevitt et al. 1995; Nevitt and Haberman 2003; Nevitt and Bonadonna 2005b).

With respect to olfactory navigation and foraging over large (hundreds and thousands of square kilometers) spatial scales, the current hypothesis driving our work is that birds build a map of their familiar surroundings through experience, first with odors in the nest, and then with odors associated with foraging experience at sea (Cunningham 2005). We have recently shown, for example, that Antarctic prion (*Pachyptila desolata*) chicks will orient to DMS and other prey-related odors at biogenic concentrations (10^{-12} M) even before fledging, and currently, we are testing the idea that this is a learned behavior through contact with parents in the nest (Bonadonna et al. 2006; Nevitt et al., unpublished). As chicks leave the nest to forage for the first time without parental assistance, we reason that knowledge of scents that are typically associated with prey prepares them to recognize productive foraging grounds (Cunningham 2005; Cunningham et al. 2006; Bonadonna et al. 2006). Once birds fledge, we hypothesize that they build up an association of potential foraging locations with scented compounds through experience, including interactions with con- and hetero-specifics (Silverman et al. 2004). Since birds routinely forage over expansive distances, this hypothesis suggests that, over time, the foraging landscape that may span thousands of square kilometers for some species becomes predictable through familiarity, despite it seeming so large from a human perspective. Because ocean features that are not strictly tied to the bathymetry (physical structure of the ocean floor) can be spatially more variable, we speculate that odor cues associated with productivity allow birds an effective means of recognizing ocean features that may vary spatially with season or weather conditions (for a more complete review, see Nevitt 2008).

Small Spatial Scales Many species of procellariiforms perform characteristic zigzag upwind search in response to odor cues. Researchers commonly have reported that procellariiforms are attracted to experimental deployments of prey-related scents from distances of several hundred meters to kilometers (for example, Grubb 1972; Hutchison and Wenzel 1980; Nevitt and Haberman 2003; Nevitt et al. 2008). Results from several studies suggest that olfactory tracking is more typical of burrow-nesting rather than surface-nesting species (Nevitt et al. 1995, 2004; Nevitt 1999) and that this behavior may be linked to differences in developmental environments in the nest (Van Buskirk and Nevitt 2008; reviewed by Nevitt 2008). However, surface-nesting procellariiforms also have large olfactory bulbs, and new evidence suggests that hunting by smell is a principal foraging strategy for at least one albatross species. This study involved examining the tracks of freely ranging wandering albatross (*Diomedea exulans*) for evidence of olfactory search (Nevitt et al. 2008). Tracks were analyzed from 19 birds equipped with miniature global positioning systems (GPS) and stomach temperature recorders (Weimerskrich et al. 2007). This combination of devices provided high-precision location data (GPS, 10-s sampling rate; Weimerskirch et al. 2002) in combination with data on the size and location of prey ingestion (Wilson et al. 1995).

On a typical foraging trip, wandering albatrosses fly in vast, looped paths, which often cover thousands of square kilometers of open ocean. Wandering albatrosses are known to forage primarily on squid, often in the form of carrion floating on the surface of the water (Croxall and Prince 1994). This suggested to us that scent might be an important cue for these birds to locate prey opportunistically. We predicted that if olfaction were used in prey capture, then wandering albatross should show a tendency to fly crosswind, since this would optimize the likelihood of encountering an odor plume and that we should see evidence for upwind, zigzag turning before prey capture (Dusenbery 1992). By contrast, if birds were using visual search, then we should see evidence of them bee-lining to prey items, irrespective of wind direction. To avoid bias, tracks were first analyzed blind with respect to wind direction in a radius of 10 km from the touchdown point. We calculated this distance to be beyond the visual range at which a bird was likely to be able to easily see a prey item or a conspecific on the water.

The analysis confirmed that wandering albatrosses tend to fly cross-wind, a behavior that had previously been shown and considered primarily as an adaptation for energetic efficiency (Weimerskirch et al. 2000; see discussion in Nevitt et al. 2008; Fig. 3). We found that initial

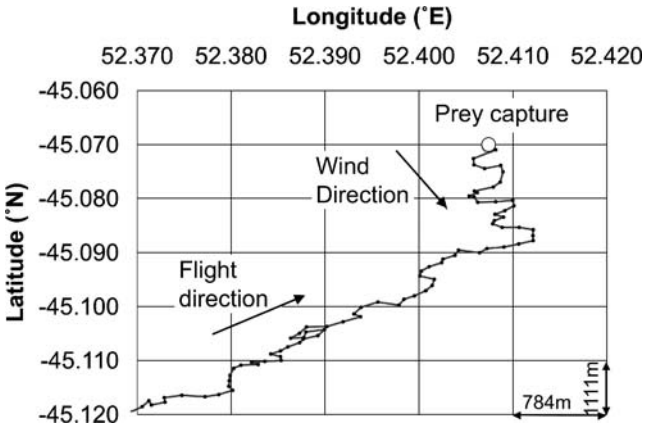

Fig. 3 GPS track of a wandering albatross equipped with a stomach temperature recorder to monitor feeding events. This track illustrates crosswind flight followed by zigzag upwind flight preceding a feeding event (*open circle*). *Arrows* refer to flight and wind direction as indicated. The horizontal and vertical distances represented by the *smallest grid rectangle* are shown in the *lower right corner*

olfactory detection was implicated in nearly half (46.8%) of all flown approaches that preceded prey capture events, thus accounting for 45.5% of the total prey mass captured by in-flight foraging. We further showed that zigzag approaches were initiated, on average, approximately 2.5 km (2,401±346 m) downwind of the prey capture site, with maximum detection distances observed at 5 km (5,002 m). This result suggested to us that, as a wandering albatross flies along a foraging transit, the area of ocean it surveys is extended, on average, approximately 2.5 km upwind via olfaction. However, when we examined tracks beyond the 10-km spatial limit of the original analysis, we occasionally observed upwind tracks that originated as far as 20 km from the point of prey capture, suggesting that prey detection may be possible from further distances. Finally, it should be noted that, while this discussion focuses on olfactory search in flight, wandering albatrosses also use sit-and-wait strategies to forage (e.g., Weimerskirch et al. 2007). Odors may be useful in this context for identifying productive areas where prey are likely to surface (see Nevitt 2000).

Apart from foraging, olfaction is used for a variety of other behaviors that operate at more localized (within 1 km) spatial scales. Within this range, several species of burrow-nesting petrels have been shown to require a sense of smell to relocate their nest, and individual-specific odors are likely to be involved in the context of nest-site recognition (reviewed by Nevitt and Bonadonna 2005b; see also Bonadonna and Nevitt 2004). When birds are displaced from their burrows at night, they typically sweep their heads from side to side in a manner consistent with olfactory search. This behavior can be elicited in chicks as well as in adults. For example, O'Dwyer et al. (2008) report that when Leach's storm petrel (*Oceanodroma leucorhoa*) chicks are presented with a choice between nest material from their own burrow and nest material from a neighboring burrow, they will sweep their heads in broad arcs around their body. This behavior is accompanied by rapid biting movements and coughing, with the bill placed close to the substrate (O'Dwyer et al. 2008; see also Cunningham et al. 2003). In simple wind tunnel experiments, we have shown in other species (thin-billed prions *Pachyptila belcheri* and blue petrels *Halobaena caerulea*) that chicks increase their turning rate in response to prey-related odors, suggesting that simple wind tunnels may provide a useful means for investigating olfactory tracking in more precocial species (e.g., Cunningham et al. 2006). We are clearly only scratching the surface of the questions that can be addressed in this fascinating order of birds.

Turkey Vultures

Turkey vultures (*Cathartes aura*) are one of the few terrestrial birds that are commonly accepted as having a remarkable sense of smell. Like procellariiforms, turkey vultures have among the largest olfactory bulbs of any bird (Bang 1960; Bang and Cobb 1968). These birds forage exclusively by scavenging for viscera and muscle tissues of dead animals, and humans have co-opted their abilities for practical purposes. In the late 1930s, for example, the Union Oil Company of California introduced ethyl mercaptan (CH_3CH_2SH), a sulfur-based compound associated with animal decay, into 40-mile gas lines so that they could use the presence of turkey vultures as an easy means to locate leaks in the lines in the remote backcountry (reviewed by Stager 1964). Similarly, black vulture (*Coragyps atratus*), a species noted for its visual rather than olfactory abilities, has been reported to use turkey vultures to find carcasses (Buckley 1997).

Although research exploring the use of olfaction by turkey vultures has been debated by scholars as notable as Audubon and Darwin, it was K. E. Stager who carried out the most comprehensive studies on this subject (Stager 1964). Working with vulture populations mainly in California and Mexico, he designed several different foraging challenges, which he carefully controlled for visual cues. These tests involved presenting birds with open containers of ethyl mercaptan or covered baits. He found that turkey vultures tended to be attracted to odor cues whether or not a visual stimulus was also present but not to visual cues (mounted specimens) in the absence of odor cues.

Pertinent to olfactory tracking, Stager (1964) also noted the flight patterns of turkey vultures in response to carcasses and ethyl mercaptan in the absence of visual cues. He reports that vultures approached odor sources from downwind and that they tended to circle the source at elevations estimated by eye to be within 100 m off the

ground. If a visual cue (in this case, an actual carcass) was also present, birds eventually tended to approach it and sometimes landed; in the absence of a visual cue, they typically circled for about 20 min and then left the area. To Stager, these observations suggested that turkey vultures located a potential prey item, first, by using olfaction, whereas visual cues allowed them to deduce its exact location.

In an effort to better understand the characteristics of the odor stimuli that are attractive to turkey vultures, other studies have suggested that the age of the carcass comes into play. Although humans often associate turkey vultures with rotting flesh, results from controlled studies suggests that they have a much more discriminating palate. For example, Owere and Northington (1961) showed that captive vultures consistently preferred freshly killed chicks over decaying food. In field studies, Houston (1986) confirmed that 1-day-old carcasses attracted more vultures than either fresh or 4-day-old carcasses, whereas putrid carcasses were ignored. Thus, for turkey vultures, olfaction may be important not only in locating potential prey but in assessing the age of the carcass.

Given the technical challenges involved, much less is known about the detailed behavioral mechanisms turkey vultures use to locate odor sources, in part because soaring altitudes typically exceed 50 m (see Estrella 1994). Unfortunately, one of the only published attempts to explain how they might accomplish this feat used a Gaussian plume model and concluded that turkey vultures should be unable to detect prey by smell above an altitude of 17 cm (Smith and Paselk 1986). The short-coming of this approach is that a Gaussian dispersion model estimates average concentration for a given distance (altitude in this case) and is thus not well suited to the biological problem. The actual situation is much more complex and requires different predictive tools (for example, large eddy simulation, or LES, Deardoff 1972). In the real atmosphere, odors from a carcass will be lifted as discrete patches that present pockets of high concentration that vultures should be able to detect. Moreover, turkey vultures typically soar on sequences of updrafts generated by thermals and ridges. Since these thermal updrafts also provide a means of vertical transport of odor patches, a more detailed examination of their use of thermals in relation to odor tracking coupled to a more rigorous treatment of atmospheric boundary layer modeling may be a starting point. We are currently exploring this problem.

Summary and Future Directions

Olfactory-mediated behaviors operate over a wide range of spatial dimensions for both fish and birds. By organizing our discussion into spatially explicit terms, specific themes emerge. In this paper, we summarize consistent findings among these seemingly disparate groups and suggest future directions to explore.

At large spatial scales, odor search reflects physical features of the fluid medium. As we have described, fish as diverse as salmon, eels, and lamprey all show patterns of vertical, oscillatory swimming as they travel through estuaries and fjords. In marine and brackish systems, water tends to be stratified by temperature or salinity gradients, and a number of studies suggest that fish actively sample different water masses as they swim through these layers. The situation is much less clear in birds, where researchers do not have as detailed an understanding of how odors might be distributed in the environment with respect to a physical substrate. Proposed mechanisms include the use of large-scale gradients for navigating distances of hundreds of kilometers. However, in some systems (procellariiforms), the physical evidence points to odors occurring as landscape features associated with sources of odor production. Following the example of Døving and others, future work with birds should be directed toward individual tracking with respect to such features.

Equally important to this problem will be developing more realistic models of atmospheric transport of odors at the spatial scales pertinent to olfactory sampling. Unlike fishes, the bird species that have been looked at do not generally fly in vertical oscillations (with the exception of the Kerguelen petrel *Lugensa brevirostris*), suggesting that birds do not need to sample air masses in the same way that fish are presumed to sample water layers (e.g., salmon and freshwater eels). This likely reflects the different stabilities of thermal or density layers in water as compared to air due to the greater degree of turbulent mixing in the atmospheric boundary layer. However, analogous to fish sampling water layers in fjords, birds that soar on thermal updrafts (e.g., vultures) may have the opportunity to sample thermals (i.e., vertical stratifications) for their odor content.

Another trend we see is that both fish and birds use olfaction in combination with other sensory cues, and attempts to study behaviors with respect to one cue in isolation are problematic. In fish, olfactory-mediated tracking has been shown convincingly to work in combination with both the lateral line and visual systems in a number of species (e.g., Baker and Montgomery 1999; Baker et al. 2002; Carton and Montgomery 2003), and recent evidence suggests involvement of the otolith system as well (Sand and Karlsen 2000; see Døving and Stabell 2003). In birds, olfaction may be used in combination with visual cues or geomagnetic information, depending on the species and the spatial scale of the behavior. The take-home message is that, in natural situations, olfaction works in combination with a range of other sensory modalities. An

understanding of how organisms interpret multimodal cues should take precedence over focusing on any one modality.

Finally, the distribution of natural cues and how these odorants disperse in turbulent environments remain largely unknown (see Zimmer-Faust et al. 1995; Moore and Crimaldi 2004), and yet the few systems where cues have been identified have led to significant advances in our understanding of how animals make sense of olfactory information in natural contexts (e.g., Nevitt et al. 1995; Carton and Montgomery 2003; Sorensen et al. 2003). Since the challenges will require understanding how cues are produced, released, and decay over time and space, progress will depend on researchers being able to think across disciplines and draw on expertise from various fields, including marine and atmospheric chemistry. Already, considerable information is available about the production and distribution of compounds not normally associated with olfaction. For example, DMSP, which is typically studied in the context of sulfur cycling in the ocean, recently has been identified as a potentially important foraging cue for fishes over coral reefs (DeBose and Nevitt 2007; DeBose et al. 2008). Gaining insight into how animals glean information from naturally occurring, biogenic odorants at appropriate biological concentrations remains a top priority for olfactory ecology.

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References

ABLE, K. P. 1991. Common themes and variations in animal orientation systems. *Am. Zool* 31:157–167.

ABLE, K. P. 1995. Orientation and navigation: a perspective on fifty years of research. *Condor* 97:592–604.

ALERSTAM, T. 2006. Conflicting evidence about long-distance animal navigation. *Science* 313:791–794.

BAKER, C. F., and MONTGOMERY, J. C. 1999. The sensory basis of rheotaxis in the blind Mexican cave fish, *Astyanax fasciatus*. *J. Comp. Physiol. A* 184:519–527.

BAKER, C. F., MONTGOMERY, J. C., and DENNIS, T. E. 2002. The sensory basis of olfactory search behavior in banded kokopu (*Galaxias fasciatus*). *J. Comp. Physiol. A* 188:553–560.

BANG, B. G. 1960. Anatomical evidence for olfactory function in some species of birds. *Nature* 188:547–549.

BANG, B. G. 1971. Functional anatomy of the olfactory system in 23 orders of birds. *Acta Anat* 58:Suppl.1–76.

BANG, B. G., and COBB, S. 1968. The size of the olfactory bulb in 108 species of birds. *Auk* 85:55–61.

BANG, B. G., and WENZEL, B. M. 1985. Nasal cavity and olfactory systems, pp. 195–223, in J. S. King, and J. McClelland (eds.). Form and Function in Birds, vol. 3 Academic, New York.

BARBIN, G. P. 1998. The role of olfaction in homing and estuarine migratory behavior of yellow-phase American eels. *Can. J. Fish. Aquat. Sci* 55:564–575.

BARBIN, G. P., PARKER, S. J., and MCCLEAVE, J. D. 1998. Olfactory cues play a critical role in the estuarine migration of silver-phase American eels. *Environ. Biol. Fish* 53:283–291.

BELANGER, J. H., and WILLIS, M. A. 1996. Adaptive control of odor-guided locomotion: behavioral flexibility as an antidote to environmental unpredictability. *Adapt. Behav* 4:217–253.

BELANGER, R. M., CORKUM, L. D., LI, W., and ZIELINSKI, B. S. 2006. Olfactory sensory input increases gill ventilation in male round gobies (*Neogobius melanostomus*) during exposure to steroids. *Comp. Biochem. Phys. A* 144:196–202.

BINGMAN, V. P., and BENVENUTI, S. 1996. Olfaction and the homing ability of pigeons in the southeastern United States. *J. Exp. Zool* 275:186–192.

BINGMAN, V. P., ALYAN, S., and BENVENUTI, S. 1998. The importance of atmospheric odours for the homing performance of pigeons in the Sonoran Desert of the southwestern United States. *J. Exp. Biol* 201:755–760.

BJERSELIUS, R., LI, W., TEETER, J. H., SEELYE, J. G., JOHNSEN, P. B., MANIAK, P. J., GRANT, G. C., POLKINGHORNE, C. N., and SORENSEN, P. W. 2000. Direct behavioral evidence that unique bile acids released by larval sea lamprey (*Petromyzon marinus*) function as a migratory pheromone. *Can. J. Fish. Aquat. Sci* 57:557–569.

BONADONNA, F., and NEVITT, G. A. 2004. Partner-specific odor recognition in an Antarctic seabird. *Science* 306:835–835.

BONADONNA, F., CARO, S., JOUVENTIN, P., and NEVITT, G. A. 2006. Evidence that blue petrel, *Halobaena caerulea*, fledglings can detect and orient to dimethyl sulphide. *J. Exp. Biol* 209:2165–2169.

BUCKLEY, N. J. 1997. Experimental tests of the information-center hypothesis with black vultures and turkey vultures. *Behav. Ecol. Sociobiol* 41:267–269.

CAPRIO, J. 1988. Peripheral filters and chemoreceptor cells in fishespp. 313–338, in J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animal-sSpringer, NewYork.

CARDÉ, R. T., and WILLIS, M. A. 2008. Navigational strategies used by insects to find distant wind-borne sources of odor. *J. Chem. Ecol.* (this issue)

CARTON, A. G., and MONTGOMERY, J. C. 2003. Evidence of a rheotactic component in the odour search behaviour of freshwater eels. *J. Fish Biol* 62:501–516.

COURTENAY, S. C., QUINN, T. P., DUPUIS, H. M. C., GROOT, C., and LARKIN, P. A. 1997. Factors affecting the recognition of population-specific odours by juvenile coho salmon. *J. Fish Biol* 50:1042–1060.

CROXALL, J. P., and PRINCE, P. A. 1994. Dead or alive, night or day-how do albatrosses catch squid? *Antarct. Sci* 6:155–162.

CUNNINGHAM, G. B. 2005. Development of olfactory behaviors in procellariiform seabirds. Dissertation in Physiology Graduate Group, University of California, Davis, pp. 155.

CUNNINGHAM, G. B., VAN BUSKIRK, R. W., BONADONNA, F., WEIMERSKIRCH, H., and NEVITT, G. A. 2003. A comparison of the olfactory abilities of three species of procellariiform chicks. *J. Exp. Biol* 206:1615–1620.

CUNNINGHAM, G. B., VANBUSKIRK, R., HODGES, M. J., WEIMERSKIRCH, H., and NEVITT, G. A. 2006. Behavioural responses of blue petrel chicks *Halobaena caerulea* to a food-related and novel odour in a simple wind tunnel. *Antarct. Sci* 18:345–352.

DAVIS, M. W., SPENCER, M. L., and OTTMAR, M. L. 2006. Behavioral responses to food odor in juvenile marine fish: acuity varies with species and fish length. *J. Exp. Mar. Biol. Ecol* 328:1–9.

DEARDORFF, J. W. 1972. Numerical investigation of neutral and unstable planetary boundary layers. *J. Atmos. Sci* 29:91–115.

DEBOSE, J. L., and NEVITT, G. A. 2007. Investigating the association between pelagic fish and DMSP in a natural coral reef system. *Mar. Freshw. Res* 58:720–724.

DEBOSE, J. L., LEMA, S. C., and NEVITT, G. A. 2008. Dimethylsulfoniopropionate as a foraging cue for reef fishes. *Science* 319:1356.

DITTMAN, A. H., and QUINN, T. P. 1996. Homing of Pacific salmon: mechanisms and ecological basis. *J. Exp. Biol* 199:83–91.

DITTMAN, A. H., QUINN, T. P., and NEVITT, G. A. 1996. Timing of imprinting to natural and artificial odors by coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish. Aquat. Sci* 53:434–442.

DØVING, K. B., and STABELL, O. B. 2003. Trails in open waters: sensory cues in salmon migration, pp. 39–52, in S. P. Collin, and N. J. Marshall (eds.). Sensory Processing in Aquatic EnvironmentsSpringer, New York.

DØVING, K. B., WESTERBERG, H., and JOHNSEN, P. B. 1985. Role of olfaction in the behavioral and neuronal responses of Atlantic salmon, *Salmo salar*, to hydrographic stratification. *Can. J. Fish. Aquat. Sci* 42:1658–1667.

DUSENBERY, D. B. 1992. Sensory Ecology. Freeman, New York.

ELLIOTT, J. K., ELLIOTT, J. M., and MARISCAL, R. N. 1995. Host selection, location, and association behaviors of anemonefishes in field settlement experiments. *Mar. Biol* 122:377–389.

ESTRELLA, R. R. 1994. Group size and flight altitude of turkey vultures in two habitats in Mexico. *Wilson Bull* 106:749–752.

FINELLI, C. M., PENTCHEFF, N. D., ZIMMER-FAUST, R. K., and WETHEY, D. S. 1999. Odor transport in turbulent flows: constraints on animal navigation. *Limnol. Oceanogr* 44:1056–1071.

GAGLIARDO, A., ODETTI, F., and IOALÈ, P. 2001a. Relevance of visual cues for orientation at familiar sites by homing pigeons: an experiment in a circular arena. *Proc. Roy. Soc. Lond* 268:2065–2070.

GAGLIARDO, A., IOALÈ, P., ODETTI, F., and BINGMAN, P. 2001b. The ontogeny of the homing pigeon navigational map: evidence for a sensitive learning period. *Proc. R. Soc. Lond. B* 268:197–202.

GRUBB, T. C. 1972. Smell and foraging in shearwaters and petrels. *Nature* 237:404–405.

HAGELIN, J. C. 2004. Short communication. Observations on the olfactory ability of the Kokapo *Strigops habroptilus*, the critically endangered parrot of New Zealand. *Ibis* 146:161–164.

HARA, T. J. 2006. Feeding behaviour in some teleosts in triggered by single amino acids primarily through olfaction. *J. Fish Biol* 68:810–825.

HARTMAN, E. J., and ABRAHAMS, M. V. 2000. Sensory compensation and the detection of predators: the interaction between chemical and visual information. *Proc. Royal Soc. London Ser. B—Biol. Sci* 267:571–575.

HASLER, A. D., SCHOLZ, A. T., and HORRALL, R. M. 1978. Olfactory imprinting and homing in salmon. *Am. Sci.* 66:374–355.

HOUSTON, D. C. 1986. Scavenging efficiency of turkey vultures in tropical forest. *Condor* 88:318–323.

HUTCHISON, L. V., and WENZEL, B. M. 1980. Olfactory guidance in foraging by procellariiforms. *Condor* 82:314–319.

JOHNSEN, P. B. 1982. Establishing the physiological and behavioral determinants of chemosensory orientation, pp. 379–386, in J. D. McCleave, G. P. Arnold, J. J. Dodson, and W. H. Neill (eds.). Mechanisms of Migration in FishesPlenum, New York.

JOHNSEN, P. B., and TEETER, J. 1985. Behavioral responses of bonnethead sharks (*Sphyrna tiburo*) to controlled olfactory stimulation. *Mar. Behav. Physiol* 11:283–291.

KEEFER, M. L., CAUDILL, C. C., PEERY, C. A., and BJORNN, T. C. 2006. Route selection in a large river during the homing migration of Chinook salmon (*Oncorhynchus tshawytscha*). *Can. J. Fish. Aquat. Sci* 63:1752–1762.

KETTLE, A. J., ANDREAE, M. O., AMOUROUX, D., ANDREAE, T. W., BATES, T. S., BERRESHEIM, H., BINGEMER, H., BONIFORTI, R., CURRAN, M. A. J., DITULLIO, G. R., HELAS, G., JONES, G. B., MCTAGGART, A. R., MIHALOPOULOS, N., NGUYEN, B. C., NOVO, A., PUTAUD, J. P., RAPSOMANIKIS, S., ROBERTS, G., SCHEBESKE, G., SHARMA, S., SIMÓ, R., STAUBES, R., TURNER, S., and UHER, G. 1999. A global database of sea surface dimethylsuflide (DMS) measurements and a procedure to predict sea surface DMS as a function of latitude, longitude and month. *Glob. Biogeochem. Cycles* 13:399–444.

KRAMER, G. 1952. Experiments on bird orientation. *Ibis* 94:265–285.

LAU, K.-K., ROBERTS, S., BIRO, D., FREEMAN, R., MEADE, J., and GUILFORD, T. 2006. An edge-detection approach to investigating pigeon navigation. *J. Theor. Biol* 239:71–78.

LEMA, S. C., and NEVITT, G. A. 2004. Evidence that thyroid hormone induces olfactory cellular proliferation in salmon during a sensitive period for imprinting. *J. Exp. Biol.* 207:3317–3327.

LØKKEBORG, S. 1998. Feeding behaviour of cod, *Gadus morhua*: activity rhythm and chemically mediated food search. *Anim. Behav* 56:371–378.

LØKKEBORG, S., and FERNÖ, A. 1999. Diel activity pattern and food search behaviour in cod, Gadus morhua. *Environ. Biol. Fish* 54:345–353.

MITAMURA, H., ARAI, N., SAKAMOTO, W., MITSUNAGA, Y., TANAKA, H., MUKAI, Y., NAKAMURA, K., SASAKI, M., and YONEDA, Y. 2005. Role of olfaction and vision in homing behaviour of black rockfish *Sebastes inermis*. *J. Exp. Mar. Biol. Ecol* 322:123–134.

MONTGOMERY, J. C., DIEBEL, C., HALSTEAD, M. B. D., and DOWNER, J. 1999. Olfactory search tracks in Antarctic fish *Trematomus bernacchii*. *Polar Biol* 21:151–154.

MOORE, P., and CRIMALDI, J. 2004. Odor landscapes and animal behavior: tracking odor plumes in different physical worlds. *J. Mar. Syst* 49:55–64.

MOORE, P. A., GRILLS, J. L., and SCHNEIDER, R. W. S. 2000. Habitat-specific signal structure for olfaction: an example from artificial streams. *J. Chem. Ecol* 26:565–584.

MURPHY, C. A., STACEY, N. E., and CORKUM, L. D. 2001. Putative steroidal pheromones in the round goby, *Neogobius melanostomus*: olfactory and behavioral responses. *J. Chem. Ecol* 27:443–470.

NEVITT, G. a. 1991. Do fish sniff? A new mechanism of olfactory sampling in pleuronectid flounders. *J. Exp. Biol* 157:1–18.

NEVITT, G. A. 1999. Olfactory foraging in Antarctic seabirds: a species-specific attraction to krill odors. *Mar. Ecol. Prog. Ser* 177:235–241.

NEVITT, G. A. 2000. Olfactory foraging by Antarctic procellariiform seabirds: life at high Reynolds numbers. *Biol. Bull* 198:245–253.

NEVITT, G. A. 2008. Sensory ecology on the high seas: investigating the odor world of the procellariiform seabirds. *J. Exp. Biol.* (in press).

NEVITT, G. A., and HABERMAN, K. L. 2003. Behavioral attraction of Leach's storm-petrels (*Oceanodroma leucorhoa*) to dimethyl sulfide. *J. Exp. Biol* 206:1497–1501.

NEVITT, G. A., and DITTMAN, A. H. 2004. Olfactory imprinting in salmon: new models and approaches, pp. 109–127, in G. Von Der Emde, J. Mogdans, and B. G. Kapoor (eds.). The Senses of Fish: Adaptations for the Reception of Natural StimuliNarosa, New Delhi.

NEVITT, G. A., and BONADONNA, F. 2005a. Seeing the world through the nose of a bird: new developments in the sensory ecology of procellariiform seabirds. *Mar. Ecol. Prog. Ser* 287:292–295.

NEVITT, G. A., and BONADONNA, F. 2005b. Sensitivity to dimethyl sulphide suggests a mechanism for olfactory navigation by seabirds. *Biol. Lett* 1:303–305.

NEVITT, G. A., VEIT, R. R., and KAREIVA, P. 1995. Dimethyl sulphide as a foraging cue for Antarctic procellariiform seabirds. *Nature* 376:680–682.

NEVITT, G. A., REID, K., and TRATHAN, P. 2004. Testing olfactory foraging strategies in an Antarctic seabird assemblage. *J. Exp. Biol* 207:3537–3544.

NEVITT, G. A., LOSEKOOT, M., and WEIMERSKIRCH, H. 2008. Evidence for olfactory search in Wandering albatross *Diomedea exulans*. *Proc. Nat. Acad. Sci. U.S.A* 105:576–4581.

NORDENG, H., and BRATLAND, P. 2006. Homing experiments with parr, smolt and residents of anadromous Arctic char *Salvelinus alpinus* and brown trout *Salmo trutta*: transplantation between neighboring river systems. *Ecol. Freshw. Fish* 15:488–499.

O'DWYER, T. W., ACKERMAN, A. L., and NEVITT, G. A. 2008. Examining the development of individual recognition in a burrow-nesting procellariiform, the Leach's storm-petrel. *J. Exp. Biol* 211:337–340.

OWRE, O. T., and NORTHINGTON, P. O. 1961. Indication of the sense of smell in the turkey vulture from feeding tests. *Am. Midland Nat* 66:200–205.

PAPI, F. 1990. Olfactory navigation in birds. *Experientia* 46:352–363.

PAPI, F. 2006. Navigation of marine, freshwater and coastal animals: concepts and current problems. *Mar. Freshw. Behav. Physiol* 39:3–12.

PAPI, F., FIORE, L., FIASHCHI, V., and BENVENUTI, S. 1971. The influence of olfactory nerve section on the homing capacity of carrier pigeons. *Monit. Zool. Ital* 5:265–267.

PLENDERLEITH, M., VAN OOSTERHOUT, C., ROBINSON, R. L., and TURNER, G. F. 2005. Female preference for conspecific males based on olfactory cues in a Lake Malawi cichlid fish. *Biol. Lett* 1:411–414.

ROPER, T. J. 1999. Olfaction in birds. *Adv. Study Behav* 28:247–332.

SAND, O., and KARLSEN, H. E. 2000. Detection of infrasound by the Atlantic cod. *R. Soc* 125:197–204.

SHERMAN, M. L., and MOORE, P. A. 2001. Chemical orientation of brown bullheads, *Ameiurus nebulosus*, under different flow conditions. *J. Chem. Ecol* 27:2301–2318.

SILVERMAN, E. D., VEIT, R. R., and NEVITT, G. A. 2004. Nearest neighbors as foraging cues: information transfer in a patchy environment. *Mar. Ecol. Prog. Ser* 277:25–35.

SMITH, S. A., and PASELK, R. A. 1986. Olfactory sensitivity of the turkey vulture (*Cathartes aura*) to three carrion-associated odorants. *Auk* 103:586–592.

SORENSEN, P. W., VRIEZE, L. A., and FINE, J. M. 2003. A multi-component migratory pheromone in the sea lamprey. *Fish Physiol. Biochem* 28:253–257.

STAGER, K. E. 1964. The role of olfaction in food location by the turkey vulture (*Cathartes aura*). *Los Angeles Cty. Mus. Contrib. Sci* 81:3–63.

UEDA, H., KAERIYAMA, M., MUKASA, K., URANO, A., KUDO, H., SHOJI, T., TOKUMITSU, Y., YAMAUCHI, K., and KURIHARA, K. 1998. Lacustrine sockeye salmon return straight to their natal area from open water using both visual and olfactory cues. *Chem. Sens* 23:207–212.

VANBUSKIRK, R., and NEVITT, G. A. 2007. Evolutionary arguments for olfactory behavior in modern birds. *ChemoSense* 10:2–6.

VANBUSKIRK, R., and NEVITT, G. A. 2008. The influence of developmental environment on the evolution of olfactory foraging behavior in procellariiform seabirds. *J. Evol. Biol* 21:67–76.

VICKERS, N. J. 2000. Mechanisms of animal navigation in odor plumes. *Biol. Bull* 198:203–212.

VRIEZE, L. A., and SORENSEN, P. W. 2001. Laboratory assessment of the role of a larval pheromone and natural stream odor in spawning stream localization by migratory sea lamprey (*Petromyzon marinus*). *Can. J. Fish Aquat. Sci* 58:2374–2385.

WALCOTT, C. 1996. Pigeon homing: observations, experiments and confusions. *J. Exp. Biol* 199:21–27.

WALLRAFF, H. G. 1989. Simulated navigation based on assumed gradients of atmospheric trace gases (models on pigeon homing. Part 3). *J. Theor. Biol* 138:511–528.

WALLRAFF, H. G. 2000. Path integration by passively displaced homing pigeons? *Anim. Behav* 60:F30–F36.

WALLRAFF, H. G. 2004. Avian olfactory navigation: its empirical foundation and conceptual state. *Anim. Behav* 67:189–204.

WALLRAFF, H. G. 2005. Beyond familiar landmarks and integrated routes: goal-oriented navigation by birds. *Connect. Sci* 17:91–106.

WALLRAFF, H. G., and ANDREAE, M. O. 2000. Spatial gradients in ratios of atmospheric trace gases: a study stimulated by experiments on bird navigation. *Tellus* 52B:1138–1157.

WARHAM, J. 1990. The Petrels: Their Ecology and Breeding Systems. Academic, London.

WARHAM, J. 1996. The Behavior, Population Biology and Physiology of the Petrels. Academic, London.

WEIMERSKIRCH, H., GUIONNET, T., MARTIN, J., SHAFFER, S. A., and COSTA, D. P. 2000. Fast and fuel efficient? Optimal use of wind by flying albatrosses. *Phil. Trans. R. Soc. Lond. B* 267:1869–1874.

WEIMERSKIRCH, H., BONADONNA, F., BAILLEUL, F., MABILLE, G., DELL'OMO, G., and LIPP, H. P. 2002. GPS tracking of foraging albatrosses. *Science* 295:1259–1259.

WEIMERSKIRCH, H., PINAUD, D., PAWLOWSKI, F., and BOST, C. A. 2007. Does prey capture induce area-restricted search? A fine scale study using GPS in a marine predator, the wandering albatross. *Am. Nat* 170:734–743.

WENZEL, B. M. 1960. The olfactory prowess of the Kiwi. *Nature* 220:1133–1134.

WENZEL, B. M. 1987. The olfactory and related systems in birds. *Ann. N.Y. Acad. Sci* 519:137–139.

WESTERBERG, H. 1982. The orientation of fish and the vertical stratification at fine- and micro-structure scales, pp. 179–204, in J. D. McCleave, G. P. Arnold, J. J. Dodson, and W. H. Neill (eds.). Mechanisms of Migration in FishesPlenum, New York.

WILSON, R. P., PUTZ, K., GREMILLET, D., CULIK, B. M., KIERSPEL, M., REGEL, J., BOST, C. A., LAGE, J., and COOPER, J. 1995. Reliability of stomach temperature changes in determining feeding characteristics of seabirds. *J. Exp. Biol* 198:1115–1135.

WILTSCHKO, W., and WILTSCHKO, R. 1998. The navigation system of birds and its development, pp. 155–159, in R. P. Balda, I. M. Pepperberg, and A. C. Kamil (eds.). Animal Congnition in Nature. Academic, San Diego.

WILTSCHKO, R., and WILTSCHKO, W. 2000. A strategy for beginners! Reply to Wallraff (2000). *Anim. Behav* 60:F37–F43.

WILLIS, M. A. 2005. Odor-modulated navigation in insects and artificial systems. *Chem. Senses* 30:i287–i288.

ZIMMER-FAUST, R. K., FINELLI, C. M., PENTCHEFF, N. D., and WETHEY, D. S. 1995. Odor plumes and animal navigation in turbulent water flow: a field study. *Biol. Bull* 188:111–116.

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REVIEW ARTICLE

Odor Detection in Insects: Volatile Codes

M. de Bruyne · T. C. Baker

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Abstract Insect olfactory systems present models to study interactions between animal genomes and the environment. They have evolved for fast processing of specific odorant blends and for general chemical monitoring. Here, we review molecular and physiological mechanisms in the context of the ecology of chemical signals. Different classes of olfactory receptor neurons (ORNs) detect volatile chemicals with various degrees of specialization. Their sensitivities are determined by an insect-specific family of receptor genes along with other accessory proteins. Whereas moth pheromones are detected by highly specialized neurons, many insects share sensitivities to chemical signals from microbial processes and plant secondary metabolism. We promote a more integrated research approach that links molecular physiology of receptor neurons to the ecology of odorants.

Keywords Insects · Olfaction · Receptors · Pheromones · *Drosophila* · Lepidoptera · Behavior · Antenna · Odor binding proteins · Evolution · Sensillum · Odor plumes · Plant volatiles

Insect Olfaction: A Renewed Challenge

In their quest for locating important resources such as mates, nutrients, oviposition, and resting sites, insects rely on their chemical senses to a large extent. While gustatory neurons signal the quality of food or mates after contact, it is the information encoded by olfactory neurons that provides cues where to look in the first place. Equally important, and often overlooked, odors can indicate toxic or other life-threatening environments. Hence, olfactory receptor neurons (ORNs) play an important role in an insect's behavioral ecology.

The past 10 years has seen a tremendous increase in our understanding of insect olfaction. The discovery of odorant receptor (OR) genes in the vinegar fly *Drosophila melanogaster* (Clyne et al. 1999a; Vosshall et al. 1999) has affected profoundly the nature of our research and has made it an important model organism. However, the behavioral ecology of odor perception has been investigated largely in other species. In light of the recent sequencing of insect genomes, it is useful to review the wealth of information on insect olfactory ecology.

Insect species are numerous and can have very different life styles and feeding ecologies. They encounter a wide range of chemicals, as each species has its special interests. Though olfaction has been studied in many insect species, comprehensive data come mainly from a few groups. Cockroaches such as *Periplaneta americana* (Boeckh et al. 1987) are ground dwellers, predominantly feeding on decaying organic matter. Its sex pheromone is a unique sesquiterpene (periplanone B, Persoons et al. 1976). Several species of large moths (Hansson 1995) mainly from the Bombicoidea (*Bombyx mori*, *Manduca sexta*) and Noctuioidea (e.g., *Heliothis* spp.) traditionally have been investigated because of their powerful long-range sex pheromones. Many moths also deposit eggs on particular host plants with differing degrees of specialization. The vinegar fly, *D. melanogaster* (Vosshall and Stocker 2007), feeds and oviposits on a variety of decaying fruits. It does not have a close relation with a particular host-plant. The

M. de Bruyne (✉)
Biological Sciences, Monash University,
Clayton, Australia
e-mail: Marien.DeBruijne@sci.monash.edu.au

T. C. Baker
Center for Chemical Ecology, Department of Entomology,
Pennsylvania State University,
University Park, PA, USA

honeybee *Apis mellifera* (Galizia and Menzel 2000) is a social insect with an intricate pheromonal communication system. Asexual females collect pollen and nectar from flowers, and they have the ability to discriminate many odorants. Mosquitoes (Takken and Knols 1999) such as *Anopheles gambiae* and *Aedes aegypti* feed on various nectar sources, but females need a blood-meal to mature their eggs. *A. aegypti* is more a generalist feeder, while *A. gambiae* prefers to bite humans.

To respond to the environment with appropriate behaviors, insect peripheral olfactory systems need to do two things: (1) Provide fast and reliable information about specific cues that induce innate behaviors, and (2) Sample salient features of the broad chemical landscape that can be learned and guide adaptive behaviors. What are the features of insect olfactory receptors that do this, and how does our newfound knowledge of molecular and neural mechanisms contribute to our understanding of the ecology of volatile signals?

Insect Noses: Organization of the Peripheral Olfactory System

Although it is the brain that transforms a set of chemical stimuli comprised of individual odorants (chemicals) into distinct odors (sensations) and associates them with a behavioral response, the ORNs are the sensors that limit what can be detected. The scope and accuracy with which odors can be identified is determined by how many different sensors there are and how they are tuned to different chemicals. Insect ORNs are bipolar neurons that develop from epithelial cells (Keil 1997). In early development, a pre-sensillum cluster of cells is formed (Ray and Rodrigues 1995; Endo et al. 2007). Some of these will move basally and become neurons, but three remain apical and form the accessory cells that will wrap themselves around the neurons. While it is the neurons that process odor information, accessory cells provide the extracellular milieu that supports their function (Park et al. 2002). These cells form sensilla, small cuticular structures where the neuronal dendrites are bathed in a lumen filled with mucus-like sensillum lymph (Zacharuk 1985). Pores in the cuticle allow odorants to pass and dissolve in the lymph (Steinbrecht 1997).

The majority of olfactory sensilla can be found on the antennae, but smaller sets are also located on other head appendages such as maxillary (de Bruyne et al. 1999) or labial palps (Stange 1992; Kwon et al. 2006). Irrespective of their location, all ORNs project to the antennal lobe of the brain. Olfactory sensilla fall into two ultrastructural categories, double walled (dw) and single walled (sw; Altner and Prillinger 1980). While the former are usually small and uniformly peg shaped, the latter can take on many different shapes (Zacharuk 1985; Steinbrecht 1997). In moths, sw sensilla can be up to 100 μm long thick-walled pointed hairs (trichoid sensilla), but in most insects (e.g., *Drosophila*), they are much smaller. Trichoid sensilla typically house neurons with unbranched dendrites (Zacharuk 1985; Shanbhag et al. 2000). Hymenoptera (e.g., honeybees) and certain Coleoptera have pored plates (placoid sensilla), some with large numbers of branched dendrites (Behrend 1971; Esslen and Kaissling 1976; Nikonov and Leal 2002). Most insects have thin-walled short (5–30 μm) blunt hairs (basiconic sensilla, Shanbhag et al. 2000). It remains unclear why insect ORNs are housed in sensilla of different shapes and sizes or how sensillum structure affects olfactory function.

In *Drosophila*, genetic experiments have shown that dw and sw sensilla develop differently, using different neurogenic genes (Gupta and Rodrigues 1997; Goulding et al. 2000). The *atonal* gene determines dw sensilla, while the *amos* gene regulates sw sensilla. The different structural subcategories of sw sensilla are regulated by patterning genes such as *lozenge* (Gupta and Rodrigues 1998; Goulding et al. 2000). In insect sensilla, usually two to four ORNs with distinct neuronal identities are combined within a single sensillum type. (Boeck et al. 1987; de Bruyne et al. 1999, 2001). The *notch* gene is involved in determining the fate of neurons within a single sensillum by being expressed in only one of them, creating *notch*-on and *notch*-off neurons (Endo et al. 2007). Neuronal identity is further determined by the *acj6* gene (Clyne et al. 1999b).

The spatial organization of ORNs in the antennal epithelium is lost upon entry of their axons into the brain. It is tempting to think of the antennal lobe as a map of olfactory information. Are ORN axon projections sorted to glomeruli according to some sort of chemical logic (a chemotopic map), transforming the intermingled distribution of ORNs in the epithelium into a functional spatial arrangement in the brain? In mosquitoes and flies, there is evidence that the antennal lobe is organized in zones for the different structural categories of sensilla and the different appendages (Anton and Rospars 2004; Couto et al. 2005; Endo et al. 2007). Within these zones, ORNs that co-inhabit a single sensillum tend to be separated based on *notch* expression with *notch*-on glomeruli clustered together on one side and *notch*-off on the other (Endo et al. 2007). It remains to be seen if the main organizing principle of the axonal projections is based on odor chemistry or the neuron's place in structures of the periphery. However, *Drosophila* ORNs that have their odor responsiveness removed by a mutation show normal projection patterns (Dobritsa et al. 2003). Hence, neural identity i.e., an ORN's place in the system, is determined independent from (and probably before) its chemical response profile. This means

that evolution of gene regulation through transcription factors can potentially reorganize odor sensitivities in the periphery, thus causing evolutionary shifts in behavioral responses.

Molecular Codes: Olfactory Genes in Neurons

In vertebrate ORNs, odor receptor (OR) genes (Buck and Axel 1991) with seven prominent transmembrane domains have been shown to be G-protein coupled receptors that mediate odor sensitivity (Firestein 2001). A large family of genes with expression in ORNs has been discovered in *Drosophila* (Clyne et al. 1999a; Vosshall et al. 1999). Although hydropathy analysis of these insect OR proteins strongly suggests they have seven transmembrane domains, they are very different from the vertebrate ORs, and indeed from any other G-protein coupled receptors. Insect ORs lack many of the characteristics of other G-protein coupled receptors (Wistrand et al. 2006), and recent evidence suggests they may insert the other way around in the membrane (Benton et al. 2006). There is also no direct evidence that they activate a G-protein-mediated transduction cascade as vertebrate ORs clearly do. Furthermore, insect ORs require the co-expression of *Or83b*, a special member of this family that is needed for correct OR transport and function in vivo (Larsson et al. 2004). The Or83b gene is the only member of this family that is highly conserved in several insect species (Krieger et al. 2003; Jones et al. 2005), indicative of its fundamental role across insects. The most recent evidence suggests that insect ORs form ligand-gated channels (Sato et al. 2008; Wicher et al. 2008), that bypass a G-protein-coupled signal transduction cascade. This would enable rapid odor processing and corroborate earlier physiological investigations (Kaissling 1987; de Bruyne et al. 1999) that highlight the fast responses of insect ORNs.

It is clear that this insect-specific family determines the response properties of most insect ORNs. OR proteins are localized to ORN dendrites (Elmore and Smith 2001). Expression of an OR transgene in *Drosophila* ORNs (Störtkuhl and Kettler 2001) or in an in vitro cell system (Wetzel et al. 2001) induces odor responses and mutations that remove OR gene expression from ORNs cause loss of odor activity (Clyne et al. 1999b; Dobritsa et al. 2003). Dobritsa et al. (2003) also demonstrated that a single OR mediates activity for the full range of odorants that excite an ORN by expressing a *Drosophila* OR transgene in a neuron from which the indigenous gene has been removed. Hallem and Carlson (2006) have shown that this applies to most *Drosophila* ORs. Interestingly, these experiments also showed that OR genes normally expressed in sensilla with a grooved peg (dw) or trichoid (sw) structure are also functional in a basiconic (sw) sensillum. Receptor-swapping is also possible among different insect species. A mosquito receptor for 2-methylphenol and a moth sex pheromone receptor detect their ligands when expressed in a *Drosophila* ORN (Hallem et al. 2004; Syed et al. 2006).

OR genes form one of the largest gene families in insects, and because they guide behavioral interactions of insects with other organisms, the analysis of their molecular evolution is of considerable interest. OR sequences show low conservation except for relatively conserved intron/exon boundaries, which suggest they arose from a common ancestor (Robertson et al. 2003). Complete sets of OR genes have been sequenced from the genomes of *Drosophila* (Robertson et al. 2003, Guo and Kim 2007), the honeybee (*A. mellifera*, Robertson and Wanner 2006), and two mosquitoes (*A. gambiae* Hill et al. 2002, and *A. aegypti*, Bohbot et al. 2007) ,and their numbers are featured in Table 1. Some OR genes have also been sequenced from the moths *B. mori* (Sakurai et al. 2004, Wanner et al. 2007) and *Heliothis virescens* (Krieger et al. 2002, 2004). If we exclude the *Or83b* gene and its homologues, *D. mela-*

Table 1 Estimates of olfactory coding units in different insects

Insect species	Order	ORs[a]	ORNs[b]	Glomeruli[c]
Drosophila melanogaster	Diptera	61	44	50±2
Anopheles gambiae	Diptera	78	>27	60±2
Aedes aegypti	Diptera	100	>10	50±2
Bombyx mori	Lepidoptera	>48	>5	60±2
Heliothis virescens	Lepidoptera	>20	>20	64±2
Apis mellifera	Hymenoptera	162	n.a.	165±2
Periplaneta americana	Blattaria	n.a.	>25	125±2

[a] Predicted from the genome, excluding pseudogenes, incomplete sequences and Or83b orthologs, including multiple splice forms
[b] Identified electrophysiologically or by gene expression (in situ)
[c] Variability is due to sexual dimorphisms and/or individual variability

nogaster has 59 OR genes that encode 61 proteins (Robertson et al. 2003). The *A. gambiae* genome reveals 78 OR genes (Hill et al. 2002), while *A. aegypti* has at least 100 (Bohbot et al. 2007). The honeybee genome has 162 of them (Robertson and Wanner 2006). This tells us that the full complement of coding units in most insect systems is likely to be ca. 10× lower than that found in most mammals. This may reflect selection pressure trade-offs between detection sensitivity and discrimination ability.

The comparison of OR genes within a species (paralogs) or between species (orthologs) shows that the OR gene family has undergone rapid evolution. Some OR genes in *Drosophila* occur in small clusters of two to three genes, and many of these clusters suggest recent gene duplications (Robertson et al. 2003; Guo and Kim 2007; Nozawa and Nei 2007). Interestingly, in the honeybee, most genes occur in large arrays with one of them encompassing 60 genes, indicating recent rapid expansion of a few subfamilies that may be bee-specific (Robertson and Wanner 2006). Comparisons between the two mosquitoes, *A. aegypti* and *A. gambiae*, separated by ca. 150 m years of evolution, confirms this species-specific expansion of subgroups but also shows orthologous subgroups that are relatively conserved (Bohbot et al. 2007). It is likely that rapid evolution of OR sequences is driven by changes in chemical ecology of these species even if the exact relation between OR sequence and ligand binding remains obscure. These comparative analyses also demonstrate the need to study the variety in insect olfactory systems.

While OR sequences are highly varied, comparative analysis of closely related *Drosophila* species shows that structural integrity of OR proteins is evolutionarily preserved (Tunstall et al. 2007). However, positive selection appears to be acting on some of these genes, at specific sites and along certain lineages. In 12 more distantly related *Drosophila* species, gene numbers are conserved (Guo and Kim 2007; Nozawa and Nei 2007). Although sequences are variable, each gene still has a clear ortholog, which suggests that over a comparable evolutionary period, insect ORs may evolve more slowly than vertebrate ones. An electrophysiological comparison of nine closely related *Drosophila* species also suggests that ORN response properties are relatively conserved (Stensmyr et al. 2003). Out of eight classes studied, only one showed changes in three species. Dekker et al. (2006) show for one of these species, *Drosophila sechellia*, that these shifts are correlated with changes in behavior. *D. sechellia*, which unlike *D. melanogaster* specializes on one particular fruit, appears to have lost OR genes and to have fixed point-mutations in the remaining genes at a higher rate than *Drosophila simulans*, which has a feeding ecology similar to *D. melanogaster* (McBride 2007). Like the higher number of OR genes in *A. aegypti* compared to *A. gambiae*, this indicates that niche specialization may lead to a loss of ORN classes. Stensmyr et al. (2003) also noted a loss of one sensillum type in *D. sechellia.*

While the loss of a sensillum type is most likely due to changes in developmental genes, changes in the regulation of OR expression can potentially reshuffle the array. Specific regulatory elements were discovered upstream of OR genes that determine whether an OR is expressed in the antenna or palp and in which neuron class (Ray et al. 2007). While most ORNs express a single OR gene, there are several cases of two or three genes per cell. In some of these cases, the two genes arise from a recent duplication, and one of them is not functional (Dobritsa et al. 2003), but in another case, both genes contribute to the response spectrum of the ORN (Goldman et al. 2005). Ray et al. (2007) showed these genes share common neuron-specific regulatory elements.

Modifying the Code: Olfactory Genes in Accessory Cells

The sensillum lymph forms the watery transition medium between volatiles and ORN dendrites, and is a mucus-like liquid packed with proteins secreted by the accessory cells. Before OR genes were identified, a group of proteins was isolated from moth olfactory sensillum lymph and dubbed pheromone-binding proteins (PBP, Vogt and Riddiford 1981) or odorant-binding proteins (OBP, Vogt et al. 1991). In moths, PBPs form a distinct subfamily of OBPs associated with the different pheromone-sensitive sensilla. In general, these proteins are differentially expressed in the different olfactory sensillum types (Vogt et al. 1991). Unlike the membrane-bound ORs, they are small, secreted hydrophilic proteins that can be extremely abundant in the lymph (Pelosi et al. 2006). Vertebrate OBPs belong to the larger family of lipocalins, but insect OBPs again define an insect-specific family of proteins. There are 51 OBP-like sequences in the *Drosophila* genome (Galindo and Smith 2001). OBPs are characterized by six cystein residues that are thought to form three disulfide bonds and a hydrophobic pocket inside (Sandler et al. 2000). A second family dubbed chemosensory proteins (CSPs) contains somewhat smaller proteins that have four disulfide bonds (Angeli et al. 1999). Neither OBPs nor CSPs are olfactory-specific genes. Many OBPs are expressed in gustatory sensilla, and several OBPs and particularly CSPs occur elsewhere in the body (Galindo and Smith 2001; Shanbhag et al. 2001; Pelosi et al. 2006).

It is thought that PBPs help solubilize hydrophobic moth pheromone molecules in the sensillum lymph, and specific binding of moth pheromone components has been demon-

strated (Du and Prestwich 1995; Leal et al. 2005). However, the presence of a specific OBP is not necessary for odor response per se. *Drosophila*, *Bombyx*, and *Anopheles* OR genes are functional in a *Drosophila* ORN where they are in an environment that does not have the OBPs that normally surround them (Hallem and Carlson 2006; Syed et al. 2006). In addition, specific responses to pheromones and other odors can be obtained in vitro from OR genes expressed in cultures of transfected cells (Wetzel et al. 2001; Nakagawa et al. 2005; Grosse-Wilde et al. 2006; Kiely et al. 2007). In vitro experiments with OR-expressing cells indicate that PBPs solubilize pheromones and can improve specificity of the receptor (Grosse-Wilde et al. 2006).

There are 51 OBP-like sequences in the *Drosophila* genome, none of which are closely related to moth PBPs. However, one of them, *obp76a*, plays a crucial role in the perception of cis-vaccenyl acetate (Z11-18:Ac), a compound quite similar in structure to moth pheromone components. The *lush* mutation removes all *obp76a* from a subset of trichoid sensilla, and its ORNs become insensitive, a defect that can be rescued by adding recombinant OBP76a protein to the preparation (Xu et al. 2005). In *Drosophila*, this compound is thought to be an aggregation pheromone produced by males, passed on to females and then to the oviposition substrate (Bartelt et al. 1985).

OBP sequence similarities across insect species are low (Vogt 2005), with only some subfamilies present across insect orders, and most with radiations within a single insect order or family. For example, the PBPs appear to have adapted specifically to a role in lepidopteran pheromone communication (Vogt 2005). This suggests that, like ORs, OBPs are under strong adaptive selection. Another line of evidence highlights this potentially important role of OBPs in insect chemical ecology, but also demonstrates their function outside the olfactory system. The feeding preference of *D. sechellia* for the toxic noni fruit (*Morinda citrifolia*) is linked to the expression pattern of *Obp57d/e* in taste sensilla on the tarsi (Matsuo et al. 2007).

Finally, in addition to OBPs, accessory cells also secrete a variety of enzymes that are likely to play a role in removing active odorants from the dendritic membranes (Vogt and Riddiford 1981; Maïbèche-Coisne et al. 2004; Ishida and Leal 2005). It is still unclear which role all these specific proteins in the sensillar lymph play in modifying ORN responses. Most likely, their roles are varied. While some PBPs appear to enhance only ORN functions, obp76a (*lush*) appears crucial. Expression of OBPs also can bridge ORN classes. *Drosophila* obp76a is also expressed in another class of sensilla that do not contain Z11-18Ac-sensitive neurons where the *lush* mutation has a much less dramatic effect (Shanbhag et al. 2001; Xu et al. 2005).

Odor Responses across ORN Populations: A Broad Olfactory Net

How many different coding units does an insect need to encode relevant odors in its environment? The number of different OR genes is a reasonable estimate, but there is no strict one-to-one OR/ORN ratio. Some ORNs express two or three genes (Couto et al. 2005; Ray et al. 2007). Some ORNs do not express any of the known OR genes. The identification of all ORN classes physiologically is informative but is tedious (Table 1). Only in *Drosophila* are we close to achieving characterization of all ORN classes (de Bruyne et al. 1999, 2001; Yao et al. 2005; van der Goes van Naters and Carlson 2007), and Table 2 shows an overview of the response properties of most of its ORNs. The number of glomeruli in the antennal lobe can be indicative because, with few exceptions, ORNs of a single class converge on a single glomerulus (Hansson and Christensen 1999; Vosshal et al. 1999). Unfortunately, glomerulus borders are difficult to visualize in some insect species, and numbers can vary slightly among individuals. Nevertheless, the estimates for all three parameters in Table 1 show that for these model species, 50–60 units appears sufficient. The ca. 160 units for honeybees (genes and glomeruli) possibly indicate the high end.

Odor stimuli contain three elements of information: odor identity, odor intensity, and a temporal component, i.e., how these two vary in time. If an odor is a mixture, its identity can change if the composition changes. Studies in which a large number of odorants are tested on many ORNs invariably show that they can be classified into response types. Most ORN classes respond to a small subset of odorants, and several odorants evoke responses from a few classes. The identity of many odors is, thus, encoded in the activity of different sets of ORN classes (Hildebrand and Shepherd 1997). However, this is dose-dependent. For instance, at relatively high doses, ethyl acetate excites three ORN classes in *Drosophila* (pb1A, ab1A, and ab2A in Table 2), but at different levels (de Bruyne et al. 1999, 2001). At lower doses, only ab1A will respond because the other two are less sensitive to this odorant but better tuned to related esters. Odor concentration in a single ORN class is usually encoded over a short range of concentrations. Dose–response curves typically show a sigmoid shape with the dynamic part covering two to three log steps (de Bruyne et al., 1999, 2001). Table 2 also outlines that each ORN class is tuned to a different odorant. What the table does not show is that there are some odorants that are promiscuous, exciting many ORNs, while others appear to be encoded by the activity of a single ORN class.

It is important to realize that the olfactory system's main function is to capture changes in an insect's odor environment and guide it toward resources or away from

Table 2 Organisation of peripheral olfactory system in Drosophila

ORN		OR	Glom.	Odorant	Sens.	Tuning
Sw sensilla (palpal basiconic)						
Pb1	A	42a	VM7d	Ethyl propionate	●●●	■■
	B	71a	VC2	4-Methylphenol	●●●	■
pb2	A	85e+33c	VC1	Fenchone	●●●	■■
	B	46aA+46aB	VA7l	4-Methylphenol	●●	■■
pb3	A	59c	VM7v	–	–	■
	B	85d	VA4	2-Heptanone	●●	■■
Sw sensilla (antennal basiconic)						
ab1	A	42b	DM1	Ethyl acetate	●●●	■■
	B	92a	VA2	3-Hydroxy-2-butanone	●●	■
	C	Gr21a	V	CO_2	●●●	■
	D	10a	DL1	Ethylbenzoate	●●●	■■
ab2	A	59b	DM4	Methyl acetate	●●●	■■
	B	85a+33b	DM5	Ethyl 3-hydroxybutanoate	●●●	■■
Ab3	A	22a+22b	DM2	Ethyl hexanoate	●●●	■■■
	B	85b	VM5d	6-Methyl-5-hepten-2-one	●●●	■■■
ab4	A	7a	DL5	E2-Hexenal	●●●	■■
	B	56a+33a	DA2	–	–	■
ab5	A	82a	VA6	Geranyl acetate	●●	■
	B	47a	DM3	Pentyl acetate	●●●	■■
ab6	A	?	?	1-Octen-3ol	●●●	■■■
	B	49b	VA5	2-Methylphenol	●●●	■
ab7	A	98a	VM5v	3-Octanol	●●	■■■
	B	67c	VC4	Ethyl lactate	●●●	■■■
ab8	A	43b	VM2	Ethyl butanoate	●●	■■■
	B	9a	VM3	2,3-Butanediol	●●●	■■■
ab9	A	69aA+69aB	D	?	?	?
	B	67b	VA3	?	?	?
Ab10	A	49a+85f	DL4	Acetophenone	●	■■
	B	Or67a	DM6	2-Phenylethanol	●●●	■■■
Sw sensilla (antennal trichoid and intermediate)[a]						
ai1	A	?	VA7m	?	?	?
	B	13a	DC2	?	?	?
at1	A	67d	DA1	Z11-octadecenyl acetate	●	■
at2	A	23a	DC3	1-Pentanol	●	■
	B	83c	DA3	?	?	?
at3	A	2a	DA4m	Iso-pentyl actetate	●	■
	B	19a+19b	DC1	1-Octen-3-ol	●●	■■■
	C	43a	DA4l	Cyclohexanol	●●	■■
at4	A	47b	VA1v	– (m/f)	–	■
	B	65a+65b+65c	DL3	– (m)	–	■
	C	88a	VA1d	– (m/f)	–	■
Dw sensilla (antennal coeloconic)[b]						
ac1	A	?	?	Ammonia	●●	■
	B	?	?	(hygroreceptor)	–	–
ac2	A	?	?	1,4-Diaminobutane	●●●	■
	B	?	?	(Hygroreceptor)	–	–
ac3	A	?	?	Propanal	●●●	■■
	B	35a	VC3m	Z3-hexenol	●●●	■■■
ac4	A	?	?	Phenylacetaldehyde	●	■
	B	?	?	–	–	■
	C	?	?	–	–	■

ORN Olfactory receptor neuron name, *OR* olfactory receptor genes expressed, *glom* glomerulus innervated, *odor* odorant known to evoke highest response, – no odorant evokes responses >50 spikes/s, *sens* sensitivity to a standard dose of that odorant in spikes/s (– < 50 ●, < 115, ●● < 180 ●●● >180), tuning, tuning width as % of odorants tested that evoke >50 Spikes/s (■ < 6%, ■■ < 22%, ■■■ > 22%).

[a] The three ORNs in at4 sensilla respond to extracts of male (m) and/or female (f) flies. [b] Most ORNs in dw sensilla do not express an OR; hence, projections to the antennal lobe have not been traced to individual glomeruli. Question marks indicate information not available. Data from de Bruyne et al. 1999, 2001; Goldman et al. 2005; Couto et al. 2005; Hallem and Carlson 2006; Endo et al. 2007; and de Bruyne and Faucher unpublished

danger. It has evolved the ability to detect key odorants with high sensitivity and to perceive a wide variety of general odorants at higher doses. Table 2 shows that *Drosophila* is particularly sensitive to a variety of esters, most likely because these are often generated by fermentation processes. Many phytophagous species have a variety of ORNs to detect terpenoids (Bruce et al. 2005), which seem to be underrepresented in the *Drosophila* ORN responses (de Bruyne et al. 2001; Hallem and Carlson 2006). The cockroach *P. americana* has several ORN classes that detect small alcohols and fatty acids (Sass 1976; Boeckh et al. 1987). While many odorants evoke responses from ORNs across insect species with very different ecologies, the part of the chemical world covered by an insect's repertoire of ORNs is likely to reflect the evolutionary history of the taxa.

In discussing tuning width of ORNs, it is important to realize that statements in the literature are rarely quantitative and depend highly on the breadth and variety of the set of odorants tested. Nevertheless, the ability of different OR proteins to bind a range of different odorant molecules varies widely (Hallem and Carlson 2006). In general, most ORNs of insects exhibit a higher degree of odorant specificity than previously thought, when they were labeled as broadly tuned odorant generalists as opposed to the more specialist receptors for pheromones (Hildebrand and Shepherd 1997). Work on heliothine moths has shown that many ORN classes respond to a relatively narrow set of plant odorants (Stranden et al. 2003; Rostelien et al. 2005). A similar reassessment of the tuning width of insect ORNs has resulted from studies performed on two species of beetle (Stensmyr et al. 2001).

ORNs that are tuned to components of insect sex pheromones exhibit some of the highest degrees of odorant specificity. Extremely specific responses to small differences in carbon chain length, functional group, or double bond positions are well known from different moth species. For instance, in *Heliothis* species, male ORNs are tuned to only one compound out of a series of closely related structural analogs (cf. Berg and Mustaparta 1995; Cossé et al. 1998; Baker et al. 2006). Highly specific responses also have been shown to stereoisomers of beetle pheromones, with one species responding only to the *R*-isomer while the other picks out the *S*-isomer (Wojtasek et al. 1998). Nevertheless, cross-reactivity does occur. A *Heliothis subflexa* ORN that mediates male attraction responds to a component of its own pheromone as well as to a different compound emitted by *H. virescens* (Baker et al. 2006), as does an ORN type in a species of *Yponomeuta* (Löfstedt et al. 1990). Genetic experiments on hybrids between the heliothine species suggest that such instances may be due to two related ORs being expressed in a single ORN (Baker et al. 2006).

Flexible Noses: Life Stages and Induced Genes

An insect's genome needs to encode several noses, each adapted to different ecological needs. The peripheral olfactory system of larval and nymphal stages can be quite different from that of the adult. Hemimetabolous insects add more olfactory sensilla with each molt, and the adult in particular has ORN classes that do not occur in earlier stages (Zacharuk and Shields 1991). In holometabolous insects, larvae often have a small set of ORNs with each individual one having different properties (Roessingh et al. 2007; Zacharuk and Shields 1991). *Drosophila* larvae have a single sensillum with 21 ORNs, each expressing a different OR gene (Fishilevich et al. 2005; Kreher et al. 2005). Some of these genes are expressed only there, while others occur in both larval and adult ORNs (Couto et al. 2005; Fishilevich et al. 2005) indicating that there is only partial overlap between the coding properties of adult and larval systems. Mosquito larvae, although aquatic, show a similar partial overlap in OR expression (Bohbot et al. 2007). Together with earlier electrophysiological analysis of olfactory perception in air and water (Behrend 1971), these results also indicate that odor detection needs no major adjustments upon transition from water to air.

Adult olfactory systems often differ between males and females. In moths, males often have larger antennae that carry many thousands of the same two or three classes of sex pheromone receptors. This presumably increases the chance of detecting the weak signal emitted by females. In females, specific ORN classes tuned to plant compounds appear to replace pheromone-sensitive ones (Heinbockel and Kaissling 1996, King et al. 2000). Consequently, small sets of moth OR genes show female-specific expression (Wanner et al. 2007). Finally, adult ORNs can adapt their response properties to external or internal conditions. Mosquito ORNs sensitive to the host chemical lactic acid are tuned down after a blood meal when females stop orienting to host odors and turn to oviposition stimuli instead (Davis 1984). Expression of an OR gene for 2-methyl phenol, a urine-related compound, is also turned off (Hallem et al. 2004). Ray et al. (2007) also provide some evidence that transcription factors can alter OR gene expression levels in adult flies.

Tuning to Mates: Pheromone Receptors

Recent rapid expansion of knowledge in insect olfaction owes a great deal to the study of pheromone perception, particularly of sex pheromones in moths. Moth sex pheromones epitomize the development of an exquisitely sensitive and fine-tuned communication system. The majority of moth sex pheromones are blends of 10- to 18-

carbon-long unbranched primary alcohols, acetates, or aldehydes, emitted from the female pheromone gland. They are synthesized from C_{16} or C_{18} fatty acids, and initial species-specific structural differences are imparted by one or more desaturases that place double bonds at specific locations (Roelofs and Rooney 2003). Species specificity is achieved even if females emit only simple blends of two or sometimes three pheromone components in precise ratios (along with other behaviorally inert compounds). Conspecific males fly upwind only to blends that closely approximate this ratio. Further specificity is imparted by males' cessation of upwind flight if the blend contains these components in the correct ratio but also contains an additional odd component emitted by heterospecific females as part of their pheromone blend (Vickers and Baker 1997, Quero et al. 2001).

Lepidopteran pheromone molecules are detected by ORNs in extra-long trichoid (sw) sensilla on the antennae of male moths (Kaissling 1987; Steinbrecht 1999). Usually there are two, but sometimes three, ORNs that co-compartmentalize within one sensillum. Pheromone-sensitive neurons are exclusive to males in most species and occur in large numbers (up to 40,000). Neurons that detect components of a pheromone blend can be together in a single sensillum (e.g., in Tortricidae and Crambidae) or in different sensilla (in Noctuidae). Their axons invariably converge on a small set of closely apposed enlarged glomeruli (macroglomerular complex or MGC) in the antennal lobe near the entrance of the antennal nerve (Hansson and Christensen 1999).

The genes that encode sex-pheromone-component-sensitive ORs of *B. mori* have been characterized and shown to respond to the two pheromone components bombykol and bombykal (Sakurai et al. 2004; Krieger et al. 2005; Nakagawa et al. 2005; Syed et al. 2006). These receptors are expressed in adjacent neurons in a single trichoid sensillum. In *H. virescens*, six related ORs have been characterized, and three were shown to respond to its pheromone components (Krieger et al. 2002, 2004; Grosse-Wilde et al. 2007). One of these is expressed in the most abundant type of trichoid sensillum, while the other two co-inhabit another type. It is likely that the choice of an OR for placement in a moth-pheromone-sensitive ORN relies upon the same genetic processes as described for *Drosophila* ORs for general odorants (Ray et al. 2007).

Flying to an Odor Source: The Need for Speed

Detailed studies of flying moths orienting to sex pheromones have taught us how insects move towards an odor source (see contribution by Cardé and Willis this issue). The activation properties of insect ORNs are in tune with the physical properties of odor strands and pockets of clean air that occur in natural odor plumes due to turbulence (Vickers et al. 2001). It is the intermittency of stimulation from the plume strands that promotes sustained upwind flight (Kennedy et al. 1981; Baker et al. 1985). Such sustained attraction and source location is based on reiterative upwind surges in response to odor strands, interrupted by cross-wind casting reversals in response to clean air pockets (Mafra-Neto and Cardé 1994; Vickers and Baker 1994; but see Justus and Cardé 2002). In-flight attraction and source contact is optimal when males discriminate the "correct" pheromone blend sufficiently rapidly in each of the pheromone plume strands they contact. Hence, the performance of insect ORNs is optimized for fast, repetitive responses to ensure accurate odor identification of strands arriving irregularly, often in intervals of less than 100 ms, but also with gaps between strands of more than ten times that (Baker and Haynes 1989; Vickers et al. 2001).

The optimal ratio of components in the blend most closely approximates the natural ratio emitted by females. Linn et al. (1986) convincingly demonstrated for several species that for such optimal blends, male behavioral thresholds are lower, and more males initiate upwind flight and from greater distances than for partial blend compositions or suboptimal ratios. Moreover, in heliothine moths blend-quality evaluations take place on a strand-by-strand basis, within fractions of a second. If components of another species' blend are perceived in a strand, male upwind flight is instantly affected. Strands of the optimal blend tainted with small amounts of such heterospecific antagonist are poorer at eliciting long upwind surges than strands of the untainted blend (Vickers and Baker 1997, Quero et al. 2001). ORNs tuned to such heterospecific pheromone component are often slightly more broadly tuned than are ORNs sensitive to a moth's own pheromone components. They may have evolved to detect pheromone components of several sympatric species, turning off attraction to plume strands that contain an alien compound and preventing orientation toward females of the wrong species (Cossé et al. 1998; Linn et al. 2007). To do this, most moths have ORNs co-compartmentalized in the same sensillum, one for these important heterospecific compounds and another for one of their own pheromone components (Cossé et al. 1998).

Why are insect ORNs generally combined in sensilla in stereotypic combinations? In *Drosophila*, the same two classes of ORNs are always found together in a single sensillum (de Bruyne et al 1999, 2001). The special ecology of moth pheromone systems suggests an answer. Blend compositions of pheromone compounds in individual plume strands that are traversed at high speed need to be assessed in split-second behavioral choices (Vickers and

Baker 1997; Quero et al. 2001). Stereotypical pairing of ORNs in the same sensillum is likely to ensure a high fidelity of on-site ratio reporting, regardless of differences in odor flux, and with the optimal spatio-temporal precision (Todd and Baker 1999). It has been shown that moths can discriminate between two overlapping pheromone plumes, due to incomplete mixing of their intertwined strands (Witzgall and Priesner 1991; Liu and Haynes 1992). Baker et al. (1998) estimated the temporal precision of blend ratio assessment to be close to 1 ms.

Nikonov and Leal (2002) suggested that in the Japanese scarab beetle *Popillia japonica* pheromone components are not only detected in parallel but processed within single sensilla before integration in the antennal lobe. On-site, within-sensillum mixture interactions can alter ORNs' reporting capabilities. However, it remains to be seen whether such cases constitute interactions within a neuron or between neurons. On-site mixture interactions were also demonstrated in *Helicoverpa zea* sensilla, in which the major pheromone component mixed with certain plant volatiles elicited greater ORN activity with a more tonic response profile than the pheromone component alone (Ochieng and Baker 2002).

It is just as crucial for the insect to respond quickly to the pockets of clean air between pheromone strands as it is to respond to the strand itself because the upwind, clean-wind direction of a large gap due to large-scale turbulence (wind-swing) does not lead to the source; a fast switch from upwind flight to cross-wind casting flight is required to increase the chance of recontacting odor-laden air (David et al. 1982). The latency of behavioral response to both the onset (upwind surges) and loss of pheromone pulses (cross-wind casting reversals) can be as fast as 0.15 s (Baker and Haynes 1987), but is usually between 0.3 and 0.6 s (Baker and Vogt 1988; Vickers and Baker 1996). This response speed does not seem specific to pheromones, as the latency to casting flight from upwind flight after the loss of host-odor is only slightly lower (0.7 s) in female moths (Haynes and Baker 1989).

Integrating Odor Identity and Intensity in Time

The biochemical, neurophysiological, and molecular processes that contribute to the propagation of behavior after contact with airborne pheromone strands all emphasize high-speed processing related to temporal resolution of the strand encounters. The frequency of action potentials in response to each pulse is significantly reduced under rapid stimulation and would make concentration coding less accurate. However, there is no evidence thus far that male moths need, or use, strand concentration information to locate females. This explains why such ORN adaptation nevertheless retains a high fidelity of reporting the temporal aspects of strand presence or absence, while losing the ability to report absolute concentration (Kaissling 1987).

There is some evidence that the speed of both ON- and OFF-responses in pheromone-sensitive ORNs is influenced by perireceptor proteins such as PBPs and pheromone degrading enzymes (PDEs; Syed et al. 2006). Some experiments indicate that binding dynamics of PBPs can be within the order of ms (Leal et al. 2005). The half-life of pheromone component molecules of *Anthaerea polyphemus* in the presence of an *A. polyphemus* PDE has been calculated to be less than 15 ms (Ishida and Leal 2005).

Pheromone-sensitive ORNs display a wide variation in the phasic or more tonic elements of their responses to pheromone components (Kaissling 1987; Berg and Mustaparta 1995). Phasic ORNs are fast to disadapt and are able to track (respond to) strands of pheromone in natural plumes (Vickers et al. 2001; Justus et al. 2005); they can also reliably respond to mechanically generated pulses at 10 Hz (Almaas et al. 1991) or even more than twice that (Bau et al. 2002). More tonically firing neurons (often called phasic–tonic) are usually slower to return to baseline firing levels, sometimes taking minutes or tens of minutes to do so (Rumbo and Kaissling 1989).

The frequency of action potentials in response to each short pulse of pheromone, normally correlated with the flux intensity of pheromone contained in each pulse, is significantly reduced under rapid stimulation, but the fidelity of response with regard to the temporal presence (pheromone strand) or absence (clean-air pocket) of the pheromone is accurate. Differential adaptation of ORNs specific to one pheromone component in each blend-strand without concomitant adaptation of ORNs specific to other components can result in a shift in ORN firing ratios being received by higher-order neurons and being registered through combinatorial coding as an off-blend, resulting in a reduction in attraction (Hansson and Baker 1991).

In *Drosophila*, there is also wide variation among different classes of ORNs for non-pheromonal odorants in the temporal response characteristics, particularly in the waning of responses after stimulus offset. Within a single ORN class, some odorants evoke responses that are cut off immediately with stimulus removal, while other odorants evoke long-lasting stimulation. This variation is independent of odorant chemistry and concentration. Instead, these temporal characteristics are dictated by the way a specific odorant interacts with a specific OR, as evidenced by experiments in which a single ORN type was transfected with many different ORs (Hallem and Carlson 2006). This variability can affect the temporal accuracy of reporting odor strands, but it may also enrich the coding possibilities for odor discrimination (de Bruyne et al. 2001).

ORNs detecting moth pheromones are the best studied examples with respect to temporal coding of natural odor stimuli. However, many insect species employ pheromones whose components can be chemically diverse, and their perception relies on a wide variety of ORN types. Many insect pheromones are related to plant volatiles, e.g., terpenoids such as periplanone (related to germacranes) in cockroaches (Persoons et al. 1976). Pheromone-sensitive neurons also reside in non-trichoid types of sensilla. For instance, in scarab beetles, placoid sensilla house the pheromone-sensitive receptor neurons (Nikonov and Leal 2002). Because of strong selective pressures, highly specialized olfactory subsystems have evolved from different elements of the general olfactory system in different insect groups, but fundamental mechanisms of olfactory coding appear to be conserved (Christensen and Hildebrand 2002)

Evolution of Pheromone Communication Systems

The evolution of pheromone communication systems is thought to be driven by changes in the biosynthesis of pheromone components that are tracked by shifts in male olfactory response (Phelan 1997; Roelofs et al. 2002). Major shifts in moth pheromone blends have been hypothesized to occur when previously unexpressed desaturase genes become expressed in the pheromone glands of some females in a population (Roelofs et al. 2002). If a few males in the population are able to respond to both the new and old blend, a new population of males and females communicating with this new blend would be formed through a process of asymmetric tracking over generations by males (Phelan 1997; Roelofs et al. 2002).

For this to occur, tuning properties of pheromone-sensitive ORNs would have to show a degree of variability for selection to act upon. It has been shown that the numbers of ORNs tuned to different pheromone components vary among European and West-Asian populations of the noctuid moth *A. segetum*, correlating with the differences in female blend ratios and male behavioral response occurring in different geographic regions (Hansson et al. 1990). Response profiles of pheromone-sensitive ORNs in hybrids between closely related heliothine species also reveal underlying genetic variability by the occurrence of new response types in addition to pure parental types (Baker et al. 2006). In some *Yponomeuta* species that use unusual pheromone components compared to their congeners, the male pheromone-sensitive ORNs display a correspondingly enlarged response spectrum (Löfstedt et al. 1990). Such cases may perhaps be due to a novel OR specific for the unusual component being co-expressed on these ORNs, or else a singly expressed, more broadly tuned OR for a wider range of compounds (Domingue et al. 2007).

The Stuff of Life: Volatiles from Primary Metabolism and Microbial Decay

While pheromones are usually produced from specific glands as secondary metabolites, many odorants that insects respond to are generated from primary metabolism of their hosts or associated microorganisms. Detection of some of these odors appears widespread across insect species.

Carbon dioxide (CO_2) is an atmospheric gas, omnipresent, and at relatively high concentrations for an odor. Insects can sense fluctuations around ambient concentrations (ca. 0.03%). Sensory neurons for its detection show modified dendrites with increased membrane surface and have been found in antenna of honeybees, ants, tsetse flies, fruit flies (Tephritidae), and *Drosophila* (Stange and Stowe 1999). In other insects, CO_2-sensitive neurons are found on the maxillary palps (mosquitoes, Grant et al. 1995) or labial palps (moths, Stange 1992). Larval stages of moths (Roessingh et al. 2007) and *Drosophila* (Faucher et al. 2006) also possess such neurons, but interestingly in both cases, they appear less sensitive than in the adults. In *Drosophila,* CO_2-sensitive neurons are found in basiconic sensilla on the antenna (de Bruyne et al. 2001; Suh et al. 2004). Although they are housed together with other ORNs, they do not express the *Or83b* gene nor any other member of the OR family. Instead, two members from the gustatory receptor (GR) family, *Gr21a* and *Gr63a*, mediate the response to CO_2 (Jones et al. 2007; Kwon et al. 2007). Orthologs of these genes have been found in the genomes of mosquitoes, moths, and beetles (Kwon et al. 2007) but not in the honeybee (Robertson and Wanner 2006). In the honeybee and other hymenopterans, CO_2-sensitive neurons are found in a different category of sensilla in deep pits on the antenna. The neurons appear less sensitive (Lacher 1964; Kleineidam et al. 2000), and may well use a different mechanism for CO_2 detection.

While CO_2 is produced in large amounts by all living cells, alkyl amines are metabolic by-products commonly formed from protein degradation. Attraction to ammonia and small amines is common in hematophagic insects such as flesh flies and mosquitoes, but other insects, such as fruit flies (Tephritidae), also need protein-rich food sources to complete egg maturation. In Carribean fruit flies, antennal responses to ammonia peak with deposition of yolk-proteins (Kendra et al. 2005). ORNs sensitive to amines commonly occur in dw sensilla. They are found in hematophagous insects such as mosquitoes (Meijerink et al. 2001) and triatiomine bugs (Taneja and Guerin 1995), but also in *Periplaneta* (Sass 1976) and even *Drosophila*. In

Drosophila, one ORN class responds to ammonia and another to 1,4-diaminobutane (putrescine; Yao et al. 2005). Like CO_2, amine perception might employ a different molecular mechanism. Neither of these neurons are known to express an identified OR gene (Table 2).

A well-documented attractant for hematophagous insects is 1-octen-3-ol. It was first associated with cattle breath, attracting tsetse flies (*Glossina* spp.). These flies bear at least two ORN classes on their antennae that detect it (van der Goes van Naters et al. 1996). Mosquitoes possess receptors on antennae and maxillary palps (Syed and Leal 2007). Actually, 1-octen-3-ol is a typical fungal odor and has been shown to signal fungal/mold infestations at oviposition sites (Steiner et al. 2007). In *Drosophila*, several ORN classes show high sensitivity to this odorant (de Bruyne et al. 2001). This volatile appears to excite different ORN classes in many insect species, and ORNs that detect it tend to be broadly tuned.

Plant Secondary Metabolites

The group of insects that are most studied because they interfere with our agriculture are those species that feed on plants. Phytophagous species make up a large proportion, and insect–plant interactions have driven evolution of sophisticated host location and defense mechanisms in insects and plants. Plants produce the largest variety of odorants as evidenced by our own use of these in spices and perfumes. The largest class of volatiles produced by plants in dazzling variety of mixtures is that of the terpenoids, all produced by well-known biosynthetic pathways. There are many ORN classes described for terpenoids, predominantly in moths. Receptors for commonly occurring terpenoids such as linalool, geraniol, β-ocimene, and β-caryophyllene are found in several moth species (Anderson et al. 1995; Shields and Hildebrand 2001; Rostelien et al. 2005). Terpenoid-sensitive ORNs are often housed in trichoid sensilla, and many of them are narrowly tuned. This highlights an important limitation in our understanding of insect olfaction. Many ORN types are not studied simply because they are tuned to natural compounds we have not tested. In several *Heliothis* species, an abundant type of ORN is tuned to the sesquiterpene germacrene-D, which occurs in many plants but cannot be obtained commercially (Stranden et al. 2003). It is likely though that narrowly tuned ORNs are also involved in a combinatorial type of coding. For instance, in the hawkmoth *M. sexta*, two narrowly tuned ORNs were found to respond to E-nerolidol and farnesol, respectively, but both also showed responses to the more common geraniol (Shields and Hildebrand 2001). Interestingly, a few of the many ORN classes described for *Drosophila* are activated by terpenoids. Instead, terpenoids such as linalool appear to inhibit some ORNs (de Bruyne et al. 2001), a phenomenon that may be more general (Kaissling et al. 1989).

The second largest group of plant volatiles, phenylpropanoids, contains an aromatic ring derived from the amino acid phenylalanine. They may serve to protect plant tissues from microorganisms. Methylsalicylate is a product of a signaling cascade that follows tissue damage (Turlings and Ton 2006) and has been shown to excite insect ORNs (de Bruyne et al. 2001; Shields and Hildebrand 2001). Methyleugenol is a powerful attractant for tephritid flies (Raghu 2004), although it is still not fully understood why. It has been suggested to be a case of pharmocophagy, where male flies pick up methyleugenol and use it to attract females.

The third group consists of derivatives of various fatty acids and contains mainly short-chain aldehydes, alcohols, and esters. E2-hexenal is a green leaf volatile (GLV), one of several compounds typically released after damage to leaves (Visser 1986) but also found in many flower and fruit odors. Highly sensitive ORNs to GLVs have been found in moths (Anderson et al. 1995) beetles (Visser 1986; Blight et al. 1995; Hansson et al. 1999) and flies (Guerin et al. 1983; de Bruyne et al. 2001).

Many insects have developed specific interactions with a select group of host plants. In theory, they could use host-specific cues to locate them. While some insects certainly use specific chemicals that identify distinct plant taxa, e.g., isothiocyanates for cruciferous plants (Blight et al. 1995), host finding more often involves mixtures of odorants that are common to many plants (Fraser et al. 2003). It seems that most phytophagous species home in on specific ratios among components (Visser 1986). As in moth pheromone systems, stereotypical pairings of ORNs in single sensilla may facilitate this, and it has been suggested that these are often excited by odorants from different biosynthetic pathways (Blight et al. 1995). Compared to pheromone systems, there are many more compounds in plant-produced mixtures, and their ratios are more variable. Here, selection probably favors robust adaptable systems rather than finely tuned specialized ones. Recent comparative work on phytophagous insect species with differing host specificities suggest that ORN response properties do not easily specialize. Closely related tephritid species show similar responses even if their behaviors towards plant odors differ considerably (Olsson et al. 2006). Studies on *Heliothis* (Rostelien et al. 2005) and *Drosophila* (Stensmyr et al. 2003) also suggest that insect noses maintain a generally broad sensitivity with small modifications. Finally, it should be borne in mind that insects probably detect many compounds or evaluate ratios between them that are indicative of non-hosts or toxic substrates.

Concluding Remarks

These are exciting times in the study of insect olfactory ecology. The convergence of genomic, physiological, and ecological data will elucidate the physiological and genetic basis of odor-mediated behaviors. The availability of newly sequenced insect genomes will shed new light on how olfactory systems evolve to fit different ecological niches. Despite the millions of years of evolution and the different chemical ecologies that separate some of the insects we have discussed, they share common sensitivities. It will be interesting to see how much of this is homology and how much is convergent evolution.

The selectivity of the olfactory system depends on processes between odor molecules and OR proteins, but how odors activate ORs is still largely unclear. Because insect ORs have evolved independently from those in vertebrates, solving this mystery in both groups may teach us what fundamental biochemical interactions link chemical ecology with molecular evolution. The visual system can provide useful analogies. Compound eyes are relatively uniform in shape and composition across insect orders. Olfactory epithelia have many ORN classes, enveloped in different sensory structures that express OR genes and some GR genes. They may employ other mechanisms as well. We know which physical properties of light dictate the uniformity of eyes and what drives variability in opsin genes, but we know little about how the chemical environment molds olfactory genomes of insects.

Photoreceptor cells are accompanied by cells that generate lenses, and absorption spectra of receptors are modified by screening pigments. By analogy, we should keep our minds open for modifications in vivo to the response properties of ORs. For instance, because OBPs and PBPs are insect-specific, extracellular, and highly expressed, they provide good targets for novel strategies in pest control. Although we have learned much about the unifying principles of olfactory function, we still have much to understand about the ecological mechanisms that drive the amazing variety in form and function of insect noses.

References

ALMAAS, T. J., CHRISTENSEN, T. A., and MUSTAPARTA, H. 1991. Chemical communication in heliothine moths I. Antennal receptor neurons encode several features of intra- and interspecific odorants in the male corn earworm moth *Helicoverpa zea*. *J. Comp. Physiol* A 169:249–258.

ALTNER, H., and PRILLINGER, L. 1980. Ultrastructure of invertebrate chemo-, thermo- and hygroreceptors and its functional significance. *Int. Rev. Cytol* 67:69–139.

ANDERSON, P., HANSSON, B. S., and LÖFQVIST, J. 1995. Plant-odour-specific receptor neurones on the antennae of female and male *Spodoptera littoralis*. *Physiol. Entomol.* 20:189–198.

ANGELI, S., CERON, F., SCALONI, A., MONIT, M., MONTEFORTI, G., MINOCCI, A., PETACCHI, R., and PELOSI, P. 1999. Purification, structural characterization, cloning and immunocytochemical localization of chemoreception proteins from *Schistocerca gregaria*. *Eur. J. Biochem.* 262:745–754.

ANTON, S., and ROSPARS, J.-P. 2004. Quantitative analysis of olfactory receptor neuron projections in the antennal lobe of the malaria mosquito, *Anopheles gambiae*. *J. Comp. Neurol* 475:315–326.

BAKER, T. C., and HAYNES, K. F. 1987. Manoeuvres used by flying male oriental fruit moths to relocate a sex pheromone plume in an experimentally shifted wind-field. *Physiol. Entomol* 12:263–279.

BAKER, T. C., and HAYNES, K. F. 1989. Field and laboratory electroantennographic measurements of pheromone plume structure correlated with oriental fruit moth behaviour. *Physiol. Entomol* 14:1–12.

BAKER, T. C., and VOGT, R. G. 1988. Measured behavioral latency in response to sex-pheromone loss in the large silk moth *Antheraea polyphemus*. *J. Exp. Biol* 137:29–38.

BAKER, T. C., WILLIS, M. A., HAYNES, K. F., and PHELAN, P. L. 1985. A pulsed cloud of sex pheromone elicits upwind flight in male moths. *Physiol. Entomol* 10:257–265.

BAKER, T. C., FADAMIRO, H. Y., and COSSÉ, A. A. 1998. Moth uses fine tuning for odor resolution. *Nature (London)* 393:530.

BAKER, T. C., QUERO, C., OCHIENG, S. A., and VICKERS, N. J. 2006. Inheritance of olfactory preferences. II. Olfactory receptor neuron responses from *Heliothis subflexa* x *Heliothis virescens* hybrid moths. *Brain Behav., Evol* 68:75–89.

BARTELT, R. J., SCHANER, A. M., and JACKSON, L. L. 1985. *cis*-Vaccenyl acetate as an aggregation pheromone in *Drosophila melanogaster*. *J. Chem. Ecol* 11:1747–1756.

BAU, J., JUSTUS, K. A., and CARDÉ, R. T. 2002. Antennal resolution of pheromone plumes in three moth species. *J. Insect Physiol* 48:433–442.

BEHREND, K. 1971. Riechen im wasser und in luft bei *Dytiscus marginalis* L. *J. Comp. Physiol* 75:108–122.

BENTON, R., SACHSE, S., MICHNICK, S. W., and VOSSHALL, L. B. 2006. Atypical membrane topology and heteromeric function of *Drosophila* odorant receptors in vivo. *PLOS Biol* 4:e20.

BERG, B. G., and MUSTAPARTA, M. 1995. The significance of major pheromone components and interspecific signals as expressed by receptor neurons in the oriental tobacco budworm moth, *Helicoverpa assulta*. *J Comp Physiol A* 177:683–694.

BLIGHT, M. M., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1995. Antennal perception of oilseed rape, *Brassica napus* (Brassicaceae), volatiles by the cabbage seed weevil *Ceutorhynchus assimilis* (Coleoptera, Curculionidae). *J. Chem. Ecol* 21:1649–1664.

BOECKH, J., ERNST, K.-D., and SELSAM, P. 1987. Neurophysiology and neuroanatomy of the olfactory pathway in the cockroach, pp. 39–43, in S. D. Roper, and J. Atema (eds.). Olfaction and taste IX. *Annals New York Academy of Sciences*, New York.

BOHBOT, J., PITTS, R. J., KWON, H. W., RUTZLER, M., ROBERTSON, H. M., and ZWIEBEL, L. J. 2007. Molecular characterization of the *Aedes aegypti* odorant receptor gene family. *Insect Mol. Biol* 16:525–537.

BRUCE, T. J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2005. Insect host location: a volatile situation. *Trends Plant Sci.* 10:269–274.

BUCK, L. B., and AXEL, R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65:175–187.

CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2002. Pheromonal and host-odor processing in the insect antennal lobe: how different? *Curr. Opin. Neurobiol* 12:393–399.

CLYNE, P. J., WARR, C. G., FREEMAN, M. R., LESSING, D., KIM, J., and CARLSON, J. R. 1999a. A novel family of divergent

seven-transmembrane proteins: candidate odorant receptors in *Drosophila*. *Neuron* 22:327–338.

CLYNE, P. J., CERTEL, S., DE BRUYNE, M., ZASLAVSKY, L., JOHNSON, W., and CARLSON, J. R. 1999b. The odor-specificities of a subset of olfactory receptor neurons are governed by acj6, a POU domain transcription factor. *Neuron* 22:339–347.

COSSÉ, A. A., TODD, J. L., and BAKER, T. C. 1998. Neurons discovered on male *Helicoverpa zea* antennae that correlate with pheromone-mediated attraction and interspecific antagonism. *J. Comp. Physiol. A* 182:585–594.

COUTO, A., ALENIUS, M., and DICKSON, B. J. 2005. Molecular, anatomical, and functional organization of the *Drosophila* olfactory system. *Curr. Biol* 15:1535–1547.

DAVID, C. T., KENNEDY, J. S., LUDLOW, A. R., PERRY, J. N., and WALL, C. 1982. A re-appraisal of insect flight towards a point source of wind-borne odor. *J. Chem. Ecol* 8:1207–1215.

DAVIS, E. E. 1984. Development of lactic acid-receptor sensitivity and host-seeking behaviour in newly emerged female *Aedes aegypti* mosquitoes. *J. Insect Physiol* 30:211–215.

DE BRUYNE, M., CLYNE, P. J., and CARLSON, J. R. 1999. Odor coding in a model olfactory organ: the *Drosophila* maxillary palp. *J. Neurosci* 19:4520–4532.

DE BRUYNE, M., FOSTER, K., and CARLSON, J. R. 2001. Odor coding in the *Drosophila* antenna. *Neuron* 30:537–552.

DEKKER, T., IBBA, I., SIJU, K. P., STENSMYR, M. C., and HANSSON, B. S. 2006. Olfactory shifts parallel superspecialism for toxic fruit in *Drosophila melanogaster* sibling, *D. sechellia*. *Curr. Biol* 16:101–109.

DOBRITSA, A., VAN DER GOES VAN NATERS, W. M., WARR, C. G., STEINBRECHT, R. A., and CARLSON, J. R. 2003. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37:827–841.

DOMINGUE, M. J., MUSTO, C. J., LINN, C. E. Jr, ROELOFS, W. L., and BAKER, T. C. 2007. Evidence of olfactory antagonistic release as a facilitator of evolutionary shifts in pheromone blend usage in *Ostrinia spp*. (Lepidoptera: Crambidae). *J. Insect Physiol* 53:488–496.

DU, G., and PRESTWICH, G. D. 1995. Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry* 34:8726–8732.

ELMORE, T., and SMITH, D. P. 2001. Putative *Drosophila* odor receptor OR43b localizes to dendrites of olfactory neurons. *Insect Biochem. Mol. Biol* 31:791–798.

ENDO, K., AOKI, T., YODA, Y., KIMURA, K., and HAMA, C. 2007. Notch signal organizes the *Drosophila* olfactory circuitry by diversifying the sensory neuronal lineages. *Nature Neurosci* 10:153–160.

ESSLEN, J., and KAISSLING, K.-E. 1976. Zahl und Verteilung antennalen Sensillen bei der Honigbiene (*Apis melifera* L.). *Zoomorphologie* 83:227–251.

FAUCHER, C., FORSTREUTER, M., HILKER, M., and DE BRUYNE, M. 2006. Behavioral responses of *Drosophila* to biogenic levels of carbon dioxide depend on life-stage, sex and olfactory context. *J. Exp. Biol.* 209:2739–2748.

FIRESTEIN, S. 2001. How the olfactory system makes sense of scents. *Nature* 413:211–218.

FISHILEVICH, E., DOMINGOS, A. I., ASAHINA, K., NAEF, F., VOSSHALL, L. B., and LOUIS, M. 2005. Chemotaxis behavior mediated by single larval olfactory neurons in *Drosophila*. *Curr. Biol* 15:2086–2096.

FRASER, A. M., MECHABER, W. L., and HILDEBRAND, J. G. 2003. Electroantennographic and behavioral responses of the sphinx moth *Manduca sexta* to host plant headspace volatiles. *J. Chem. Ecol.* 29:1813–1833.

GALINDO, K., and SMITH, D. P. 2001. A large family of divergent *Drosophila* odorant-binding proteins expressed in gustatory and olfactory sensilla. *Genetics* 159:1059–1072.

GALIZIA, C. G., and MENZEL, R. 2000. Probing the olfactory code. *Nature Neurosci* 3:853–854.

GOLDMAN, A. L., VAN DER GOES VAN NATERS, W. M., LESSING, D., WARR, C. G., and CARLSON, J. R. 2005. Coexpression of two functional odor receptors in one neuron. *Neuron* 45:661–666.

GOULDING, S. E., ZUR LAGE, P., and JARMAN, A. P. 2000. *amos*, a proneural gene for *Drosophila* olfactory sense organs that is regulated by *lozenge*. *Neuron* 25:69–78.

GRANT, A. J., WIGTON, B. E., AGHAJANIAN, J. G., and O'CONNELL, R. J. 1995. Electrophysiological responses of receptor neurons in mosquito maxillary palp sensilla to carbon dioxide. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 177:389–396.

GROSSE-WILDE, E., SVATOS, A., and KRIEGER, J. 2006. A pheromone-binding protein mediates the bombykol-induced activation of a pheromone receptor in vitro. *Chem. Senses* 31:547–555.

GROSSE-WILDE, E., GOHL, T., BOUCHÉ, E., BREER, H., and KRIEGER, J. 2007. Candidate pheromone receptors provide the basis for the response of distinct antennal neurons to pheromonal compounds. *Eur. J. Neurosci* 25:2364–2373.

GUERIN, P. M., STÄDLER, E., and BUSER, H. R. 1983. Identification of host plant attractants for the carrot fly, *Psilae rosae*. *J. Chem. Ecol*. 9:843–861.

GUO, S., and KIM, J. 2007. Molecular evolution of *Drosophila* odorant receptor genes. *Mol. Biol. Evol* 24:1198–1207.

GUPTA, B. P., and RODRIGUES, V. 1997. *Atonal* is a proneural gene for a subset of olfactory sense organs in *Drosophila*. *Genes to Cell* 2:225–233.

GUPTA, B. P., FLORES, G. V., BANERJEE, U., and RODRIGUES, V. 1998. Patterning an epidermal field: *Drosophila Lozenge*, a member of the AML-1/Runt family of transcription factors, specifies olfactory sense organ type in a dose-dependent manner. *Dev.Biol* 203:400–411.

HALLEM, E. A., and CARLSON, J. R. 2006. Coding of odors by a receptor repertoire. *Cell* 125:143–160.

HALLEM, E. A., FOX, A. N., ZWIEBEL, L. J., and CARLSON, J. R. 2004. Mosquito receptor for human sweat odorant. *Nature* 427:212–213.

HANSSON, B. S. 1995. Olfaction in lepidoptera. *Cell. Mol. Life Sci* 51:1003–1027.

HANSSON, B. S., and BAKER, T. C. 1991. Differential adaptation rates in a male moth's sex pheromone receptor neurons. *Naturwiss* 78:517–520.

HANSSON, B. S., and CHRISTENSEN, T. A. 1999. Functional characteristics of the antennal lobe, pp. 126–162, in B. Hansson (ed.). Insect OlfactionSpringer, Berlin.

HANSSON, B. S., TOTH, M., LÖFSTEDT, C., SZÖCS, G., SUBCHEV, M., and LÖFQVIST, J. 1990. Pheromone variation among eastern European and a western Asian population of the turnip moth *Agrotis segetum*. *J Chem Ecol* 16:1611–1622.

HANSSON, B. S., LARSSON, M., and LEAL, W. S. 1999. Green leaf volatile-detecting olfactory receptor neurones display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol* 24:121–126.

HAYNES, K. F., and BAKER, T. C. 1989. An analysis of anemotactic flight in female moths stimulated by host odour and comparison with the males' response to sex pheromone. *Physiol. Entomol* 14:279–289.

HEINBOCKEL, T., and KAISSLING, K.-E. 1996. Variability of olfactory receptor neuron responses of female silkmoths (*Bombyx mori* L.) to benzoic acid and (+/−)-linalool. *J. Insect Physiol* 42:565–578.

HILDEBRAND, J. G., and SHEPHERD, G. M. 1997. Mechanisms of olfactory discrimination: converging evidence for common principles across phyla. *Annu Rev Neurosci* 20:595–631.

HILL, C. A., FOX, A. N., PITTS, R. J., KENT, L. B., TAN, P. L., CHRYSTAL, M. A., CRAVCHIK, A., COLLINS, F. H., ROBERTSON, H. M., and ZWIEBEL, L. J. 2002. G protein-coupled receptors in *Anopheles gambiae*. *Science* 298:176–178.

ISHIDA, Y., and LEAL, W. S. 2005. Rapid inactivation of a moth pheromone. *Proc. Natl. Acad. Sci. USA* 102:14075–14079.

JONES, W. D., NGUYEN, T.-A. T., KLOSS, B., LEE, K. J., and VOSSHALL, L. B. 2005. Functional conservation of an insect odorant receptor gene across 250 million years of evolution. *Curr. Biol* 15:R119–R121.

JONES, W. D., CAYIRLIOGLU, P., KADOW, I. G., and VOSSHALL, L. B. 2007. Two chemosensory receptors together mediate carbon dioxide detection in *Drosophila*. *Nature* 445:86–90.

JUSTUS, K. A., and CARDÉ, R. T. 2002. Flight behaviour of two moths, *Cadra cautella* and *Pectinophora gossypiella*, in homogeneous clouds of pheromone. *Physiol. Entomol* 27:67–75.

JUSTUS, K. A., CARDÉ, R. T., and FRENCH, A. S. 2005. Dynamic properties of antennal responses to pheromone in two moth species. *J. Neurophysiol* 93:2233–2239.

KAISSLING, K. E. 1987. R. H. Wright lectures on insect olfaction, in K. Colbow (ed.). Simon Fraser University Press, Burnaby, BC, Canada.

KAISSLING, K.-E., MENG, L. Z., and BESTMANN, H. J. 1989. Responses of the bombykol receptor cells to (Z.E.)-4,6-hexadecadiene and linalool. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 165:147–154.

KEIL, T. 1997. Comparative morphogenesis of sensilla: a review. *Int. J. Insect Morphol. Embryol* 26:151–160.

KENDRA, P. E., MONTGOMERY, W. S., MATEO, D. M., PUCHE, H., EPSKY, N. D., and HEATH, R. R. 2005. Effect of age on EAG response and attraction of female *Anastrepha suspensa* (Diptera: Tephritidae) to ammonia and carbon dioxide. *Environ. Entomol* 34:584–490.

KENNEDY, J. S., LUDLOW, A. R., and SANDERS, C. J. 1981. Guidance of flying male moths by wind-borne sex pheromone. *Physiol. Entomol* 6:395–412.

KIELY, A., AUTHIER, A., KRALICEK, A., WARR, C. G., and NEWCOMB, C. D. 2007. Functional analysis of a *Drosophila melanogaster* olfactory receptor expressed in Sf9 cells. *J. Neurosci. Methods* 159:189–194.

KING, J. R., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2000. Response characteristics of an identified, sexually dimorphic olfactory glomerulus. *J. Neurosci* 20:2391–2399.

KLEINEIDAM, C., ROMANI, R., TAUTZ, J., and ISIDORO, N. 2000. Ultrastructure and physiology of the CO_2 sensitive sensillum ampullaceum in the leaf-cutting ant *Atta sexdens*. *Anthropod Struct. Dev.* 29:43–55.

KREHER, S. A., KWON, J. Y., and CARLSON, J. R. 2005. The molecular basis of odor coding in the *Drosophila* Larva. *Neuron* 46:445–456.

KRIEGER, J., RAMING, K., DEWER, Y., BETTE, S., CONZELMANN, S., and BREER, H. 2002. A divergent gene family encoding candidate olfactory receptors of the moth *Heliothis virescens*. *Eur. J. Neurosci* 16:619–628.

KRIEGER, J., KLINK, O., MOHL, C., and RAMING, K. 2003. A candidate odorant receptor subtype highly conserved across different insect orders. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 189:519.

KRIEGER, J., GROSSE-WILDE, E., GOHL, T., DEWER, Y., RAMING, K., and BREER, H. 2004. Genes encoding candidate pheromone receptors in a moth (*Heliothis virescens*). *Proc. Natl. Acad. Sci. USA* 101:11845–11850.

KRIEGER, J., GROSSE-WILDE, E., GOHL, T., and BREER, H. 2005. Candidate pheromone receptors of the silkmoth *Bombyx mori*. *Eur. J. Neurosci* 21:2167–2176.

KWON, H. W., RUTZLER, M., and ZWIEBEL, L. J. 2006. Olfactory responses in a gustatory organ of the malaria vector mosquito *Anopheles gambiae*. *Proc. Natl. Acad. Sci. USA* 103:13526–13531.

KWON, J. Y., DAHANUKAR, A., WEISS, L. A., and CARLSON, J. R. 2007. The molecular basis of CO_2 reception in *Drosophila*. *Proc. Natl. Acad. Sci. USA* 104:3574–3578.

LACHER, V. 1964. Elektrophysiologische Untersuchungen an einzelnen Rezeptoren für Geruch, Kohlendioxyd, Luftfeuchtigkeit und Temperatur auf den Antennen der Arbeitsbiene und der Drohne (*Apis mellifira* L.). *Z. vergl. Physiol.* 48:587–623.

LARSSON, M., DOMINGOS, A. I., JONES, W. D., CHIAPPA, M. E., AMREIN, H., and VOSSHALL, L. B. 2004. Or83b encodes a broadly expressed odorant receptor essential for *Drosophila* olfaction. *Neuron* 43:703–714.

LEAL, W. S., CHEN, A. M., ISHIDA, Y., CHIANG, V. P., ERICKSON, M. L., MORGAN, T. L., and TSURUDA, J. M. 2005. Kinetics and molecular properties of pheromone binding and release. *Proc. Natl. Acad. Sci. USA* 102:5386–5391.

LINN, C. E. Jr, CAMPBELL, M. G., and ROELOFS, W. L. 1986. Male moth sensitivity to multicomponent pheromones: critical role of female-released blend in determining the functional role of components and active space of the pheromone. *J. Chem. Ecol* 12:659–668.

LINN, C. E. Jr, MUSTO, C. J., DOMINGUE, M. J., BAKER, T. C., and ROELOFS, W. L. 2007. Support for (*Z*)-11-hexadecanal as a pheromone antagonist in *Ostrinia nubilalis*: flight tunnel and single sensillum studies with a New York population. *J. Chem. Ecol* 33:909–921.

LIU, Y. B., and HAYNES, K. F. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage looper moth, *Trichoplusia ni*. *J.Chem.Ecol* 18:299–307.

LÖFSTEDT, C., HANSSON, B. S., DIJKERMAN, H. J., and HERREBOUT, W. M. 1990. Behavioral and electrophysiological activity of unsaturated analogues of the pheromone tetradecenyl acetate in the small ermine moth *Yponomeuta rorellus*. *Physiol. Entomol* 15:47–54.

MAIBECHE-COISNE, M., NIKONOV, A. A., ISHIDA, Y., JACQUIN-JOLI, E., and LEAL, W. S. 2004. Pheromone anosmia in a scarab beetle induced by in vivo inhibition of a pheromone-degrading enzyme. *Proc. Natl. Acad. Sci. USA.* 101:11459–11464.

MAFRA-NETO, A., and CARDÉ, R. T. 1994. Fine-scale structure of pheromone plumes modulates upwind orientation of flying moths. *Nature* 369:142–144.

MATSUO, T., SUGAYA, S., YASUKAWA, J., AIGAKI, T., and FUYAMA, F. 2007. Odorant-binding proteins OBP57d and OBP57e affect taste Perception and host-plant preference in *Drosophila sechellia*. *PLoS Biol.* 5:5118Epub, May.

MCBRIDE, C. S. 2007. Rapid evolution of smell and taste receptor genes during host specialization in *Drosophila sechellia*. *Proc. Natl. Acad. Sci. USA* 104:4996–5001.

MEIJERINK, J., BRAKS, M. A. H., and VAN LOON, J. J. A. 2001. Olfactory receptors on the antennae of the malaria mosquito *Anopheles gambiae* are sensitive to ammonia and other sweat-borne components. *J. Insect Physiol*. 47:455–464.

NAKAGAWA, T., SAKURAI, T., NISHIOKA, T., and TOUHARA, K. 2005. Insect sex-pheromone signals mediated by specific combinations of olfactory receptors. *Science* 307:1638–1642.

NIKONOV, A., and LEAL, W. S. 2002. Peripheral coding of sex pheromone and a behavioral antagonist in the Japanese beetle, *Popillia japonica*. *J. Chem. Ecol* 28:1075–1089.

NOZAWA, M., and NEI, M. 2007. Evolutionary dynamics of olfactory receptor genes in *Drosophila* species. *Proc. Natl. Acad. Sci. USA* 104:7122–7127.

OCHIENG', S. A., and BAKER, T. C. 2002. Host plant volatiles synergize responses of sex pheromone-specific olfactory receptor neurons in male *Helicoverpa zea*. *J. Comp. Physiol. A* 188:325–333.

OLSSON, S. B., LINN, C. E. Jr, and ROELOFS, W. L. 2006. The chemosensory basis for behavioral divergence involved in sympatric host shifts. I. Characterizing olfactory receptor neuron classes responding to key host volatiles. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 192:279–288.

PARK, S.-K., SHANBHAG, S. R., DUBIN, A. E., DE BRUYNE, M., WANG, Q., YU, P., SHIMONI, N., D'MELLO, S., CARLSON, J. R., HARRIS, G. L., STEINBRECHT, R. A., and PIKIELNY, C. W. 2002. Inactivation of olfactory sensilla of a single morphological type differentially affects the response of *Drosophila* to odors. *J. Neurobiol* 51:248–260.

PELOSI, P., ZHOU, J. J., BAN, L. P., and CALVELLO, M. 2006. Soluble proteins in insect chemical communication. *Cell. Mol. Life. Sci* 63:1658–1676.

PERSOONS, C. J., VERWIEL, P. E., RITTER, F. J., TALMAN, E., NOOIJEN, P. J. F., and NOOIJEN, W. J. 1976. Sex pheromones of the American cockroach, *Periplaneta americana*: A tentative. structure of periplanone B. *Tetrahedron Lett* 24:2055–2058.

PHELAN, P. L. 1997. Genetics and phylogenetics in the evolution of sex pheromones, pp. 563–579, in R. T. Cardé, and A. K. Minks (eds.). Insect Pheromone Research: New DirectionsChapman & Hall, New York.

QUERO, C., FADAMIRO, H. Y., and BAKER, T. C. 2001. Responses of male *Helicoverpa zea* to single pulses of sex pheromone and behavioural antagonist. *Physiol. Entomol* 26:106–115.

RAGHU, S. 2004. Functional significance of phytochemical lures to dacine fruit flies (Diptera: Tephritidae): an ecological and evolutionary synthesis. *Bull. Entomol. Res* 94:385–399.

RAY, K., and RODRIGUES, V. 1995. Cellular events during development of olfactory sense organs in *Drosophila melanogaster*. *Dev. Biol* 167:426–438.

RAY, A., VAN DER GOES VAN NATERS, W. M., SHIRAIWA, T., and CARLSON, J. R. 2007. Mechanisms of odor receptor gene choice in *Drosophila*. *Neuron* 53:553–569.

ROELOFS, W. L., and ROONEY, A. P. 2003. Molecular genetics and evolution of pheromone biosynthesis in Lepidoptera. *Proc. Nat. Acad. Sci* 100:9179–9184.

ROELOFS, W. L., LIU, W., HAO, G., JIAO, H., ROONEY, A. P., and LINN, C. E. Jr 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci. USA* 99:13621–13626.

ROBERTSON, H. M., and WANNER, K. W. 2006. The chemoreceptor superfamily in the honey bee, *Apis mellifera*: expansion of the odorant, but not gustatory, receptor family. *Genome Res* 16:1395–1403.

ROBERTSON, H. M., WARR, C. G., and CARLSON, J. R. 2003. Molecular evolution of the insect chemoreceptor gene superfamily in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 100:14537–14542.

ROESSINGH, P., SEN, X., and MENKEN, S. B. J. 2007. Olfactory receptors on the maxillary palps of small ermine moth larvae: Evolutionary history of benzaldehyde sensitivity. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 193:635–647.

ROSTELIEN, T., STRANDEN, M., BORG-KARLSON, A. K., and MUSTAPARTA, H. 2005. Olfactory receptor neurons in two heliothine moth species responding selectively to aliphatic green leaf volatiles, aromatic compounds, monoterpenes and sesquiterpenes of plant origin. *Chem Senses* 30:443–461.

RUMBO, E. R., and KAISSLING, K.-E. 1989. Temporal resolution of odor pulses by three types of pheromone receptor cells in *Antheraea polyphemus*. *J Comp Physiol A* 165:281–291.

SAKURAI, T., NAKAGAWA, T., MITSUNO, H., MORI, H., ENDO, Y., ANOUE, S., YASUKOCHI, Y., TOUHARA, K., and NISHIOKA, T. 2004. Identification and functional characterization of a sex pheromone receptor in the silkmoth *Bombyx mori*. *Proc. Natl. Acad. Sci. USA* 101:16653–16658.

SANDLER, B. H., NIKINOVA, L., LEAL, W. S., and CLARDY, J. 2000. Sexual attraction in the silkworm moth: structure of the pheromone-binding-protein-bombykol complex. *Chem. Biol* 7:143–151.

SASS, H. 1976. Zur nervoesen codierung von geruchreizen bei *Periplaneta americana*. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 107:49–65.

SATO, K. ,PELLEGRIN, M., NAKAGAWA, T., VOSSHALL, L. B., and TOUHARA, K. 2008. Insect olfactory receptors are heteromeric ligand-gated ion channels. *Nature* 452:1002–1006.

SHANBHAG, S. R., MÜLLER, B., and STEINBRECHT, R. A. 2000. Atlas of olfactory organs of *Drosophila melanogaster* 2. Internal organization and cellular architecture of olfactory sensilla. *Arthropod Struct. Dev* 29:211–229.

SHANBHAG, S. R., HEKMAT-SCAFE, D., KIM, M. S., PARK, S. K., CARLSON, J. R., PIKIELNY, C., SMITH, D. P., and STEINBRECHT, R. A. 2001. Expression mosaic of odorant-binding proteins in *Drosophila* olfactory organs. *Microsc. Res. Tech* 55:297–306.

SHIELDS, V. D. C., and HILDEBRAND, J. G. 2001. Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 186:1135–1151.

STANGE, G. 1992. High resolution measurement of atmospheric carbon dioxide concentration changes by the labial palp organ of the moth *Heliothis armigera* (Lepidoptera:Noctuidae). *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 171:317–324.

STANGE, G., and STOWE, S. 1999. Carbon-dioxide sensing structures in terrestrial arthropods. *Microsc. Res. Tech* 47:416–427.

STEINBRECHT, R. A. 1997. Pore structures in insect olfactory sensilla: A review of data and concepts. *Int. J. Insect Morphol. Embryol* 26:229–245.

STEINBRECHT, R. A. 1999. Olfactory receptors, pp. 156–176, in E. Eguchi, and Y. Tominaga (eds.). Atlas of arthropod sensory receptorsSpringer-Verlag, Tokyo.

STEINER, S., ERDMANN, D., STEIDLE, J. L. M., and RUTHER, J. 2007. Host habitat assessment by a parasitoid using fungal volatiles. *BMC Frontiers Zool* 4:3.

STENSMYR, M. C., LARSSON, M. C., BICE, S., and HANSSON, B. S. 2001. Detection of fruit- and flower-emitted volatiles by olfactory receptor neurons in the polyphagous fruit chafer *Pachnoda marginata* (Coleoptera: Cetoniinae). *J Comp Physiol [A]* 187:509–519.

STENSMYR, M. C., DEKKER, T., and HANSSON, B. S. 2003. Evolution of the olfactory code in the *Drosophila melanogaster* subgroup. *Proc. Natl. Acad. Sci. USA* 270:2333–2340.

STÖRTKUHL, K. F., and KETTLER, R. 2001. Functional analysis of an olfactory receptor in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* 98:9381–9385.

STRANDEN, M., LIBLIKAS, I., KONIG, W. A., ALMAAS, T. J., BORG-KARLSON, A. K., and MUSTAPARTA, H. 2003. (−)-Germacrene D receptor neurones in three species of heliothine moths: structure-activity relationships. *J Comp Physiol A Neuroethol Sens Neural Behav Physiol* 189:563–577Epub 2003 Jun 25.

SUH, G. S., WONG, A. M., HERGARDEN, A. C., WANG, J. W., SIMON, A. F., BENZER, S., AXEL, R., and ANDERSON, D. J. 2004. A single population of olfactory sensory neurons mediates an innate avoidance behaviour in *Drosophila*. *Nature* 431:854–859Epub 2004 Sep 15.

SYED, Z., and LEAL, W. S. 2007. Maxillary palps are broad spectrum odorant detectors in *Culex quinquefasciatus*. *Chem Senses* 32:727–7382007.

SYED, Z., ISHIDA, Y., TAYLOR, K., KIMBRELL, D. A., and LEAL, W. S. 2006. Pheromone reception in fruit flies expressing a moth's odorant receptor. *Proc. Natl. Acad. Sci. USA* 103:16538–16543.

TANEJA, J., and GUERIN, P. M. 1995. Oriented responses of the triatomine bugs *Rhodnius prolixus* and *Triatoma infestans* to vertebrate odours on a servosphere. *J. Comp. Physiol. A Sens. Neural Behav. Physiol* 176:455–464.

TAKKEN, W., and KNOLS, B. G. J. 1999. Odor-mediated behavior of Afrotropical malaria mosquitoes. *Annu. Rev. Entomol* 44:131–157.

TODD, J. L., BAKER, and T. C. 1999. Function of peripheral olfactory organs, pp. 67–96, in B. S. Hansson (ed.). Insect olfaction-Springer-Verlag, Berlin.

TUNSTALL, N. E., SIREY, T., NEWCOMB, R. D., and WARR, C. G. 2007. Selective pressures on *Drosophila* chemosensory receptor genes. *J. Mol. Evol* 64:628–636.

TURLINGS, T. C., and TON, J. 2006. Exploiting scents of distress: the prospect of manipulating herbivore-induced plant odours to enhance the control of agricultural pests. *Curr. Opin. Plant Biol*. 9:421–427.

VAN DER GOES VAN NATERS, W. M., and CARLSON, J. R. 2007. Receptors and neurons for fly odors in *Drosophila*. *Curr. Biol* 17:606–612.

VAN DER GOES VAN NATERS, W. M., BOOTSMA, L., DEN OTTER, C. J., and BELEMTOUGRI, R. G. 1996. Search for tsetse attractants: A structure–activity study on 1-octen-3-ol in *Glossina fuscipes fuscipes* (Diptera: Glossinidae). *J. Chem. Ecol* 22:343–355.

VICKERS, N. J., and BAKER, T. C. 1994. Reiterative responses to single strands of odor promote sustained upwind flight and odor source location by moths. *Proc. Nat. Acad Sci. USA* 91:5756–5760.

VICKERS, N. J., and BAKER, T. C. 1996. Latencies of behavioral response to interception of filaments of sex pheromone and clean air influence flight track shape in *Heliothis virescens*(F.) males. *J. Comp. Physiol*. *A* 178:831–847.

VICKERS, N. J., and BAKER, T. C. 1997. Chemical communication in heliothine moths. VII. Correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol* 180:523–536.

VICKERS, N. J., CHRISTENSEN, T. A., BAKER, T. C., and HILDEBRAND, J. G. 2001. How do odour plume dynamics influence the brain's olfactory code. *Nature* 410:466–470.

VISSER, J. H. 1986. Host odour perception in phytophagous insects. *Annu. Rev. Entomol* 31:121–144.

VOGT, R. G. 2005. Molecular basis of pheromone detection in insects, pp. 753–804, in L. I. Gilbert, K. Iatro, and S. Gill (eds.). Comprehensive insect physiology, biochemistry, pharmacology and molecular biology. Volume 3Endocrinology. Elsevier, London.

VOGT, R. G., and RIDDIFORD, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163.

VOGT, R. G., PRESTWICH, G. D., and LERNER, M. R. 1991. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol* 22:74–84.

VOSSHALL, L. B., and STOCKER, R. F. 2007. Molecular architecture of smell and taste in *Drosophila*. *Annu. Rev. Neurosci* 30:505–533.

VOSSHALL, L. B., AMREIN, H., MOROZOV, P. S., RZHETSKY, A., and AXEL, R. 1999. A spatial map of the olfactory receptor expression in the *Drosophila* antenna. *Cell* 96:725–736.

WANNER, K. W., ANDERSON, A. R., TROWELL, S. C., THEILMANN, D. A., ROBERTSON, H. M., and NEWCOMB, R. D. 2007. Female-biased expression of odourant receptor genes in the adult antennae of the silkworm, *Bombyx mori*. *Insect Mol. Biol* 16:107–119.

WETZEL, C. H., BEHRENDT, H.-J., GISSELMANNN, G., STÖRTKUHL, K. F., HOVEMANN, B. T., and HATT, H. 2001. Functional expression and characterization of a *Drosophila* odorant receptor in a heterologous cell system. *Proc. Natl. Acad. Sci. USA* 98:9377–9380.

WICHER, D., SCHÄFER, R., BAUERNFEIND, R., STENSMYR, M.C., HELLER, R., HEINEMANN, S.H., and HANSSON, B. S. 2008. *Drosophila* odorant receptors are both ligand-gated and cyclic-nucleotide-activated cation channels. *Nature* 452:1007–1011.

WISTRAND, M., KÄLL, L., and SONNHAMMER, E. L. 2006. A general model of G protein-coupled receptor sequences and its application to detect remote homologs. *Protein Sci* 15:509–521.

WITZGALL, P., and PRIESNER, E. 1991. Wind-tunnel study on attraction inhibitor in male *Coleophora laricella* Hbn. (Lepidoptera:Coleophoridae). *J. Chem. Ecol* 17:1355–1362.

XU, P., ATKINSON, R., JONES, D. N., and SMITH, D. P. 2005. *Drosophila* OBP LUSH is required for activity of pheromone-sensitive neurons. *Neuron* 45:193–200.

YAO, C. A., IGNELL, R., and CARLSON, J. R. 2005. Chemosensory coding by neurons in the coeloconic sensilla of the *Drosophila* antenna. *J. Neurosci* 25:8359–8367.

ZACHARUK, R. Y. 1985. Antennae and sensilla, pp. 1–69, in G. A. Kerkut, and L. I. Gilbert (eds.). Comprehensive insect physiology biochemistry and pharmacology, vol.6, Nervous system: sensory. Pergamon press, Oxford.

ZACHARUK, R. Y., and SHIELDS, V. D. C. 1991. Sensilla of immature insects. *Annu. Rev. Entomol* 36:331–354.

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REVIEW ARTICLE

Neural Processing, Perception, and Behavioral Responses to Natural Chemical Stimuli by Fish and Crustaceans

Charles D. Derby · Peter W. Sorensen

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Abstract This manuscript reviews the chemical ecology of two of the major aquatic animal models, fish and crustaceans, in the study of chemoreception. By necessity, it is restricted in scope, with most emphasis placed on teleost fish and decapod crustaceans. First, we describe the nature of the chemical world perceived by fish and crustaceans, giving examples of the abilities of these animals to analyze complex natural odors. Fish and crustaceans share the same environments and have evolved some similar chemosensory features: the ability to detect and discern mixtures of small metabolites in highly variable backgrounds and to use this information to identify food, mates, predators, and habitat. Next, we give examples of the molecular nature of some of these natural products, including a description of methodologies used to identify them. Both fish and crustaceans use their olfactory and gustatory systems to detect amino acids, amines, and nucleotides, among many other compounds, while fish olfactory systems also detect mixtures of sex steroids and prostaglandins with high specificity and sensitivity. Third, we discuss the importance of plasticity in chemical sensing by fish and crustaceans. Finally, we conclude with a description of how natural chemical stimuli are processed by chemosensory systems. In both fishes and crustaceans, the olfactory system is especially adept at mixture discrimination, while gustation is well suited to facilitate precise localization and ingestion of food. The behaviors of both fish and crustaceans can be defined by the chemical worlds in which they live and the abilities of their nervous systems to detect and identify specific features in their domains. An understanding of these worlds and the sensory systems that provide the animals with information about them provides insight into the chemical ecology of these species.

Keywords Chemical ecology · Chemoreception · Gustation · Olfaction · Pheromone

C. D. Derby (✉)
Brains and Behavior Program, Center for Behavioral Neuroscience, Department of Biology, Georgia State University, Atlanta, GA, USA
e-mail: cderby@gsu.edu

P. W. Sorensen
Department of Fisheries and Wildlife, University of Minnesota, St. Paul, MN, USA

Chemical Senses in the Aquatic Medium

Aquatic organisms evolved in a world of dissolved chemicals, and this resulted in the appearance of numerous types of chemosensory systems. As in terrestrial organisms, the senses of olfaction and gustation are usually identified as the main chemosensory systems in aquatic organisms, although the distinctions between them are somewhat blurred. This is because the primary distinction between these senses in terrestrial organisms—the physical medium through which chemical molecules are delivered to animals (air for olfaction and water for gustation)—does not apply in aquatic systems. Thus, a second basis for distinguishing between olfaction and gustation—neuroanatomy—becomes particularly relevant for aquatic animals. However, this distinction is more useful for vertebrates than invertebrates, whose neuroanatomy differs. In aquatic vertebrates, like all vertebrates, olfaction is defined as the sense mediated by neurons with axons in the olfactory nerve (cranial nerve I). In addition, a common feature of olfaction in vertebrates and many invertebrates is that the first-order processing regions in their brains are organized into glomeruli, which contain the synapses between the olfactory receptor neurons

and interneurons, and that these brain regions have a chemotopic organization such that different odorants generate distinctive patterns of glomerular activity (Hildebrand and Shepherd 1997; Eisthen 2002; Ache and Young 2005). Gustation in vertebrates, on the other hand, is mediated by non-neuronal, modified epithelial cells that are innervated by the facial (cranial nerve VII), glossopharyngeal (cranial nerve IX), and vagal (cranial nerve X) nerves that project to different brain structures and appear to have different functions (Atema 1977; Caprio et al. 1993). Another way that olfaction and gustation can be distinguished is by their function: Gustation is more apt to mediate simple and reflexive behaviors, food consummatory behaviors in particular, whereas olfaction tends to mediate more complex behaviors such as searching for distant sources of chemicals, courtship behavior, and learning about odors (Atema 1977).

Besides olfaction and gustation, both fish and crustaceans have a diversity of other, less understood chemical senses. Fish have a trigeminal system and solitary chemoreceptor cells that cover their bodies (Kapoor and Finger 2003), whose functions are not yet clearly established. Crustaceans have a diversity of chemoreceptor neurons that differ in their packaging within sensilla, their connections and organization in the central nervous system, and the behaviors that they mediate (Horner et al. 2006, 2008b). One pathway of crustaceans—the aesthetasc sensilla and the olfactory lobe pathway—is considered 'olfactory' because of organizational similarities between it and the olfactory pathways of vertebrates and insects. While other crustacean chemosensors are typically packaged with mechanosensors into sensilla, these sensilla are extremely diverse in structure and distributed differently across the animal's body surface where they serve different behavioral functions (see "Processing of Natural Chemical Stimuli"). Regardless of how chemosensory systems are defined, it is important to recognize that aquatic organisms have a variety of chemosensory systems whose neuroanatomical structures and functions vary dramatically.

The Chemical World Perceived by Fish and Crustaceans

Aquatic animals detect, discriminate, and respond to a wealth of chemicals in their natural environment. This diversity is immense, as aquatic organisms in general release literally thousands of small and soluble products that can carry information. Notably, most of the compounds found in aquatic environments are relatively unspecialized metabolic products (Atema 1988; Carr 1988). With the possible exception of some pheromones (Sorensen and Stacey 1999), there is little evolutionary pressure for organisms to produce specialized chemicals that facilitate their discrimination. Chemoreception is basic to meeting most biological needs of organisms, including those related to reproduction, social interactions, acquiring food and shelter, and defense from predators. Of course, this is particularly true in waters with low light levels. For the most part, chemoreception in aquatic ecosystems requires the detection of small differences in mixture composition in complex backgrounds, as opposed to detection of a few specialized compounds. In the next section, we give select examples of the discriminatory abilities of fish and crustaceans that focus on the nature of their chemical worlds and their abilities to detect, discriminate, and respond to them. A discussion of the molecular identity of important chemicals and how they are neurally processed follows later.

Fish

Fish use chemicals that mediate many key aspects of their lives, most of which are poorly understood but appear highly complex. Among the most important are habitat recognition, food finding, conspecific identification, and predator avoidance. Fish are the most diverse group of vertebrates, represented by more than 26,000 species that live in an immense variety of habitats. The type, concentration, and distribution of chemicals in their environments are all important factors in determining the chemical ecology and life history strategies. Because of this vast diversity, our review considers only a small number of fish species and situations, so we have selected representative examples.

Most species of fish are highly mobile and exhibit a variety of complex behaviors, many of which depend on them having information about their environment. Ablation studies often demonstrate that the type and location of habitat is determined by using environmental chemicals as cues. Although some species, such as freshwater bass, live in and compete for limited territories, other species are migratory and travel great distances only to return to very specific locations to reproduce. For example, Pacific salmon are born in small inland streams and then migrate to oceans to feed but eventually return 'home' by using olfactory cues (Hasler and Scholz 1983). A variety of studies demonstrate that the chemical nature of 'home stream' odor is learned. Salmon select between proximate streams when returning home by choosing the stream in which they were raised many years earlier. Given the proximity and ecological similarities of the streams among which Pacific salmon must choose, it has been suggested that the odor that they learn is complex and comprised of mixtures of compounds from minerals, plants, and animals (Hasler and Wisby 1951; Nordeng 1977). Salmon can identify stream odors after many years at sea and

environmental change, demonstrating their remarkable ability to recognize patterns. Another interesting example of olfactory-driven migration is the parasitic sea lamprey, which has a similar life history to salmon except that it does not return to home streams but rather selects streams that contain young lamprey. Lamprey recognize home streams by using innately recognized pheromones released by larval lamprey in combination with other unknown compounds found in all stream waters (Sorensen et al. 2003, 2005; Sorensen and Hoye 2007). A third example is the use of olfactory cues by non-migratory fish to identify home ranges within streams—difference in the odor mixtures they discriminate must be subtle (Gunning 1959; Arnesen and Stabell 1992). These three examples demonstrate that fish discriminate complex matrices of waterborne chemicals and can remember and track them through space and time by using their olfactory systems that demonstrate sophisticated perceptual abilities.

As most fishermen know, fish are also adept at using chemicals to identify and locate food, even in turbid, deep, or dark waters. This highlights the outstanding ability of fish to perceive chemicals associated with food and may even be partially responsible for the evolutionary success of fish in exploiting diverse feeding niches (Moyle and Cech 2000). Both olfaction and gustation are used in distinguishing between similar types of food. Most fish are 'feeding generalists' with keenly developed abilities to identify and locate a range of foods based on their nutritive values, even in changing environments in which the relative abundance of specific prey may dramatically shift. Notably, fish have evolved multiple suites of feeding behaviors, including appetitive and consummatory behaviors (Jones 1992; Valentinčič 2005). These behaviors are mediated by both olfaction and gustation, which can detect overlapping sets of relatively common metabolic products. L-Amino acids are the most important of these, but other classes are known (see below). Species differences in sensitivity to these classes of chemical stimuli are common, and almost certainly, there are innate abilities to learn certain types of stimuli (Jones 1992). These abilities may be mediated by the gustatory sense, which is generally more narrowly tuned and often linked to mechanistic responses. Nevertheless, in the natural world, most feeding chemical stimuli appear to be discriminated as complex mixtures. For example, Carr (1982) reports that mixtures of 17 to 22 amino acids plus betaine could account for the majority, though not all, of the feeding activity elicited by four natural food items for the pigfish but not the related pinfish. Finally, as one might expect, selection of food by at least some marine fish is influenced by their detection of deterrent molecules, such as alkaloids, tetrodotoxin, and acids. This subject has received considerable attention from chemical ecologists but, unfortunately, little work from neuroscientists (Hara 1994; Hay 1996; Kicklighter et al. 2005; Hayden et al. 2007; Kamio et al. 2007; Cohen et al. 2008).

Of course, fish do more than hide and eat. Pheromones, here defined as sets of chemicals that convey information about an individual's identity and condition to other members of its species, play essential roles in the sexual and social life histories of most fishes (Stacey and Sorensen 2005). Olfactory blocking studies consistently demonstrate that olfaction mediates the perception of sex pheromones, which are often so important that anosmic fish simply fail to mate (Stacey and Kyle 1983). Finding and identifying conspecifics of appropriate maturity in complex natural environments can be challenging, so mating in even highly visual species such as swordtails is almost always assisted by pheromones (Wong et al. 2005). Intraspecific cues and pheromones can have multiple functions. Among these, species recognition for the purpose of aggregation or schooling is paramount. Although the chemical basis of aggregation is not clear, variations in metabolite production—rather than production of novel, species-specific compounds—are likely responsible (Sorensen and Stacey 1999). Odor recognition systems tuned to small variations in mixture composition should provide species-specific information. In addition to using olfaction for species recognition, some fish, including sticklebacks and salmon, use odors to determine kinship. These odors may include major histocompatibility complex-related peptides (Reusch et al. 2002; Ward and Hart 2002). Finally, almost all fish are able to discriminate reproductive state by using sex pheromones that they detect with high sensitivity and specificity (Stacey and Sorensen 2005). Hormonally derived signals are especially important, presumably because of their inherent relevance (Stacey and Sorensen 2005). However, hormone systems are highly conserved, thus providing little latitude for the evolution of novel hormonal products and their receptors, and some species have evolved to use a wider range of products (Li et al. 2002; Yambe et al. 2007).

In addition to sex pheromones, many fish can detect intraspecific chemicals that indicate danger, in particular, chemicals from conspecifics injured by predators (Smith 1992; Døving et al. 2005). However, injured fish release a variety of chemicals, some of which (e.g., amino acids) may be food cues in some contexts. Since the first description of the alarm response in European minnows in the field by von Frisch (1938), at least six types of compounds or their mixtures have been proposed to mediate alarm responses, with the purine hypoxanthine-3-*N*-oxide receiving the most attention (Pfeiffer et al. 1985; Brown et al. 2000, 2003). However, none of the suggested bioactive molecules has received supporting evidence from electrophysiological recordings or chemical measurements.

Interestingly, several studies suggest that the alarm cues can be learned (Wisenden 2000), raising the possibility that multiple types or mixtures of chemicals may be involved. The observation that fish recognize chemicals released by predators that have eaten conspecifics—presumably conspecific alarm cues that remain functional after being digested by the predators (Brown et al. 1993)—supports this possibility. Alarm cues from injured conspecifics are mediated by the olfactory system (Maniak et al. 2000; Døving et al. 2005).

In summary, fish live in complex environments wherein they face extreme challenges in finding shelter, mates, and food, while at the same time avoiding predators. They use their chemical senses in these behaviors by discriminating complex (though, at present, often incompletely defined) chemical mixtures of relatively common molecules. These abilities have allowed fish to succeed and diversify, becoming the majority of planet's vertebrate biomass and biodiversity.

Crustaceans

Crustaceans, like fish, rely on combinations of sophisticated chemosensory systems to identify and locate food, mates, and predators in noisy chemical environments filled with a multitude of products. The best studied chemosensory behavior in crustaceans is the selection and acquisition of food. Crustaceans use antennular chemoreception to identify attractive food (Derby 2000; Derby et al. 2001) and locate it from a distance (Atema 1996; Zimmer and Butman 2000; Grasso and Basil 2002; Weissburg et al. 2002; Keller and Weissburg 2004). Amino acids and nucleotides are two major sets of molecules that they use. Once near the food, ingestion is based on input from their gustatory systems on legs and mouthparts (Derby 2000; Derby et al. 2001). Food selection and ingestion is influenced by the blend of attractive and deterrent compounds, although we know more about the former than the latter (Derby et al. 2001; Prusak et al. 2005; Kamio et al. 2007). Some crustaceans can learn to avoid food associated with gastric malaise (Wight et al. 1990).

Crustaceans make use of chemical signals in most aspects of their reproduction. They use sex pheromones to identify and locate conspecifics of the opposite sex. Copepods, amphipods, shrimp, crabs, lobsters, and crayfish are leading examples (Gleeson 1991; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002, 2008; Stebbing et al. 2003; Ting and Snell 2003; Caskey and Bauer 2005; Ekerholm and Hallberg 2005; Belanger and Moore 2006; Atema and Steinbach 2007). Some sex pheromones are detected from a distance, others seem to be used in close range, even requiring contact. Chemical cues are also used in other aspects of reproduction. For example, many female crustaceans incubate their fertilized eggs, and chemicals released from hatching eggs induce abdominal pumping, fanning, and other behaviors from the females that facilitate the rapid and synchronized hatching of eggs and release of larvae (Tankersley et al. 2002; Rittschof and Cohen 2004).

Crustaceans use chemical cues during intraspecific interactions and social behavior. These chemicals are often in their urine and under controlled release so that they can be used at appropriate times during behavioral interactions (Breithaupt 2001; Breithaupt and Atema 2000; Breithaupt and Eger 2002; Moore and Bergman 2005; Moore 2007). Some crustaceans, such as lobsters and hermit crabs, use cues to recognize individual conspecifics (Johnson and Atema 2005; Gherardi et al. 2005). Others, such as crayfish, use cues to determine social status (Moore and Bergman 2005; Moore 2007). Lobsters use chemical information in aggressive interactions with conspecific (Breithaupt and Atema 2000). Spiny lobsters, which are highly social animals that often live in aggregations, use chemicals to identify each other and find safe shelter (Zimmer-Faust et al. 1985; Nevitt et al. 2004; Briones-Fourzán and Lozano-Álvarez 2005; Horner et al. 2006, 2008b). Spiny lobsters even recognize diseased conspecifics through chemical cues and avoid aggregating with them (Behringer et al. 2006). Young crayfish, which associate with their mother for some days after hatching, can locate her, as well as the shelter that she provides, by means of chemicals that she releases around the time of hatching (Little 1975). This cue appears not be specific to mother but is sex specific (Little 1976).

Crustaceans use chemicals to locate high-quality shelter or places to live. A well-known and long-studied example is the selection of sites to settle by larval barnacles (Dreanno et al. 2006a, b). Crustaceans such as pea crabs that live as commensals or symbionts with other organisms use chemical cues to locate their future hosts (Grove and Woodin 1996). Chemical cues are also used by hermit crabs to recognize shells as future homes (Rittschof and Cohen 2004).

Crustaceans also use chemoreception to avoid predators. Some can sense predators from a distance and thereby avoid them. Examples include crayfish and spiny lobsters (Berger and Butler 2001; Bouwma and Hazlett 2001). Predator avoidance can also be mediated through a less direct mechanism. Some crustaceans release chemicals when damaged, via leakage of body fluids, or when disturbed, via controlled release in urine, and these chemicals are avoided by conspecifics. Examples of species that use alarm cues include crayfish, spiny lobsters, and hermit crabs (Hazlett 1994; Zimmer-Faust et al. 1985; Rittschof et al. 1992; Nevitt et al. 2000; Zulandt Schneider and Moore 2000; Shabani et al. 2006; Bouwma 2007).

The Molecular Identity of Chemical Cues and Signals

Methods of Identification

The identity of bioactive molecules can be elucidated by using natural products chemistry techniques together with bioassays, based on any of several experimental approaches. Bioassay-guided fractionation is a standard technique that makes no assumptions about the nature of the bioactive substances. By this method, a natural product is separated into fractions based on any of a number of properties, including solubility in solvents of different polarity, molecular mass, and molecular charge. One method is a Kupchan partition scheme or modifications thereof, which is based on partitions differing in solubility—hexanes, chloroform, ethyl acetate, butanol, or water (Kupchan et al. 1975). With each separation, the resultant fractions are tested for bioactivity, usually with behavioral or electrophysiological assays. Comparison of the bioactivity of the fractions vs. the original material and a negative control allows identification of fractions that contain active molecules. When a natural product has more than one active ingredient, more than one fraction may have activity. It is often possible to separate bioactive molecules to sufficient purity to identify them through mass spectroscopy, nuclear magnetic resonance (NMR), or other analytical procedures. Databases such as Marinlit (http://www.chem.canterbury.ac.nz/marinlit/marinlit.shtml) and Chenomx NMR suite (http://www.chenomx.com), which contain known molecules, can be searched using known features of the bioactive molecules to identify possible molecular structures. Potential problems with this approach include degradation of bioactive molecules during separation and purification, and synergistic interactions among bioactive molecules that partition into different fractions such that their activity cannot be followed.

A second experimental approach to the molecular identification of chemical cues and signals is to determine which molecules are in relatively high concentration in the natural extracts that contain them. For example, when seeking a female sex pheromone in crustaceans, one might identify molecules in higher concentration in water from reproductive females compared to water from conspecifics that do not produce the pheromone. An example of this is the use of metabolomics to identify sex pheromones in blue crabs (Kamio et al. 2006). Metabolomics is a high-throughput approach to identify molecules enriched in or unique to one stimulus vs. another, usually focusing on small metabolites. Metabolomics has the advantage of not requiring purification of a component but can be based on spectra from mixtures (Daviss 2005). It can use data from either mass spectroscopy or NMR. This approach does not guarantee identification of bioactive molecules, in part because the bioactive molecules are not necessarily those in high concentrations, especially in the fishes whose pheromones can be common hormonal products (Sorensen and Scott 1994).

A third approach is searching for specific types of chemicals based on knowledge of the chemistry or biology of a system. For example, when ink secretions from sea hares were found to excite lobster chemosensory neurons, knowing that many of those neurons are sensitive to amino acids prompted amino acid analysis of the sea hare secretions and the eventual demonstration that amino acids in those secretions play an important defensive role (Kicklighter et al. 2005; Derby et al. 2007).

Fish

Fish perceive complex mixtures that contain a diversity of types of chemicals. Although there is presently no complete explanation for these perceptual abilities, electrophysiological studies have identified seven major classes of chemical stimuli that explain some of these abilities. These classes are amino acids, amines, nucleotides, bile acids (reduced steroids produced by the liver), aminosterols (a special class of bile steroids conjugated with amines), sex steroids, and prostaglandins. These compounds are all small (<8 kD), polar, and ubiquitous (Carr 1988; Hara 1994, 2007; Carr et al. 1996; Caprio and Derby 2007). There is overlap among classes—for example, both bile acids and sex steroids are steroids, and the relationship between olfactory receptor type and ligand class is not yet clear, so these categories of chemicals may mean more to biochemists than they do to the fish. Almost certainly, more classes of chemical stimuli await identification. Here, we briefly review our current understanding of known stimuli and how they are perceived by fish.

The chemical nature of food cues is best understood. Amino acids appear to be universally used by fish in this regard, likely because most are predators and have high protein requirements, and thus, amino acids are good indicators of high-quality food. Laboratory studies consistently demonstrate that specific mixtures of L-amino acids can attract many fish species and that single amino acids sometimes trigger reflexive snapping and biting behaviors, which at least on occasion are linked with the gustatory sense (Mackie 1982; Caprio et al. 1993; Hara 1994; Carr et al. 1996). Electrophysiological recordings demonstrate that fish olfactory systems detect all primary L-amino acids with high (nanomolar) sensitivity and specificity (Hara 1994, 2007). Fish external gustatory systems also detect L-amino acids, although often with much narrower ranges of sensitivity and, generally, in species-specific manners (Hara 2007; see below). This difference in the sensitivity of fish olfactory and gustatory systems is correlated with the

different but overlapping functions of these systems (see "Processing of Natural Chemical Stimuli").

Although amino acids are critical to food recognition, other molecules also are employed. The detailed studies on food recognition and ingestion show that L-amino acids rarely can explain all behaviors (Atema et al. 1980; Carr 1982; Carr et al. 1996). Indeed, in the case of marine fishes, betaine (a methylated amino acid) and D-amino acids, both present in marine environments, are important gustatory stimulants that can enhance the actions of amino acids (Carr 1982; Carr et al. 1996; Sorensen and Caprio 1998). Polyamines, which are protein breakdown products, are powerful stimulants of feeding arousal and search in goldfish due to their activation of specific olfactory pathways (Rolen et al. 2003). Nucleotides, which stimulate the olfactory and gustatory systems of many fish, have roles too (Carr 1988; Carr et al. 1996). Other bioactive chemicals will almost certainly come to light with further investigation. The blend of chemicals in a mixture is important to its efficacy, especially in olfactory-driven arousal and search behaviors. In summary, a wide variety of nitrogenous products serve as feeding cues for fish, although other bioactive chemicals, some yet to be identified, surely contribute.

With the exception of the sea lamprey (see below), little is known about the identities of chemical cues employed by fish to recognize the location or type of habitat. For species such as landlocked masu salmon that learn locational odors, the challenge is especially complex, although behavioral tests suggest that mixtures of L-amino acids are an important part of home stream odor recognition (Shoji et al. 2003). It is difficult to envision that amino acids are the sole contributors to home stream odor in more complex ecosystems because they are ubiquitous and their concentrations change with time and location. Suggestions that bile acids that originate from fish contribute to home stream odor (Døving et al. 1980) are intriguing, especially because they are detected by fish olfactory systems with great sensitivity and specificity. However, they are relatively generic and vary little among species (Sorensen, unpublished). Complex mixtures that contain many classes of chemicals are most likely to be important. Even for the relatively simple sea lamprey, mixtures are important. Using bioassay-guided fractionation, Sorensen et al. (2005) discovered three new sulfated steroids—petromyzonamine disulfate, petromyzosterol disulfate, and petromyzonol sulfate—that are released by larval lamprey and attract adults at concentrations below 10^{-13} M. All three components stimulate the olfactory system with great specificity, and while they will attract migratory adults on their own, they synergize each other's actions, especially in the context of stream water (Sorensen et al. 2003). Other unidentified minor components exist in this mixture (Fine et al. 2004, unpublished). Why the ancient and relatively simple lamprey has evolved a multi-component pheromone is unclear, but it may relate to the challenges they face in locating home streams at great distances.

The vast majority of fish species appear able to detect and discriminate sex steroids and F-series prostaglandins as hormonal pheromones (Stacey and Sorensen 2005). Indeed, the olfactory systems of dozens of species from a variety of taxa (cyprinids, salmonids, and gobids) are able to detect hormonal products with high sensitivity and specificity, and in about half a dozen instances, biological responses have been noted as well (Stacey and Sorensen 2005). Critical to the detection of sex steroids is the number of carbons in the steroid nucleus, absence or presence of a conjugating group, and side-chain position and structure (Sorensen et al. 1990; Stacey and Sorensen 2005). Most sex steroid products are relatively common, and for the most part, species from the same genus share the same sensitivities. Thus, sex steroids alone cannot be responsible for species specificity. To date, only prostaglandin F2α and its immediate metabolites have been shown to be especially active stimulants for an entire taxonomic group (e.g., minnows; Stacey and Sorensen 2005). We speculate that signal specificity resides with contextual cues such as body odor and acute sensitivity to the composition of odorant mixtures,

To address how fish employ sex pheromones, we briefly review the goldfish, the best understood example. Similar to many external-fertilizing temperate freshwater fish, goldfish ovulate in the spring in response to a surge in a luteinizing hormone, which triggers a series of gonadal hormonal surges whose by-products function as potent sex pheromones. This system may have evolved because males that could predict female spawning by detecting released hormonal metabolites had a reproductive advantage. Three sets of female cues are known. The first is released by mature females and is associated with estradiol (Kobayashi et al. 2002). The second is released 12 h before spawning by ovulatory females and functions as a preovulatory primer with endocrinological actions. This pheromone is comprised of a mixture of at least two common sex steroid hormones, androstenedione and 17,20β-dihdroxy-4-pregnen-3-one (17,20βP), and one metabolite, 17,20β-dihdroxy-4-pregnen-3-one-20-sulfate (17,20βP-S), the ratio of which shifts during the course of the reproductive cycle during which time dozens of steroids are released (Sorensen and Scott 1994). All three components are potent olfactory stimulants and detected by different, specific receptor sites. Male fish identify these chemicals as a mixture and exhibit a strong endocrine response (leading to sperm production) when 17,20βP is highest and a behavioral response when 17,20βP-S is highest (Sorensen et al. 1995; Poling et al. 2001). The third, post-ovulatory pheromone is a mixture of

F prostaglandins that is released by ovulated females in their urine in pulses, thus suggesting that odor concentration and context are also important to cue function (Sorensen et al. 1988; Appelt and Sorensen 2007). Recent data also suggest that cue specificity is determined by critical but unknown components in goldfish body odor, for if this odor is modified, responses to hormonal pheromones are greatly reduced (Sorensen et al. 2000). The reliance of goldfish on common hormonal mixtures together with context is so striking that we believe that this strategy is probably commonplace in fish.

Finally, several studies have investigated the chemistry of alarm signals, without clear resolution. Hypoxanthine-3-*N*-oxide, peptides, purines, proteins, and their mixtures have all been suggested to function as alarm signals (Brown et al. 2000, 2003; Døving et al. 2005). Some evidence exists that mixtures of nitrogen oxides may mediate alarm responses in somewhat taxon-specific manners (Brown et al. 2003), but data on release of these chemicals by fish and chemosensitivity to them are lacking.

In summary, substantial subsets of various metabolic products, some specialized and most not, are identified by fish olfactory and gustatory systems as meaningful chemicals that are then used to mediate an array of behaviors. Other unidentified chemicals are undoubtedly important. These stimuli occur and only have biological relevance as mixtures.

Crustaceans

Feeding stimulants are some of the most studied and best understood molecules in the chemical ecology of crustaceans. Trophic level accounts for some of the differences in the identity of feeding stimuli of crustaceans. Carnivores such as lobsters (*Homarus*) and spiny lobsters (*Panulirus*) respond best to small, nitrogen-containing compounds that are prevalent in tissues of their animal prey. These compounds include many that are also feeding stimulants for fish, including amino acids, amines, nucleotides, and peptides (Zimmer-Faust and Case 1982a; Carr 1988; Derby and Atema 1988; Zimmer-Faust 1993). Carnivorous crustaceans are relatively insensitive to carbohydrates and sugars. Herbivores and omnivores, such as fiddler crabs, ghost crabs, kelp crabs, and crayfish, are often sensitive to sugars common to plants, bacteria, and diatoms, as well as to some amino acids (Robertson et al. 1981; Zimmer-Faust and Case 1982b; Trott and Robertson 1984; Weissburg and Zimmer-Faust 1991; Archdale and Anraku 2005; Corotto et al. 2007). Selection of food by crustaceans is also controlled by the presence of feeding deterrents, yet the molecular identity of these deterrents is much more poorly understood than for the stimulants. An example of identified feeding deterrents is in crayfish, which are deterred from ingesting plants that contain phenylpropanoid-based natural products and a C-18 acetylenic acid (Prusak et al. 2005; Lane and Kubanek 2006; Parker et al. 2007).

Larval settlement factors have been studied with great interest for decades due to their economic impact. Therefore, the recent molecular identification of the factor was highly anticipated and gratefully received (Dreanno et al. 2006a, b). The bioactive molecule is a 169-kD glycoprotein called settlement-inducing protein complex, which has 30% similarity to α_2-macroglobulins. The observation that small and specific peptides evoke settlement (Rittschof and Cohen 2004) makes sense in light of knowing the identity of the protein.

Sex pheromones in crustaceans also have been intensely investigated but without much success in identifying the active molecules. The pheromone used by male copepods (*Tigriopus japonicus*) to recognize females has been partially characterized—and interestingly, it too has similarity to α_2-macroglobulin (Ting and Snell 2003). Efforts to identify sex pheromones in decapod crustaceans, such as crabs, crayfish, clawed lobsters, and spiny lobsters, have not led to the conclusive identification of the molecular structures (Gleeson 1991; Asai et al. 2000; Hardege et al. 2002; Kamio et al. 2002, 2006; Atema and Steinbach 2007). Efforts to identify pheromones on these and other species continue (J. Hardege, personal communication; M. Kamio, personal communication).

Plasticity

The Importance of Plasticity

The ability to adaptively modify chemically mediated behaviors through experience is critical to the survival of most animals. This is particularly true for animals that are long-lived, mobile, and omnivorous, which is the case for many fish and crustaceans. Plasticity occurs in several contexts in chemoreception of fish and crustaceans, including selection of food, social interactions with conspecifics, and even in responses to pheromones. Some examples are given in the next sections.

Fish

Fishes have well-developed abilities to recognize and learn natural chemical cues. The most impressive example is that of olfactory learning in migratory pacific salmonids, which imprint to bouquets of stream odors during sensitive developmental periods (Dittman et al. 1996). Although this ability is partially attributable to developmental changes in their olfactory epithelia (Nevitt et al. 2004), the ability of

adults to recall olfactory memories many years later presumably involves higher brain centers. Surely pattern recognition is paramount to this process, which appears sophisticated enough that fish can discriminate and remember the presence of trace amounts of chemicals to which they are relatively insensitive, such as morpholine for salmon (Hasler and Scholz 1983; Hara et al. 1984). It is also apparent that combinations of odorants can be identified as distinct 'bouquets' that may give the impression of mixture synergism. For example, goldfish cannot be trained to respond to morpholine alone, but they can learn to respond to odorant mixtures that contain morpholine (Dodson and Bitterman 1989). Mixture perception and the ability to learn unique odors clearly are important to place location in salmon, which do not have a particularly notable olfactory system from an anatomical perspective. Presumably, other fishes are similar, as suggested by the examples of food recognition in pinfish and sex pheromone discrimination in goldfish. Glomerular processing in the olfactory bulb probably plays a role in this process as described below.

Feeding studies also provide evidence of the ability of fish to discriminate and learn complex chemical mixtures. Fishermen and behavioral ecologists enumerate examples of fish that develop the ability to recognize and select especially desirable food items as their abundance fluctuates (Jones 1992). Olfaction plays a major role in this process. For example, responses of yellowfin tuna to prey odors shift as their diet changes (Atema et al. 1980). Ablation studies suggest that olfaction alone mediates this response through formation of 'chemical search images' (Atema et al. 1980). In this case, olfaction appears to guide arousal and search, and vision assumes the final role of food location. In other species, gustation rather than vision plays the consummatory role. This plasticity does not signify a lack of innate predisposition to respond to particular types of prey. There are several anecdotal examples of naive young fish responding to specific food items (Jones 1992), a phenomenon once termed 'specific appetite,' which may be a gustatory attribute (see below). The interplay between these senses warrants specific investigation by using natural cues.

Not surprisingly, laboratory studies of associative and instrumental learning show that some fish are adept at learning to discriminate among different amino acids. The channel catfish is the best understood in this regard, and many studies demonstrate its ability to learn to discriminate among amino acids when presented individually or in mixtures (Valentinčič et al. 2000; Valentinčič 2005). Channel catfish can identify binary mixtures as unique entities and as the sum of their components. Other studies suggest that channel catfish can discriminate mixtures that contain up a half a dozen amino acids (Valentinčič 2005), an important ability in the natural world where feeding stimuli are complex. While two behavioral studies suggest these abilities are mediated by the olfactory system alone (Atema 1977; Valentinčič and Caprio 1994), another that used different techniques suggests that fish can learn to recognize single amino acids via their external gustatory system (Holland and Teeter 1981). Nevertheless, many young fish that have not fed before respond to individual amino acids with increased biting and swallowing, suggesting at least that the internal gustatory system is innately responsive (Jones 1992; Valentinčič and Caprio 1997; Hara 2007). Catfish also can learn to avoid eating palatable food items that have been previously associated with distasteful chemicals (Little 1977). Olfactory-blocked goldfish can be trained to preferentially acquire more nutritious food (Bitterman 1982) and to touch funnels through which amino acids are injected, although in the latter case, their sensitivity is reduced (Zippel et al. 1993). In summary, the roles of olfaction and gustation in feeding can differ with the former being more flexible. However, how experience modifies responsiveness to natural odors is incompletely known at present.

Finally, a few studies suggest that olfaction can mediate learning to a wide variety of relevant stimuli. For example, wild minnows recall the odor of predators more than 1 year after experiencing them (Smith 1992). Goldfish may be able to associate simple organic molecules, acids, and even steroidal sex pheromones with feeding (Zippel et al. 1993; Sorensen 2007).

Crustaceans

Crustaceans provide many interesting cases of experience-dependent plasticity and learning (Krasne 1973), including several varieties of chemosensory learning. These include habituation, classical and operant conditioning, aversive associative learning, and one-trial flavor avoidance learning (Abramson and Feinman 1988; Daniel and Derby 1988; Fine-Levy et al. 1988; Wight et al. 1990; Derby 2000; Shuranova et al. 2005). Such learning can influence the selection of food by crustaceans.

Learning is involved in identifying and remembering the odor of conspecifics, including individual recognition in crayfish, lobsters, hermit crabs, and mantis shrimp (Caldwell and Dingle 1985; Karavanich and Atema 1998; Breithaupt and Atema 2000; Hazlett 2003; Gherardi et al. 2005; Atema and Steinbach 2007). The formal nature of this learning has not been fully characterized, but memories of these odors can last for many days.

Experience can also influence selection of hosts by commensal crabs (Derby and Atema 1980). This ability might be useful for these crabs to relocate hosts after leaving them to mate.

The ability of spiny lobsters to learn food odors has been explored intensively, largely within the context of defining the neural basis of olfactory discrimination of mixtures (Derby 2000; Derby et al. 2001). Spiny lobsters are able to learn to associate food odors with danger and subsequently to avoid searching for those odors. Differential conditioning shows that spiny lobsters can learn to discriminate chemical mixtures that differ only in the ratios of their components. They can remember these odors and perform discrimination tasks for several days (Fine-Levy et al. 1988).

A form of plasticity in chemical communication in blue crabs has recently been demonstrated (Kamio et al. 2008). This is not experience-dependent plasticity but context-dependent plasticity. In this case, male crabs detect reproductive females, move toward them, and quickly pair-bond with them if they are immediately accessible. If they can sense but not grab the female, however, the males will perform a courtship display behavior that directs his pheromone toward her. This context-dependent chemical signaling is a form of behavioral plasticity that should enhance reproductive success.

Processing of Natural Chemical Stimuli

Many studies of neural processing in fish and crustaceans have used natural products and their components as chemical stimuli. Tested stimuli include single compounds of known biological importance and synthetic mixtures of these components in varying degrees of complexity (Derby 2000; Valentinčič 2005). Much is known about processing of individual food-related chemicals because their molecular identity is defined. Coding of natural products other than food has been less thoroughly examined for sex pheromones and chemical deterrents. Little work has been conducted on natural mixtures. In this section, we describe the organization of the chemosensory systems of fish and crustaceans, and how they code natural stimuli.

Fish

The olfactory and gustatory systems of fish are distinctly different senses that mediate different aspects of chemical recognition and drive different behaviors. Olfaction, not gustation, is used to identify and locate pheromones. On the other hand, both olfaction and gustation mediate aspects of feeding, though the specific roles of each appear to depend on the species, feeding experience, and other factors. Information from the olfactory and gustatory systems is probably integrated at higher neural levels, driving what to an outside observer might seem to be a single continuum of behaviors. Exactly where this occurs in the brain is not yet known. Here, we describe the neurobiology of these systems, elements of which are recently reviewed by Caprio and Derby (2007), with the aim of outlining how natural chemical mixtures are perceived by fish.

The basic neural structure of the fish olfactory sense is fundamentally similar to that of other vertebrates. It is comprised of three elements: olfactory receptor neurons (ORNs) located in the olfactory epithelium, glomeruli in the olfactory bulb into which ORNs converge, and output neurons (mitral cells) that convey information from the glomeruli to the forebrain where further processing occurs (Michel 2006; Caprio and Derby 2007). Fish have three major types of ORNs: ciliated, microvillar, and crypt cells. These cells express particular types of olfactory receptor molecules, second messengers, channels, and other molecules associated with transduction, suggesting that they have specific and different roles. Current evidence suggests that microvillar cells detect amino acids and that ciliated cells detect all other cues and perhaps some amino acids. Nevertheless, the coding of olfactory information commences with the olfactory receptors, which are seven-transmembrane G-protein linked proteins, and the receptor cells containing them (Caprio and Derby 2007; Saraiva and Korsching 2007). Although the process of receptor discovery is ongoing, fish species are thought to express over 100 members from three families of receptors (ORs, V1Rs, and V2Rs; Ngai et al. 1993; Saraiva and Korsching 2007). Of these, only one class has been functionally expressed to date. It detects amino acids, with a specific bias to arginine, and its activity can be predictably altered with site-directed mutations (Luu et al. 2004). The current view is that fish olfactory systems encode most chemical stimuli by using a combinatorial code, whereby particular attributes of molecules are discriminated by the activity of specific combinations of receptors, the pattern of which conveys identity of the molecules. Such a code should be both versatile and specific, but it would require neural mechanisms capable of encoding complex patterns of receptor binding—an attribute of the olfactory bulb. In addition to a combinational scheme for identifying general odors, such as those associated with food, a simpler scheme based on one or a few receptor types may be used to identify sex pheromones (Friedrich and Korsching 1998; Sorensen and Sato 2005).

All ORNs that share the same receptor type converge on the same glomeruli in the olfactory bulb, where the neural signal is enhanced and modulated by interneurons that extend from other glomeruli or by centrifugal input from higher levels of the central nervous system. Because glomeruli with similar chemosensitivity are located close to each other, the result is a systematic three-dimensional chemotopic map of chemical identities across the bulb (Friedrich and Korsching 1998; Nikonov and Caprio 2001). Odorants such as sex pheromones appear to be detected by fewer, specialized neurons (Friedrich and Korsching 1998),

although electroencephalogram recordings from goldfish bulb suggest that these units receive significant inhibitory input (Hanson 2001), so they are not true 'labeled lines.' Indeed, glomeruli in the olfactory bulb are extensively interconnected, as mitral cells project to more than one glomerulus and local interneuronal connections are extensive. Thus, there is considerable potential for complex but orderly processing of mixture information, perhaps from multiple classes of odorants, in the fish olfactory bulb (Hara 1970), likely creating distinctive olfactory 'fingerprints' for natural odor sources including conspecifics, as occurs in the mouse (Schaefer et al. 2001)

Chemical information is passed from the olfactory bulb to the forebrain through a series of lateral and medial olfactory tracts whose functions have been studied in the carps by using single odorants, as described below. Behavioral and electrophysiological studies in the goldfish show that the medial tracts convey sex pheromone information (Stacey and Kyle 1983; Sorensen et al. 1991), and the lateral tracts convey amino acid and food odor information (Stacey and Kyle 1983; Hamdani et al. 2001). Responses to alarm cues appear to be conveyed by special parts of the medial tract in the closely related crucian carp (Hamdani et al. 2000). Interestingly, some amino acid information is also conveyed by the medial tracts in the goldfish (Sorensen et al. 1991), suggesting that amino acids might be perceived as part of the background body odor component of pheromones. The forebrain of catfish contains a chemotopic map (Nikonov et al. 2005). Connections from the forebrain to higher brain regions would allow integration of olfactory information with that from visual and other systems, including gustatory, as is required for driving multimodal behaviors such as feeding and mating. In summary, the olfactory system contains a network of connections that encodes the identities of complex odor mixtures and perhaps their concentrations in highly precise manners that should permit learning and memory. Tests of natural odors are now needed to ascertain the full potential of this system and whether it can explain all of the behaviors observed in wild animals to natural stimuli.

The anatomy and neural functions of the gustatory system in fish are remarkably different from the olfactory system except that the chemosensitivities of the two overlap for food stimuli. These differences emphasize what seem to be important distinctions in their respective roles in food procurement. Gustation is mediated by taste buds, groupings of receptor cells that are derived from epithelial tissue. These cells synapse onto primary gustatory neurons, with each neuron typically receiving input from cells in many buds. Several classes of genes for gustatory receptors have been cloned in fish (Ishimaru et al. 2005), and their chemosensitivity is beginning to be understood (Oike et al. 2007). Some taste cells express more than one type of receptor, which distinguishes them from olfactory cells (Ishimaru et al. 2005). The expression and connectivity properties of taste cells result in gustatory fibers that respond to multiple classes of chemical stimuli but still with specificity. Responses are concentration-dependent, and stimulus quality seems to be encoded by overall firing rate. There is no evidence of special processing of mixture information (Caprio and Derby 2007). Taste bud abundance and distribution vary enormously with fish species, especially for the external gustatory system (facial nerve; VII), which innervates the barbels (if present) and/or the lips, and projects to the highly structured facial lobe. Taste buds in the oropharyngeal cavities and gill arches of all fish are innervated by the vagal (X) and glossopharyngeal nerves (IX), which project to the vagal lobe and other regions of the hindbrain. This topographic mapping scheme appears to reflect an emphasis on processing the spatial distribution of chemical concentrations, an attribute correlated with catfish's ability to track chemical plumes by using gustation alone (Bardach et al. 1967). Similar topographic maps are found in the carp vagal lobe and appear to mediate food sorting in the mouth (Finger and Morita 1985; Finger 2008). Swallowing and ingestion do not appear to be highly specific processes, as most detected amino acid will evoke the response (Caprio and Derby 2007).

In summary, the olfactory and gustatory systems are both designed to process natural chemical mixtures but in quite different manners. While olfaction is organized to adaptively integrate information associated with a wide range of chemical stimuli for various functions, gustation is specialized for sensitivity and localization of food source. Tests of the natural function of these neural systems in free-swimming animals to complex, natural stimuli await. It will be particularly important to examine function in species other than catfish, which are somewhat unusual in their possession of a highly developed and broadly tuned external gustatory system but for which sex pheromones are not yet available for testing.

Crustaceans

A Diversity of Organs Mediating a Diversity of Behaviors The chemical sensors of crustaceans differ in their sensillar organization, central connections, and behavioral role. One pathway that is conserved in many crustaceans is that of the aesthetasc sensilla and olfactory lobe. This is considered an 'olfactory' pathway because the aesthetascs are innervated only by chemosensory (and not mechanosensory) neurons and because of structural similarities with the insect and vertebrate olfactory pathways (Schachtner et al. 2005). All other sensilla that contain chemosensory neurons also contain mechanosensory neurons. But these bi-modal

chemosensilla are extremely diverse in structure, are distributed differently across the animal's body surface, and serve different behavioral functions. This suggests that crustacean chemoreceptors cannot be categorized according to a dichotomous olfactory–gustatory classification.

Functions of Different Chemosensory Pathways Together, the diverse chemosensors across the body of crustaceans gather information about conspecifics, predators, attractive and defensive properties of prey, and more. These sensors connect differentially within the central nervous system, leading to the control of different aspects of behavior. The olfactory (aesthetasc) and gustatory pathways differ in the behaviors controlled. In spiny lobsters, the antennules drive detection of and orientation to distant, attractive chemicals, including food-related chemicals and intraspecific chemicals such as aggregation or alarm cues (Derby et al. 2001; Horner et al. 2004, 2008b; Shabani et al. 2006). In blue crabs, however, leg chemoreceptors can play a major role in mediating orientation to distant odors, as the wide spacing between legs on the animal's two sides provides spatial information that helps the crabs locate the plume's boundaries (Keller and Weissburg 2004). In lobsters, chemosensors on the legs of lobsters are used to locate food once the animal has reached the source (Derby and Atema 1982). Mouthpart chemoreceptors then assess the food for phagostimulants and phagodeterrents that lead to its ingestion or rejection (Derby and Atema 1982; Derby et al. 2001; Garm et al. 2005; Kicklighter et al. 2005; Garm and Høeg 2006).

The antennules are the most intensively studied of all of the crustacean chemosensory pathways. The two major antennular chemosensory pathways—the aesthetasc/olfactory lobe pathway and non-aesthetasc/lateral antennular lobe pathway—of spiny lobsters have some functional redundancy: Both assess the quality of food, both mediate olfactory learning, and both enable orientation to distant food odors (Derby et al. 2001; Steullet et al. 2001, 2002; Horner et al. 2004). Unique functions of the aesthetasc pathway include encoding intraspecific cues used in sexual and social interactions (Gleeson 1982, 1991; Johnson and Atema 2005; Horner et al. 2008a, b). Unique functions of the non-aesthetasc pathway include mediating antennular sensory-motor tasks. One example is the class of non-aesthetasc sensilla—the asymmetric sensilla—that are necessary and sufficient for mediating chemically evoked antennular grooming behavior (Schmidt and Derby 2005). Detectors of pheromones and social cues are not exclusively located in the aesthetasc/olfactory pathway, however. For example, male crayfish have sensors on their claws that detect female odors (Belanger and Moore 2006).

How are mixtures coded by the peripheral olfactory system of decapod crustaceans? Information about different classes of chemicals is carried by different types of chemoreceptor neurons (CRNs). CRNs have different specificities, with some being most sensitive to one or several amino acids, others most sensitive to one or several adenine nucleotides, others most sensitive to ammonium ions, and so on (Voigt and Atema 1992; Derby 2000; Garm et al. 2005). Single CRNs can be stimulated by compounds that belong to different classes of molecules. For example, taurine-best CRNs can also be sensitive to other amino acids, AMP, ammonium, and other compounds, although the thresholds for taurine are 100 to 10,000 times lower than other stimuli (Cromarty and Derby 1997). Stimulus quantity, at least for food-related chemicals and their mixtures, is coded by CRN population responses or across-fiber patterns rather than labeled lines (Derby 2000; Caprio and Derby 2007). Such codes enable discrimination of highly similar mixtures, such as binary mixtures or 30+ component mixtures that contain the same components but at different blend ratios (Steullet and Derby 1997; Derby 2000; Derby et al. 2001).

Our understanding of how crustaceans process sex pheromones, social cues, anti-feeding compounds, and other chemical stimuli besides feeding stimulants is in its infancy. Not knowing the identity of these bioactive molecules is a major reason, and such identification is urgently needed. One example of how chemical defenses are processed is a set of recent studies on spiny lobsters' responses to defensive secretions of sea hares (Kicklighter et al. 2005; Kamio et al. 2007). The studies suggest that these secretions function by 'phagomimicry,' in which the defensive secretion stimulates the feeding pathway to deceive spiny lobsters into attending to a false food stimulus, and by sensory disruption, in which the sticky and potent secretions cause high-amplitude, long-lasting sensory stimulation. Distasteful feeding deterrents also occur in these secretions, although their identity and neural processing are just now being studied (Kamio et al. 2007). Thus, chemical defenses may act in complex ways, even against a single predatory species.

General Conclusions and Future Directions

The chemical milieu of aquatic environments shapes how animals living there perceive their chemical world. Our paper discussed the chemosensory abilities of two major groups of aquatic animals—fish and crustaceans—within the context of the chemical ecology of the aquatic environment. Fish and crustaceans use chemoreception in many of their inter- and intraspecific interactions, including identifying and locating high-quality food, mates, and

suitable habitat and shelters, avoiding predators and low-quality or toxic food, and interacting with conspecifics in social and agonistic encounters. The compounds that mediate these interactions are diverse but typically are mixtures of small, water-soluble compounds. The responses of many fish and crustaceans to some chemicals are somewhat hard wired, but others, particularly for species that live in complex and diverse habitats, and may live for decades, show enormous plasticity. Aquatic animals have a diversity of chemical senses to detect these chemicals, including but not limited to those that can be placed into the classical categories of 'olfaction' and 'gustation.' In general, olfactory systems are well adapted for discrimination of complex stimuli, and gustatory systems control proximate identification, location, and ingestion of palatable food and rejection of distasteful or toxic food.

A conceptualization of the approaches to studying the chemical ecology of chemoreception is presented in Fig. 1. Individual disciplines can be used to make important contributions. For example, current techniques in analytical chemistry can determine what molecules are present in organisms and their environment and, thus, might serve as chemical cues or signals. Neuroscience brings molecular, cellular, and systems level analyses that can be used to ascertain what chemicals are detected, as well as mechanisms of reception and integration of these chemicals. Ethology allows evaluation of the behavioral relevance of detected chemicals. Evolutionary ecological approaches allow investigations of phylogenetic relationships, and how the habitat in which organisms live can influence the nature of their chemical senses. Reliance upon only one of these approaches can lead to, at best, partial understanding of a system and often leads to misunderstandings. We and others (e.g., Zimmer and Derby 2007; Zimmer and Zimmer 2008) advocate using multiple approaches and working at the intersection of these disciplines using the integrated approaches of neuroethology, neuroecology, and chemical ecology.

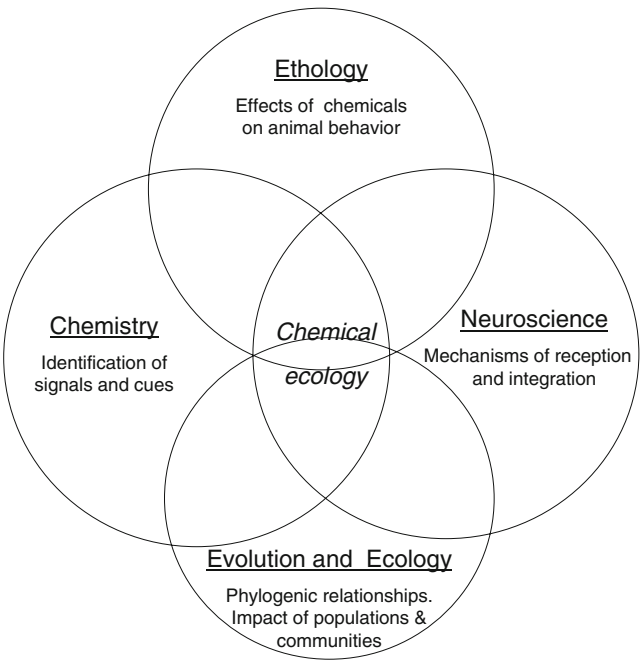

Fig. 1 A visual depiction of approaches to the study of chemical ecology. Disciplinary approaches are shown in the *circles*. We advocate using integrated and multidisciplinary approaches to most effectively understand issues in chemical ecology. See the text for further explanation

Although our understanding of neural processing of fish and crustaceans has advanced in recent years, we still have many challenges facing us. We have almost no information on how entire categories of chemicals are processed. For example, how pheromones and other intraspecific cues are processed by crustacean nervous systems is virtually unknown, and scant information exists for fishes. In the former case, this is largely a result of not knowing the molecules involved. In general, our knowledge of the identity of ecologically relevant chemicals is limited to a few contexts (especially feeding) and a few species (often the commercially important ones). However, with the advent of sensitive and sophisticated analytical chemical equipment and techniques, the challenge of identifying bioactive chemicals is not nearly so daunting, and we expect major advances to be made in this area.

Advances in molecular techniques also have given us tools to elucidate mechanisms of sensory processing. These tools have helped in identifying olfactory and gustatory receptors and other molecules involved in sensory transduction, especially in animals whose genomes have been sequenced, most notably zebrafish (Oike et al. 2007; Saraiya and Korsching 2007). The first crustacean with a sequenced genome is *Daphnia pulex* (Colbourne et al. 2005; see Daphnia Water Flea Genome Database at http://wfleabase.org), which provides a useful tool for studying the chemical senses of this and other crustaceans. Nonetheless, having a sequenced genome for a more established model organism, such as a decapod crustacean, will be a prized contribution. In the meantime, other molecular tools will have to suffice to identify molecules involved in chemical sensing (McClintock et al. 2006). Advances in methods of simultaneously recording from ensembles of neurons by using multielectrode arrays or imaging with voltage- and calcium dyes opens up exciting possibilities for understanding how sensory systems process and discriminate mixtures.

On a final note, we urge that mechanistic approaches to the study of chemical sensing be applied within the framework of chemical ecology and with an eye toward broader contributions of this type of work. What we learn about neural processing of ecologically and economically important

species, such as salmon, lamprey, lobsters, and crabs, can and should have broader applications to be used by community and systems ecologists and fisheries scientists.

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References

ABRAMSON, C. I., and FEINMAN, R. D. 1988. Classical conditioning of the eye withdrawal reflex in the green crab. *J. Neurosci* 8:2907–2912.

ACHE, B. W., and YOUNG, J. M. 2005. Olfaction: diverse species, conserved principles. *Neuron* 48:417–430.

APPELT, C. A., and SORENSEN, P. W. 2007. Female goldfish signal spawning readiness by altering when and where they release a urinary pheromone. *Anim. Behav* 74:1329–1338.

ARCHDALE, M. V., and ANRAKU, K. 2005. Feeding behavior in Scyphozoa, Crustacea and Cephalopoda. *Chem. Senses* 30:i303–i304.

ARNESEN, A. M., and STABELL, O. B. 1992. Behaviour of stream-dwelling brown trout towards odours present in home stream water. *Chemoecology* 3:94–100.

ASAI, N., FUSETANI, N., MATSUNAGA, S., and SASAKI, J. 2000. Sex pheromones of the hair crab *Erimacrus isenbeckii*. Part 1: Isolation and structures of novel ceramides. *Tetrahedron* 56:9895–9899.

ATEMA, J. 1977. Functional separation of smell and taste in fish and Crustacea, pp. 165–174, in J. Le Magnen, and P. Mac Leod (eds.). Olfaction and Taste VI. Information Retrieval, Paris.

ATEMA, J. 1988. Distribution of chemical stimuli, pp. 29–56, in J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animals. Springer, New York.

ATEMA, J. 1996. Eddy chemotaxis and odor landscapes: exploration of nature with animal sensors. *Biol. Bull* 191:129–138.

ATEMA, J., and STEINBACH, M. A. 2007. Chemical communication in the social behavior of the lobster, *Homarus americanus*, and other decapod Crustacea, pp. 115–144, in E. Duffy, and M. Thiel (eds.). Ecology and Evolution of Social Behavior: Crustaceans as Model Systems. Oxford University Press, Oxford, UK.

ATEMA, J., HOLLAND, K., and IKEHARA, W. 1980. Olfactory responses of yellowfin tuna (*Thunnus albacares*) are prey odors: chemical search image. *J. Chem. Ecol* 6:457–465.

BARDACH, J. E., TODD, J. H., and CRICKMER, R. 1967. Orientation by taste in fish of the genus *Ictalurus*. *Science* 155:1276–1278.

BEHRINGER, D. C., BUTLER, M. J., and SHIELDS, J. D. 2006. Avoidance of disease by social lobsters. *Nature* 441:421.

BELANGER, R. M., and MOORE, P. A. 2006. The use of the major chelae by reproductive male crayfish (*Orconectes rusticus*) for discrimination of female odours. *Behaviour* 143:713–731.

BERGER, D. K., and BUTLER, M. J. 2001. Octopuses influence den selection by juvenile Caribbean spiny lobster. *Mar. Freshw. Res* 52:1049–1053.

BITTERMAN, M. E. 1982. Migration and learning in fishes, pp. 397–420, in J. D. McCleave, G. P. Arnold, J. J. Dodson, and W. Neill (eds.). Mechanisms of Migration in Fishes. Plenum, New York.

BOUWMA, P. 2007. Should I stay or should I go? The ontogeny of spiny lobster responses to alarm odor cues. Abstract at the 2007 Benthic Ecology meeting.

BOUWMA, P., and HAZLETT, B. A. 2001. Integration of multiple predator cues by the crayfish *Orconectes propinquus*. *Anim. Behav* 61:771–776.

BREITHAUPT, T. 2001. The fan organs of crayfish enhance chemical information flow. *Biol. Bull* 200:150–154.

BREITHAUPT, T., and ATEMA, J. 2000. The timing of chemical signaling with urine in dominance fights of male lobsters (*Homarus americanus*). *Behav. Ecol. Sociobiol* 49:67–78.

BREITHAUPT, T., and EGER, P. 2002. Urine makes the difference: chemical communication in fighting crayfish made visible. *J. Exp. Biol* 205:1221–1231.

BRIONES-FOURZÁN, P., and LOZANO-ÁLVAREZ, E. 2005. Seasonal variations in chemical response to conspecific scents in the spotted spiny lobster, *Panulirus guttatus*. *N. Z. J. Mar. Freshw. Res* 39:383–390.

BROWN, G. E., CHIVERS, D. P., and SMITH, R. J. F. 1993. Localized defecation by pike: a response to labeling by cyprinid alarm pheromone? *Behav. Ecol. Sociobiol* 36:105–110.

BROWN, G. E., ADRIAN, J. C., SMYTH, E., LEET, H., and BRENNAN, S. 2000. Ostariophysan alarm pheromones: laboratory and field tests of the functional significance of nitrogen oxides. *J. Chem. Ecol* 26:139–154.

BROWN, G. E., ADRIAN, J. C., NADERI, N. T., HARVEY, M. C., and KELLY, J. M. 2003. Nitrogen oxides elicit antipredator responses in juvenile catfish but not convict cichlids or rainbow trout: conservation of the ostariophysan alarm pheromone. *J. Chem. Ecol* 29:1781–1796.

CALDWELL, R., and DINGLE, J. 1985. A test of individual recognition in the stomatopod *Gonodactylus festae*. *Anim. Behav* 33:101–106.

CAPRIO, J., and DERBY, C. D. 2007. Aquatic animal models in the study of chemoreception, in A. Basbaum, M. Bushnell, D. Smith, G. Beauchamp, S. Firestein, P. Dallos, D. Oertel, R. Masland, T. Albright, J. Kaas, and E. Gardner (eds.). The Senses: A Comprehensive Reference, Six-Volume Set. Elsevier, New York.

CAPRIO, J. C., BRAND, J. B., TEETER, J. H., VALENTINČIČ, T., KALINOSKI, D. L., KOHBARA, J., KAMAZAWA, T., and WEGERT, S. 1993. The taste system of the channel catfish: from biophysics to behavior. *Trends Neurosci* 16:192–197.

CARR, W. E. S. 1982. Chemical stimulation of feeding behavior, pp. 259–274, in T. J. Hara (ed.). Chemoreception in Fishes. Elsevier, New York.

CARR, W. E. S. 1988. The molecular nature of chemical stimuli in the aquatic environment, pp. 3–27, in J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animals. Springer, New York.

CARR, W. E. S., NETHERTON, J. C. III, GLEESON, R. A., and DERBY, C. D. 1996. Stimulants of feeding behavior in fish: analyses of tissues of diverse marine organisms. *Biol. Bull* 190:149–160.

CASKEY, J. L., and BAUER, R. T. 2005. Behavioral tests for a possible contact pheromone in the caridean shrimp *Palaemonetes pugio*. *J. Crustac. Biol* 25:571–576.

COHEN, S. A. P., HATT, H., KUBANEK, J., and MCCARTY, N. A. 2008. Reconstitution of a chemical defense signaling pathway in a heterologous system. *J. Exp. Biol* 211:599–605.

COLBOURNE, J. K., SINGAN, V. R., and GILBERT, D. G. 2005. wFleaBase: the *Daphnia* genome database. *BMC Bioinformatics* 6:45.

COROTTO, F. S., MCKELVEY, M. J., PARVIN, E. A., ROGERS, J. L., and WILLIAMS, J. M. 2007. Behavioral responses of the crayfish *Procambarus clarkii* to single chemosensory stimuli. *J. Crustac. Biol* 27:24–29.

CROMARTY, S. I., and DERBY, C. D. 1997. Multiple receptor types on individual excitatory olfactory neurons: implications for coding of mixtures in the spiny lobster. *J. Comp. Physiol. A* 180:481–492.

DANIEL, P. C., and DERBY, C. D. 1988. Behavioral olfactory discrimination of mixtures in the spiny lobster (*Panulirus argus*) based on a habituation paradigm. *Chem. Senses* 13:385–395.

DAVISS, B. 2005. Growing pains for metabolomics. *Scientist* 19:25–28.

DERBY, C. D. 2000. Learning from spiny lobsters about chemosensory coding of mixtures. *Physiol. Behav* 69:203–209.

DERBY, C. D., and ATEMA, J. 1980. Induced host odor attraction in the pea crab *Pinnotheres maculatus*. *Biol. Bull* 158:26–33.

DERBY, C. D., and ATEMA, J. 1982. The function of chemo- and mechanoreceptors in lobster (*Homarus americanus*) feeding behaviour. *J. Exp. Biol* 98:317–327.

DERBY, C. D., and ATEMA, J. 1988. Chemoreceptor cells in aquatic invertebrates: peripheral mechanisms of chemical signal processing in decapod crustaceans, pp. 365–385, in J. Atema, R. R. Fay, A. N. Popper, and W. N. Tavolga (eds.). Sensory Biology of Aquatic Animals. Springer, New York.

DERBY, C. D., STEULLET, P., HORNER, A. J., and CATE, H. S. 2001. The sensory basis to feeding behavior in the Caribbean spiny lobster *Panulirus argus*. *Mar. Freshw. Res* 52:339–1350.

DERBY, C. D., KICKLIGHTER, C. E., JOHNSON, P. M., and ZHANG, X. 2007. Chemical composition of inks of diverse marine molluscs suggests convergent chemical defenses. *J. Chem. Ecol* 33:1105–1113.

DITTMAN, A. D., QUINN, T. P., and NEVITT, G. A. 1996. Timing of imprinting to natural and artificial odors by coho salmon (*Oncorhynchus kisutch*). *Can. J. Fish Aquat. Sci* 53:434–442.

DODSON, J. J., and BITTERMAN, M. E. 1989. Compound uniqueness and the interactive role of morpholine in fish chemoreception. *Biol. Behav* 14:13–27.

DØVING, K. B., SELSET, R., and THOMMESEN, G. 1980. Olfactory sensitivity to bile acids in salmonid fishes. *Acta Physiol. Scand* 108:123–131.

DØVING, K. B., HAMDANI, E. H., HOGLUND, E., KASUMYAN, A., and TUVIKENE, A. O. 2005. Review of the chemical and physiological basis of alarm reactions in cyprinids, pp. 131–163, in G. von der Emde, J. Mogdans, and B. G. Kapoor (eds.). Senses of Fish. Narosa, New Delhi.

DREANNO, C., MATSUMURA, K., DOHMAE, N., TAKIO, K., HIROTA, H., KIRBY, R. R., and CLARE, A. S. 2006a. An a_2-macroglobulin-like protein is the cue to gregarious settlement of the barnacle *Balanus amphitrite*. *Proc. Natl. Acad. Sci. U.S.A* 103:14396–14401.

DREANNO, C., KIRBY, R. R., and CLARE, A. S. 2006b. Smelly feet are not always a bad thing: the relationship between cyprid footprint protein and the barnacle settlement pheromone. *Biol. Lett* 2:423–425.

EISTHEN, H. L. 2002. Why are olfactory systems of different animals so similar? *Brain Behav. Evol* 59:273–293.

EKERHOLM, M., and HALLBERG, E. 2005. Primer and short-range releaser pheromone properties of pre-moult female urine from the shore crab *Carcinus maenas*. *J. Chem. Ecol* 31:1845–1864.

FINE, J. M., VRIEZE, L. A., and SORENSEN, P. W. 2004. Evidence that petromyzontid lampreys employ a common migratory pheromone that is partially comprised of bile acids. *J. Chem. Ecol* 30:2091–2110.

FINE-LEVY, J. B., GIRARDOT, M.-N., DERBY, C. D., and DANIEL, P. C. 1988. Differential associative conditioning and olfactory discrimination in the spiny lobster *Panulirus argus*. *Behav. Neural Biol* 49:15–331.

FINGER, T. A. 2008. Sorting food from stones: the vagal taste system in goldfish *Carassius auratus*. *J. Comp. Physiol*. *A* 194:135–143.

FINGER, T. A., and MORITA, Y. 1985. Two gustatory system: facial and vagal gustatory nuclei have different brainstem connections. *Science* 227:776–778.

FRIEDRICH, R. W., and KORSCHING, S. I. 1998. Chemotopic, combinatorial, and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer. *J. Neurosci* 18:9977–9900.

GARM, A., and HØEG, J. T. 2006. Ultrastructure and functional organization of mouthpart sensory setae of the spiny lobster *Panulirus argus*: new features of putative mechanoreceptors. *J. Morphol* 267:464–476.

GARM, A., SHABANI, S., HØEG, J. T., and DERBY, C. D. 2005. Chemosensory neurons in the mouthparts of the spiny lobsters *Panulirus argus* and *Panulirus interruptus* (Crustacea: Decapoda). *J. Exp. Mar. Biol. Ecol* 314:175–186.

GHERARDI, F., TRICARICO, E., and ATEMA, J. 2005. Unraveling the nature of individual recognition by odor in hermit crabs. *J. Chem. Ecol* 31:2877–2896.

GLEESON, R. A. 1982. Morphological and behavioral identification of the sensory structures mediating pheromone reception in the blue crab *Callinectes sapidus*. *Biol. Bull* 163:162–171.

GLEESON, R. A. 1991. Intrinsic factors mediating pheromone communication in the blue crab, *Callinectes sapidus*, pp. 17–32, in R. T. Bauer, and J. W. Martin (eds.). Crustacean Sexual Biology. Columbia University Press, New York.

GRASSO, F. W., and BASIL, J. A. 2002. How lobsters, crayfishes, and crabs locate sources of odor: current perspectives and future directions. *Curr. Opin. Neurobiol* 12:721–727.

GROVE, M. W., and WOODIN, S. A. 1996. Conspecific recognition and host choice in a pea crab, *Pinnixa chaetopterana* (Brachyura: Pinnotheridae). *Biol. Bull* 190:359–366.

GUNNING, G. E. 1959. The sensory basis of homing in the longear sunfish, *Lepomis megalotis megalotis* (Rafinsque). *Invest. Indiana Lakes Streams* 5:103–130.

HAMDANI, E. H., STABELL, O. B., ALEXANDER, G., and DØVING, K. B. 2000. Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. *Chem. Senses* 25:103–109.

HAMDANI, E. H., ALEXANDER, G., and DØVING, K. B. 2001. Projection of sensory neurons with microvilli to the lateral olfactory tract indicates their participation in feeding behaviour in the crucian carp. *Chem. Senses* 26:1134–1144.

HANSON, L. H. 2001. Coding of pheromonal information in the goldfish olfactory bulb. Ph.D. dissertation, University of Minnesota.

HARA, T. J. 1970. An electrophysiological basis for olfactory discrimination in homing salmon: a review. *J. Fish. Res. Board Can* 27:565–586.

HARA, T. J. 1994. The diversity of chemical stimulation in fish olfaction and gustation. *Rev. Fish Biol. Fish.* 4:1–35.

HARA, T. J. 2007. Gustation, pp. 45–96, in T. J. B. S. HaraZielinski (ed.). Fish Physiology Series (A. P. Farrell and C. J. Brauner, eds.). Sensory Systems Neuroscience. Elsevier, New York.

HARA, T. J., MACDONALD, S., EVANS, R. E., MARUI, T., and ARAI, S. 1984. Morpholine, bile acids, and skin mucous as possible chemical cues in salmonid homing: electrophysiological re-evaluation, pp. 363–378, in J. D. McCleave, G. P. Arnold, J. J. Dodson, and W. Neill (eds.). Mechanisms of Migration in Fishes. Plenum, New York.

HARDEGE, J. D., JENNINGS, A., HAYDEN, D., MULLER, C. T., PASCOE, D., BENTLEY, M. G., and CLARE, A. S. 2002. Novel behavioural assay and partial purification of a female-derived sex pheromone in *Carcinus maenas*. *Mar. Ecol. Prog. Ser* 244:179–189.

HASLER, A. D., and WISBY, W. J. 1951. Discrimination of stream odors by fishes and relation to parent stream behavior. *Am. Nat* 85:223–238.

HASLER, A. D., and SCHOLZ, A. T. 1983. Olfactory Imprinting and Homing in Salmon. Investigations in the Mechanism of the Imprinting Process. p. 134. Springer, Berlin.

HAY, M. E. 1996. Marine chemical ecology: what's known and what's next? *J. Exp. Mar. Biol. Ecol* 200:103–134.

HAYDEN, D., JENNINGS, A., MÜLLER, C., PASCOE, D., BUBLITZ, R., WEBB, H., BREITHAUPT, T., WATKINS, L., and HARDEGE, J. 2007. Sex-specific mediation of foraging in the shore crab, *Carcinus maenas*. *Horm. Behav* 52:162–168.

HAZLETT, B. A. 1994. Alarm responses in the crayfish *Orconectes virilis* and *Orconectes propinquus*. *J. Chem. Ecol* 20:1525–1535.

HAZLETT, B. A. 2003. Predator recognition and learned irrelevance in the crayfish *Orconectes virilis*. *Ethology* 109:765–780.

HILDEBRAND, J. G., and SHEPHERD, G. M. 1997. Mechanisms of olfactory discrimination: converging evidence for common principals across phyla. *Annu. Rev. Neurosci* 20:595–561.

HOLLAND, K. M., and TEETER, J. H. 1981. Behavioral and cardiac reflex assays of the chemosensory acuity of channel catfish to amino acids. *Physiol. Behav* 27:699–707.

HORNER, A. J., WEISSBURG, M. J., and DERBY, C. D. 2004. Dual chemosensory pathways can mediate orientation of spiny lobsters to distant food odors. *J. Exp. Biol* 207:3785–3796.

HORNER, A. J., NICKLES, S. P., WEISSBURG, M. J., and DERBY, C. D. 2006. Source and specificity of chemical cues mediating shelter preference of Caribbean spiny lobsters (*Panulirus argus*). *Biol. Bull* 211:128–139.

HORNER, A. J., SCHMIDT, M., EDWARDS, D. H., and DERBY, C. D. 2008a. Role of the olfactory pathway in agonistic behavior of crayfish *Procambarus clarkii*. *Invertebr. Neurosci* 8:11–18.

HORNER, A. J., WEISSBURG, M. J., and DERBY, C. D. 2008b. The olfactory pathway mediates sheltering behavior of Caribbean spiny lobsters, *Panulirus argus*, to conspecific urine signals. *J. Comp. Physiol. A* 194:243–253.

ISHIMARU, Y., OKADA, S., NAITO, H., NAGAI, T., YASUOKA, A., MATSUMOTO, I., and ABE, K. 2005. Two families of candidate taste receptors in fishes. *Mech. Dev* 122:1310–1321.

JOHNSON, M. E., and ATEMA, J. 2005. The olfactory pathway for individual recognition in the American lobster *Homarus americanus*. *J. Exp. Biol* 208:2865–2872.

JONES, K. A. 1992. Food search and behaviour in fish and the use of chemical lures in commercial and sports fishing, pp. 288–320, in T. J. Hara (ed.). Fish Chemoreception. Chapman and Hall, New York.

KAMIO, M., MATSUNAGA, S., and FUSETANI, N. 2002. Copulation pheromone in the crab *Telmessus cheiragonus* (Brachyura: Decapoda). *Mar. Ecol. Prog. Ser* 234:183–190.

KAMIO, M., KUBANEK, J., and DERBY, C. 2006. *N*-Acetylglucosamino-1,5-lactone is a candidate sex pheromone in female blue crabs. *Chem. Senses* 31:A82.

KAMIO, M., KICKLIGHTER, C., KO, K.-C., NUSNBAUM, M., AGGIO, J., HUTCHINS, M., and DERBY, C. 2007. Defense through chemoreception: an L-amino acid oxidase in the ink of sea hares deters predators through their chemical senses. *Chem. Senses* 32:A37.

KAMIO, M., REIDENBACH, M. A., and DERBY, C. D. 2008. To paddle or not: context dependent courtship display by male blue crabs, *Callinectes sapidus*. *J. Exp. Biol* 211:1243–1248.

KAPOOR, B. G., and FINGER, T. E. 2003. Taste and solitary chemoreceptor cells, pp. 753–769, in G. Arratia, B. G. Kapoor, M. Chardon, and R. Diogo (eds.). Catfishes, vol. 2. Science, Enfield, New Hampshire.

KARAVANICH, C., and ATEMA, J. 1998. Individual recognition and memory in lobster dominance. *Anim. Behav* 56:1553–1560.

KELLER, T. A., and WEISSBURG, M. J. 2004. Effects of odor flux and pulse rate on chemosensory tracking in turbulent odor plumes by the blue crab, *Callinectes sapidus*. *Biol. Bull* 207:44–55.

KICKLIGHTER, C. E., SHABANI, S., JOHNSON, P. M., and DERBY, C. D. 2005. Sea hares use novel antipredatory chemical defenses. *Curr. Biol* 15:549–554.

KOBAYASHI, M., SORENSEN, P. W., and STACEY, N. E. 2002. Hormonal and pheromonal control of spawning in goldfish. *Fish Physiol. Biochem* 26:71–84.

KRASNE, F. B. 1973. Learning in Crustacea, pp. 49–130, in W. C. Corning, J. A. Dyal, and A. O. D. Willows (eds.). Invertebrate Learning. Vol. 2: Arthropods and Gastropod Mollusks. Plenum, New York.

KUPCHAN, S. M., BRITTON, R. W., LACADIE, J. A., ZIEGLER, M. F., and SIGEL, C. W. 1975. The isolation and structural elucidation of bruceantin and cruceantinol. *J. Org. Chem* 40:648–654.

LANE, A. L., and KUBANEK, J. 2006. Structure–activity relationship of chemical defenses from the freshwater plant *Micranthemum umbrosum*. *Phytochemistry* 67:1224–1231.

LI, W., SCOTT, A. P., SIEFKAS, M. J., YAN, H., LIU, Q., YUN, S.-S., and GAGE, D. A. 2002. Bile acid secreted by male sea lamprey that acts as sex pheromone. *Science* 296:138–141.

LITTLE, E. E. 1975. Chemical communication in maternal behaviour of crayfish. *Nature* 255:400–401.

LITTLE, E. E. 1976. Ontogeny of maternal behavior and brood pheromone in crayfish. *J. Comp. Physiol. A* 112:133–142.

LITTLE, E. E. 1977. Conditioned aversion to amino acid flavors in the catfish, *Ictalurus punctatus*. *Physiol. Behav* 19:743–747.

LUU, P., ACHNER, F., BERTRAND, H.-O., FAN, J., and NGAI, J. 2004. Molecular determinants of ligand selectivity in a vertebrate odorant receptor. *J. Neurosci* 24:10128–10137.

MACKIE, A. M. 1982. Identification of the gustatory feeding stimulants, pp. 275–291, in T. J. Hara (ed.). Chemoreception in Fishes. Elsevier, New York.

MANIAK, P. J., LOSSING, R., and SORENSEN, P. W. 2000. Injured Eurasian ruffe, *Gymnocephalus cernuss*, release an alarm pheromone which may prove useful in their control. *J. Great Lakes Res* 26:183–195.

MCCLINTOCK, T. S., ACHE, B. W., and DERBY, C. D. 2006. Lobster olfactory genomics. *Integr. Comp. Biol* 46:940–947.

MICHEL, W. C. 2006. Chemoreception, pp. 471–497, in D. H. Evans, and J. B. Claiborne (eds.). The Physiology of Fishes. 3rd edn. CRC, Boca Raton, FL.

MOORE, P. A. 2007. Agonistic behavior in freshwater crayfish: the influence of intrinsic and extrinsic factors on aggressive behavior and dominance, pp. 90–114, in J. E. Duffy, and M. Thiel (eds.). Evolutionary Ecology of Social and Sexual Systems: Crustacea as Model Organisms. Oxford University Press, Oxford, UK.

MOORE, P. A., and BERGMAN, D. A. 2005. The smell of success and failure: the role of intrinsic and extrinsic chemical signals on the social behavior of crayfish. *Integr. Comp. Biol* 45:650–657.

MOYLE, P. B., and CECH, J. J. 2000. Fishes: An Introduction to Ichthyology. 3rd edn.Prentice Hall, New York.

NEVITT, G., PENTCHEFF, N. D., LOHMANN, K. J., and ZIMMER, R. K. 2000. Den selection by the spiny lobster *Panulirus argus*: testing attraction to conspecific odors in the field. *Mar. Ecol. Prog. Ser* 203:225–231.

NEVITT, G. A., DITTMAN, A. D., QUINN, T. P., and MOODY, W. J. 2004. Evidence for a peripheral olfactory memory in imprinted salmon. *Proc. Nat Acad. Sci. U.S.A.* 91:4288–4292.

NGAI, J., DOWLING, M. M., BUCK, L., AXEL, R., and CHESS, A. 1993. The family of genes encoding odorant receptors in the channel catfish. *Cell* 72:657–666.

NIKONOV, A. A., and CAPRIO, J. 2001. Electrophysiological evidence for a chemotopy of naturally relevant odors in the channel catfish. *J. Neurophysiol* 86:1869–1876.

NIKONOV, A. A., FINGER, T. A., and CAPRIO, J. 2005. Beyond the olfactory bulb: an odotopic map in the forebrain. *Proc. Natl. Acad. Sci. U.S.A* 102:18688–18693.

NORDENG, H. 1977. A pheromone hypothesis for homeward migration in anadromous salmonids. *Nature* 233:411–413.

OIKE, H., NAGAI, T., FURUYAMA, A., OKADA, S., AIHARA, Y., ISHIMARU, Y., MARUI, T., MATSUMOTO, I., MISAKA, T., and ABE, K. 2007. Characterization of ligands for fish taste receptors. *J. Neurosci* 27:5584–5592.

PARKER, J. D., BURKEPILE, D. E., COLLINS, D. O., KUBANEK, J., and HAY, M. E. 2007. Stream mosses as chemically-defended refugia for freshwater macroinvertebrates. *Oikos* 116:302–312.

PFEIFFER, W., RIEGELBAUER, G., MEIER, G., and SCHEIBLER, B. 1985. Effect of hypoxanthine-3-*N*-oxide on central nervous excitation of the black tetra, *Gymnocorymbus ternetzi* (Characidae, Ostariophysi, Pisces) indicated by dorsal light response. *J. Chem. Ecol* 11:507–523.

POLING, K. R., FRASER, E. J., and SORENSEN, P. W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol* 129B:645–651.

PRUSAK, A. C., O'NEAL, J., and KUBANEK, J. 2005. Prevalence of chemical defenses among freshwater plants. *J. Chem. Ecol* 31:1145–1160.

REUSCH, T. B., HABERLI, M. A., AESCHLIMANN, P. B., and MILINSKI, M. 2002. Female sticklebacks in a strategy of sexual selection explaining MHC polymorphism. *Nature* 414:300–302.

RITTSCHOF, D., and COHEN, J. H. 2004. Crustacean peptide and peptide-like pheromones and kairomones. *Peptides* 25:1503–1516.

RITTSCHOF, D., TSAI, D. W., MASSEY, P. G., BLANCO, L., KUEBER, G. L., and HAAS, R. J. 1992. Chemical mediation of behavior in hermit crabs: alarm and aggregation cues. *J. Chem. Ecol* 18:959–984.

ROBERTSON, J. R., FUDGE, J. A., and VERMEER, G. K. 1981. Chemical and live feeding stimulants of the sand fiddler crab, *Uca pugilator* (Bose). *J. Exp. Mar. Biol. Ecol* 53:47–64.

ROLEN, S. H., SORENSEN, P. W., MATTSON, D., and CAPRIO, J. 2003. Polyamines as olfactory stimuli in the goldfish, *Carassius auratus*. *J. Exp. Biol* 206:1683–1696.

SARAIVA, L. R., and KORSCHING, S. I. 2007. A novel olfactory receptor gene family in teleost fish. *Genome Res* 17:1448–1457.

SCHACHTNER, J., SCHMIDT, M., and HOMBERG, U. 2005. Organization and evolutionary trends of primary olfactory brain centers in Tetraconata (Crustacea plus Hexapoda). *Arthropod. Struct. Dev* 34:257–299.

SCHAEFER, M. L., YOUNG, D. A., and RESTREPO, D. 2001. Olfactory fingerprints for major histocompatibility body odors. *J. Neurosci* 21:2481–2487.

SCHMIDT, M., and DERBY, C. D. 2005. Non-olfactory chemoreceptors in asymmetric setae activate antennular grooming behavior in the Caribbean spiny lobster *Panulirus argus*. *J. Exp. Biol* 208:233–248.

SHABANI, S., KAMIO, M., and DERBY, C. D. 2006. Chemicals released by injured or disturbed conspecifics mediate defensive behaviors via the aesthetasc pathway in the spiny lobster *Panulirus argus*. *Chem. Senses* 31:A81–A82.

SHOJI, T., YAMAMOTO, Y., NISHIKAWA, D., KURIHARA, K., and UEDA, H. 2003. Amino acids in stream waters are essential for salmon homing migration. *Fish Physiol. Biochem* 28:249–251.

SHURANOVA, Z., BURMISTROV, Y., and ABRAMSON, C. I. 2005. Habituation to a novel environment in the crayfish (*Procambarus cubensis*). *J. Crustac. Biol* 25:488–494.

SMITH, R. J. F. 1992. Alarm signals in fish. *Rev. Fish Biol. Fish* 2:33–63.

SORENSEN, P. W. 2007. Goldfish can be conditioned to respond to a sex pheromone. *Chem. Senses* 32:A19.

SORENSEN, P. W., and SCOTT, A. P. 1994. The evolution of hormonal sex pheromones in teleost fish: poor correlation between the pattern of steroid release by goldfish and olfactory sensitivity suggests that these cues evolved as a result of chemical spying rather than signal specialization. *Acta Scand. Physiol* 152:191–205.

SORENSEN, P. W., and CAPRIO, J. 1998. Chemoreception in fish, pp. 375–406, in R. E. Evans (ed.). The Physiology of Fishes. 2nd edn.CRC, Florida.

SORENSEN, P. W., and STACEY, N. E. 1999. Evolution and specialization in fish hormonal pheromones, pp. 15–48, in R. E. Johnston, D. Müller-Schwarze, and P. W. Sorensen (eds.). Advances in Chemical Signals in Vertebrates. Plenum, New York.

SORENSEN, P. W., and SATO, K. 2005. Second messenger systems mediating sex pheromone and amino acid sensitivity in goldfish olfactory receptor neurons. *Chem. Senses* 30:Suppl. 1315–316.

SORENSEN, P. W., and HOYE, T. R. 2007. A critical review of the discovery and application of a migratory pheromone in an invasive fish, the sea lamprey *Petromyzon marinus* L. *J. Fish Biol* 71:100–114.

SORENSEN, P. W., HARA, T. J., STACEY, N. E., and GOETZ, F. W. 1988. F prostaglandins function as potent olfactory stimulants that comprise the postovulatory female sex pheromone in goldfish. *Biol. Reprod.* 39:1039–1050.

SORENSEN, P. W., HARA, T. J., STACEY, N. E., and DULKA, J. G. 1990. Extreme olfactory specificity of male goldfish to the preovulatory pheromone 17a,20b-dihydroxy-4-pregnen-3-one. *J. Comp. Physiol. A* 166:373–385.

SORENSEN, P. W., HARA, T. J., and STACEY, N. E. 1991. Sex pheromones selectively stimulate the medial olfactory tracts of male goldfish. *Brain Res* 558:343–347.

SORENSEN, P. W., SCOTT, A. P., STACEY, N. E., and BOWDIN, L. 1995. Sulfated 17,20b-dihydroxy-4-pregnen-3-one functions as a potent and specific olfactory stimulant with pheromonal actions in the goldfish. *Gen. Comp. Endocrinol* 100:128–142.

SORENSEN, P. W., SCOTT, A. P., and KIHSLINGER, R. L. 2000. How common hormonal metabolites function as specific pheromones in the goldfish, pp. 125–129, in B. Norberg, O. S. Kjesbu, G. L. Taranger, E. Andersson, and S. O. Stefansson (eds.). Proceedings of the Sixth International Symposium on the Reproductive Physiology of Fish. Bergen, Norway.

SORENSEN, P. W., VRIEZE, L. A., and FINE, J. 2003. A multicomponent migratory pheromone in the sea lamprey. *Fish Physiol. Biochem* 28:253–257.

SORENSEN, P. W., FINE, J. M., DVORNIKOVS, V., JEFFREY, C. S., SHAO, F., WANG, J., VRIEZE, L. A., ANDERSON, K. R., and HOYE, T. R. 2005. Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nat. Chem. Biol* 1:324–328.

STACEY, N. E., and KYLE, A. L. 1983. Effects of olfactory tract lesions on sexual and feeding behavior in the goldfish. *Physiol. Behav* 30:621–628.

STACEY, N. E., and SORENSEN, P. W. 2005. Hormones, pheromones, and reproductive behaviors, pp. 359–412, in K. A. Sloman, S. Balshine, and R. W. Wilson (eds.). Behaviour: Interactions with Fish Physiology, vol. 24 in Fish Physiology (W. S. Hoar, D. J. Randall, and A. P. Farrell, series eds.). Academic, New York.

STEBBING, P. D., BENTLEY, M. G., and WATSON, G. J. 2003. Mating behaviour and evidence for a female released courtship pheromone in the signal crayfish *Pacifastacus leniusculus*. *J. Chem. Ecol* 29:465–475.

STEULLET, P., and DERBY, C. D. 1997. Coding of blend ratios of binary mixtures by olfactory neurons in the Florida spiny lobster, *Panulirus argus*. *J. Comp. Physiol. A* 180:123–135.

STEULLET, P., DUDAR, O., FLAVUS, T., ZHOU, M., and DERBY, C. D. 2001. Selective ablation of antennular sensilla on the Caribbean spiny lobster *Panulirus argus* suggests that dual antennular chemosensory pathways mediate odorant activation of searching and localization of food. *J. Exp. Biol* 204:4259–4269.

STEULLET, P., KRUETZFELDT, D. R., HAMIDANI, G., FLAVUS, T., NGO, V., and DERBY, C. D. 2002. Dual parallel antennular chemosensory pathways mediate odor-associative learning and odor

discrimination in the Caribbean spiny lobster *Panulirus argus*. *J. Exp. Biol* 205:851–867.

TANKERSLEY, R. A., BULLOCK, T. M., FORWARD, R. B., and RITTSCHOF, D. 2002. Larval release behaviors in the blue crab *Callinectes sapidus*: role of chemical cues. *J. Exp. Mar. Biol. Ecol* 273:1–14.

TING, J. H., and SNELL, T. W. 2003. Purification and sequencing of a mate-recognition protein from the copepod *Tigriopus japonicus*. *Mar. Biol* 143:1–8.

TROTT, T. J., and ROBERTSON, J. R. 1984. Chemical stimulants of cheliped flexion behavior by the western Atlantic ghost crab *Ocypode quadrata* (Fabricius). *J. Exp. Mar. Biol. Ecol* 78:237–252.

VALENTINČIČ, T. 2005. Taste and olfactory stimuli and behavior in fishes, pp. 65–85, in G. von der Emde, J. Mogdans, and B. G. Kapoor (eds.). Senses of FishNarosa, New Delhi.

VALENTINČIČ, T. S., and CAPRIO, J. C. 1994. Consummatory behavior in intact and anosmic channel catfish *Ictalurus punctatus* to amino acids. *Physiol. Behav* 55:857–863.

VALENTINČIČ, T. S., and CAPRIO, J. C. 1997. Visual and chemical release of feeding behavior in adult rainbow trout. *Chem. Senses* 22:375–382.

VALENTINČIČ, T. S., KRALJ, M., STENOVEC, A., and CAPRIO, J. C. 2000. The behavioral detection of binary mixtures of amino acids and their individual components by catfish. *J. Exp. Biol* 203:3307–3317.

VOIGT, R., and ATEMA, J. 1992. Tuning of chemoreceptor cells of the second antenna of the American lobster (*Homarus americanus*) with a comparison of four of its other chemoreceptor organs. *J. Comp. Physiol. A* 171:673–683.

VON FRISCH, K. 1938. Zur Psychologie des Fisch-schwarmes. *Naturwissenschaften* 226:601–606.

WARD, A. J. W., and HART, P. J. B. 2002. The effects of kin and familiarity on interactions between fish. *Fish & Fisheries* 4:348–358.

WEISSBURG, M. J., and ZIMMER-FAUST, R. K. 1991. Ontogeny *versus* phylogeny in determining patterns of chemoreception: initial studies with fiddler crabs. *Biol. Bull* 181:205–215.

WEISSBURG, M. J., FERNER, M. C., PISUT, D. P., and SMEE, D. L. 2002. The ecology of chemically-mediated prey perception. *J. Chem. Ecol* 28:1933–1950.

WIGHT, K., FRANCIS, L., and ELDRIDGE, D. 1990. Food aversion learning by the hermit crab *Pagurus granosimanus*. *Biol. Bull* 178:205–209.

WISENDEN, B. D. 2000. Olfactory assessment of predation risk in the aquatic environment. *Phil. Trans R. Soc. Lond. B* 355:1205–1208.

WONG, B. B. M., FISHER, H. S., and ROSENTHAL, G. G. 2005. Species recognition of male swordtails by chemical cues. *Behav. Ecol* 16:818–821.

YAMBE, Y., KITAMURA, S., KAMIO, M., YAMADA, M., MATSUNAGA, M., FUSETANI, N., and YAMAZAKI, F. 2007. L-Kynurenine, an amino acid identified as a sex pheromone in the urine of ovulated female masu salmon. *Proc. Nat. Acad. Sci. U.S.A* 103:15370–15374.

ZIMMER, R. K., and BUTMAN, C. A. 2000. Chemical signaling processes in the marine environment. *Biol. Bull* 198:168–187.

ZIMMER, R. K., and DERBY, C. D. 2007. Biological Bulletin virtual symposium: the neuroecology of chemical defense. *Biol. Bull* 213:205–207.

ZIMMER, R. K., and ZIMMER, C. A. 2008. Dynamic scaling in chemical ecology. *J. Chem. Ecol.* this issue

ZIMMER-FAUST, R. K. 1993. ATP: a potent prey attractant evoking carnivory. *Limnol. Oceanogr* 38:1271–1275.

ZIMMER-FAUST, R. K., and CASE, J. F. 1982a. Odors influencing foraging behavior of the California spiny lobster, *Panulirus interruptus*, and other decapod Crustacea. *Mar. Behav. Physiol* 9:35–58.

ZIMMER-FAUST, R. K., and CASE, J. F. 1982b. Organization of food search in the kelp crab, *Pugettia producta* (Randall). *J. Exp. Mar. Biol. Ecol* 57:237–255.

ZIMMER-FAUST, R. K., TYRE, J. E., and CASE, J. F. 1985. Chemical attraction causing aggregation in the spiny lobster, *Panulirus interruptus* (Randall), and its probable ecological significance. *Biol. Bull* 169:106–118.

ZIPPEL, H. P., VOIGT, R., KNAUST, M., and LUAN, Y. 1993. Spontaneous behaviour, training, and discrimination training in goldfish using chemosensory stimuli. *J. Comp. Physiol. A* 172:81–90.

ZULANDT SCHNEIDER, R. A., and MOORE, P. A. 2000. Urine as a source of conspecific disturbance signals in the crayfish *Procambarus clarkii*. *J. Exp. Biol* 203:765–771.

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REVIEW ARTICLE

Central Processing of Natural Odor Mixtures in Insects

Hong Lei · Neil Vickers

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Abstract In nature, virtually all olfactory stimuli are mixtures of many single odorants. Behavioral experiments repeatedly have demonstrated that an animal's olfactory system is capable of discriminating behaviorally relevant from irrelevant odor mixtures. However, the sensory mechanisms that underlie such discriminative capability remain elusive. The limited anatomical and physiological evidence collected from both insect and vertebrate models that pertains to this topic is scattered in the literature dating back to early 1980s. Thus, a synthesis of this information that includes more recent findings is needed in order to provide a basis for probing the fundamental question from a new angle. In this review, we discuss several proposed models for mixture processing, along with experimental data gathered from both the initial stage of olfactory processing (i.e., antennal lobe in insects or olfactory bulb in vertebrates) and higher areas of the brain, with an emphasis on how the lateral circuits in the antennal lobe or olfactory bulb may contribute to mixture processing. Based on empirical data as well as theoretical modeling, we conclude that odor mixtures may be represented both at the single-neuron level and at the population level. The difference between these two types of processing may reside in the degree of plasticity, with the former being hard-wired and the latter being more subjected to network modulation.

H. Lei (✉)
ARL-Neurobiology, University of Arizona,
Tucson, AZ 85721, USA
e-mail: hlei@email.arizona.edu

N. Vickers
Department of Biology, University of Utah,
Salt Lake City, UT 84112, USA

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Introduction

Virtually all organisms are capable of detecting and orienting to chemical stimuli. For many animals, odorous signals represent a rich source of information about the environment. Odorant molecules are detected by peripheral sensory cells and transduced into electrical potentials that can be conveyed to central processing structures in the brain. Over the past 20 years, there has been a rapid expansion of our knowledge of the olfactory system both at the peripheral and central levels. In this review, we focus on the coding of olfactory stimuli that occurs within the central nervous system. We discuss how the brain utilizes its anatomical resource to organize peripheral inputs and how this facilitates the recognition and discrimination of behaviorally relevant odor blends. A variety of approaches have been employed to investigate the neural basis of olfactory coding in insects. These include single-cell electrophysiology, multi-unit recording, and population-level studies that use voltage or calcium-sensitive dyes. In some instances, these neurobiological paradigms have been augmented by the use of modeling. We consider the evidence that these different techniques have provided for various coding schemes with a primary focus on insect systems. Where informative, comparisons between olfactory information processing in vertebrate and invertebrate systems are made.

Encoding Odor Mixtures at the Early Stage of Central Processing

Environmental odorous signals such as sex pheromones and volatiles emitted from a flower are first detected by olfactory receptor cells (ORCs) located at the periphery and then processed in central circuits across multiple synaptic levels, ultimately leading to specific olfaction-based behaviors. Unlike the pheromone-responsive ORCs that are strictly tuned to single pheromone components (but see Takanashi et al. 2006), ORCs that respond to plant odorants can be classified roughly into two categories, narrowly tuned or broadly tuned, when comparing their response spectra (Hansson et al. 1999; Hillier and Vickers 2007; Hillier et al. 2006; Rostelien et al. 2000; Shields and Hildebrand 2001a, b). Depending on the chemical identity, some plant odorants may activate a few specialized types of ORCs, whereas others may activate a large group of ORC types. ORCs that express the same olfactory receptor protein converge onto a single glomerulus in the antennal lobe (AL) of insects or olfactory bulb (OB) of vertebrates, the first processing stage along the olfactory pathway (Mombaerts et al. 1996; Ressler et al. 1994; Vosshall et al. 2000), thus extending the diversity of ORC tuning properties to the AL circuitry that includes glomerular output neurons or projection neurons (PNs) that are the equivalent of mitral/tufted (M/T) cells in OB.

Natural odor stimuli are almost always mixtures of numerous single components at certain concentrations and relative proportions, e.g., floral volatiles emitted from a flower. Detection, encoding, and discrimination of such complex stimuli are important functions for an animal's olfactory system. The anatomical organization of olfactory systems determines that information about natural odor stimuli is first deconstructed by the receptor array located at the periphery, then processed and distributed in the circuits of OB/AL, and finally reconstructed as a unitary odor percept at second- or higher-order synaptic centers, such as the olfactory cortex in vertebrates or the lateral protocerebrum in insects (Davis 2004). Little is known about the mechanisms by which the distributed information channels are combined to produce a perceived image of an odor mixture. In the following sections, we discuss several possibilities regarding mixture-coding mechanisms based on recent advances mainly in insect olfaction research but inevitably including some vertebrate literature.

Elemental and Configural Processing of Odor Blends

Overwhelming evidence from animal taxa ranging from insects to mammals indicates that a basic function of ORCs is to analyze the chemical composition of a natural odor by transforming the information of a complex chemical mixture into the combinatorial activation patterns of different types of ORCs, i.e., a so-called receptor code (Duchamp-Viret et al. 2000; Hallem and Carlson 2006; Korsching 2001; Ma and Shepherd 2000; Touhara 2002; Yao et al. 2005). Once the information about an odor mixture is distributed into different ORC channels, a process of regrouping all or a subset of these channels would conceivably create a unique perception of the original blend. This regrouping process may occur as early in processing pathway as the OB/AL.

The insect AL and vertebrate OB consist of a species-specific number of glomeruli, which collect the converging axonal inputs of ORCs expressing the same olfactory receptor protein (Mombaerts et al. 1996; Vosshall et al. 2000). The combinatorial receptor-code for odors is, therefore, also transmitted to the first processing stage and represented by odor-specific patterns of glomerular activity (Malnic et al. 1999). Imaging studies in both vertebrates (Friedrich and Korsching 1998; Rubin and Katz 1999; Uchida et al. 2000; Meister and Bonhoeffer 2001; Fried et al. 2002) and insects (Joerges et al. 1997; Galizia et al. 1999; Wang et al. 2003; Skiri et al. 2004; Carlsson et al. 2005) have provided ample evidence that supports this view. However, only a handful of physiological studies have been conducted to compare specifically the glomerular-activity patterns evoked by odor blends with that by the blend constituents. Nonetheless, it is generally accepted that for survival, animals must be able to discriminate behaviorally important odor mixtures, which may vary in their composition, blend ratio, and overall concentrations from other similar but behaviorally irrelevant odor mixtures in the animal's environment.

A general model to describe how an animal's olfactory system discriminates odor blends relates to information processing of blend constituents. A binary blend (A+B), for example, may be perceived as a linear summation of the individual representation for A or B alone or as a non-linear integration of the individual representations that discards information about the individual components but gives rise to a new percept of the odor mixture. The former type of processing has been labeled dissociative, analytical, or elemental and the latter associative, synthetic, or configural (Westbrook and Charnock 1985; Erickson et al. 1990; Alvarado and Rudy 1992; Kay et al. 2003, 2005). Elemental and configural processing are not mutually exclusive. Therefore, central processing of an odor mixture may result in a unique representation, while still preserving information about single components.

Evidence from Single-Cell Studies Evidence from several lines of research has revealed that the elemental and/or configural processing of odor blends may occur at the earliest processing stage of olfactory pathway (the OB or

AL). In the OB of the channel catfish, extracellular recordings have shown that most of the OB neurons respond to binary mixtures in a way that is similar to one of the components; however, when mixing one excitatory compound with an inhibitory compound, the response to the mixture became less predictable (Kang and Caprio 1995). Component dominancy, a form of elemental processing, also has been reported in a study on rat OB, where 90% of the binary mixtures were found to evoke responses in M/T cells if one of the two components also evoked responses (Giraudet et al. 2002). In a recent electrophysiological study on mouse OB, M/T cells typically responded to the effective compounds presented both as single compounds and in mixtures, although the mixture-evoked responses were often partially suppressed (Davison and Katz 2007). The overall conclusion from these studies was that OB circuits maintain elemental representations of single components when processing odor blends. However, a study on zebrafish OB demonstrated that the M/T cells displayed elemental or configural representations of odor mixtures depending on the compounds that were present in the odor mixture (Tabor et al. 2004). The responses of M/T cells to mixtures of amino acids often were dominated by one of the component responses, i.e., component dominance. In contrast, responses to mixtures of food extracts, which have more complicated compositions than amino acid mixtures, were significantly different from the responses to any of the individual food extracts that comprise the mixtures. This phenomenon was thought to be related to the degree of overlap between the representations of blend constituents (Tabor et al. 2004). In their experiment (Tabor et al. 2004), the food extracts contained many more components than the mixture of amino acids. Afferent inputs to M/T cells were, thus, more overlapping when using a mixture of food extracts versus a mixture of amino acids. With such highly overlapped inputs, the representation of the mixture (food extract 1+food extract 2) became less related to those of the constituents (food extract 1 or food extract 2) due to a strong activation of the lateral inhibitory pathways. Thus, the M/T cells appeared to acquire novel response properties. This model predicts that the more components used to make an odor blend, the less likely the blend representation would be to preserve the identities of the single components—in other words, trending away from an elemental toward a configural representation—because the degree of overlapping representation increases with the number of components. Indeed, results from human psychophysical experiments have demonstrated that discrimination of single components from an odor mixture become nearly impossible when the number of components exceeds 3 or 4 (Laing and Francis 1989; Livermore and Laing 1996). This model also predicts that the perception of odor mixtures should acquire more novel qualities with the increasing similarity of the components. Associative conditioning experiments in rats support this notion (Wiltrout et al. 2003). Animals in this experiment could not reliably identify the components in binary mixtures of similar, but not dissimilar, compounds.

Both elemental and configural representations of odor blends by the second-order output neurons have been reported in the insect AL (Christensen et al. 1989a, b, 1991, 1995; Hartlieb et al. 1997; Anton and Hansson 1995; Wu et al. 1996; Anton et al. 1997). The macroglomerular complex (MGC) of the male moth AL provides advantages in studying this question because it is devoted to processing female-produced pheromone blends, odor mixtures with obvious biological relevance. In the hawk moth *Manduca sexta*, for example, the uniglomerular projection neurons (uPNs) that arborize in one of the MGC glomeruli, the cumulus, show excitatory responses to the converging input from the ORCs that are specialized for one of the major pheromone components (*E*10,*E*12,*Z*14-hexadecatrienal) and show inhibitory responses to the input of another population of ORCs selective for a secondary pheromone component (*E*10,*Z*12-hexadecadienal) that specifically activates an adjacent glomerulus, the toroid (Christensen and Hildebrand 1997; Lei et al. 2002; Heinbockel et al. 2004). When combined, the two-component blend evokes excitatory responses in the uPNs despite the presence of the inhibitory component in the blend, a phenomenon that can be described as "elemental," because the blend-evoked response resembles the component-evoked response (Fig. 1; also see Heinbockel et al. 1999). However, examples of configural processing also have been reported in insect AL. In *Helicoverpa zea* male moths, some AL neurons were found that showed no or weak response to *Z*11-hexadecenal and *Z*9-tetradecenal, but the mixture of these two pheromone components evoked long-lasting-excitation that exceeded stimulus duration six times (Christensen et al. 1991). In contrast, some pheromone-responsive neurons in the AL of male *Agrotis segetum* were excited by each of the four pheromonal compounds but inhibited by the mixture of all these components (Hartlieb et al. 1997). These mixture-selective neurons, however, were not morphologically characterized. Single-cell studies on identified neuron types in the honeybee AL, including the homo-/hetero-local interneurons and uni-/multi-glomerular projection neurons, supported the hypothesis of configural processing of odor mixtures, i.e., responses to mixtures do not resemble the responses to single components. Particularly, mixtures tended to evoke stronger responses on multi-glomerular projection neurons (mPNs) and suppressed responses on uPNs in comparison with single-component-evoked responses on these two types of neurons (Sun et al. 1993).

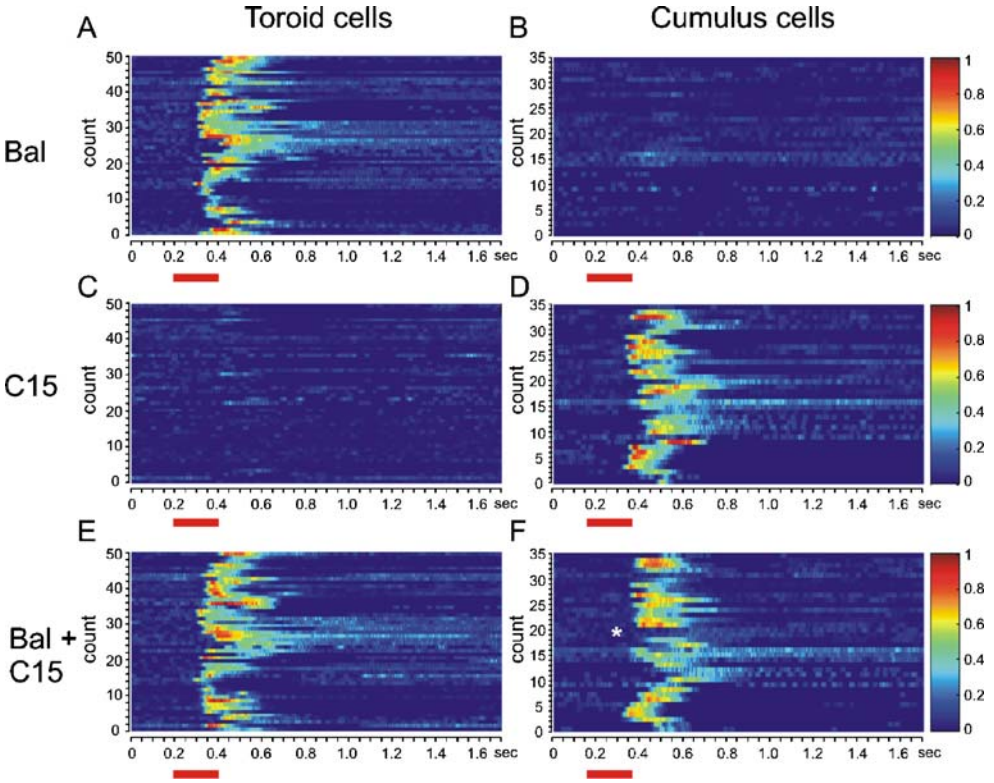

Fig. 1 Response specificity of MGC neurons—50 toroid cells (**a**, **c**, **e**) and 35 Cumulus cells (**b**, **d**, **f**)—recorded from the antennal lobe of male *M. sexta*. Toroid cells showed specific excitatory responses to Bal stimuli (**a**), inhibitory or no response to C15 stimuli (**c**), and excitatory responses to the binary mixture of Bal and C15 (**e**). Similarly, the cumulus cells were specifically excited by the C15 stimuli (**d**) and inhibited or not affected by the Bal stimuli (**b**). The binary mixture evoked responses that were similar to that evoked by C15 (**f**). Both types of cells exhibited elemental processing of the binary mixture, as demonstrated by the similarity of the active-compound-evoked responses and the mixture-evoked responses. However, some cells were exceptional (for example, the few cumulus cells indicated with *asterisk* in **f**), which were responsive to a single component but not to the mixture. The stimulus concentration was not consistent for all cells. The *bar below each panel* indicates the odor stimulation. The response magnitude is normalized to the maximum within cells

What type of neurons are likely the candidates in AL or OB that perform the configural processing of odor blends? Based on discoveries from the honeybee (Sun et al. 1993), we infer that a neuron serving such a function should possess two morphological features: (1) innervation of multiple glomeruli to gather information from different receptor channels and (2) termination at a higher synaptic center to supply the integrated information for further processing. Although no such type of neurons has been found in the OB of vertebrates, mPNs have been described in crustaceans (Wachowiak and Ache 1994) and insects (Homberg et al. 1988; Kanzaki et al. 1989; Stocker et al. 1990; Fonta et al. 1993; Malun et al. 1993; Abel et al. 2001; Ro et al. 2007). In *Drosophila*, unilateral mPNs bypass the mushroom body (MB) calyx, to which the uPNs send collateral branches, and directly terminate in the lateral protocerebrum (Stocker et al. 1990). The mPNs of honeybees, on the other hand, target a variety of places in the protocerebrum, including the MB calyx and the lateral protocerebrum (LP; Fonta et al. 1993). In *M. sexta*, the number of mPNs approximately comprise 50% of the total number of PNs (Homberg et al. 1988). Some of these mPNs densely innervate one or two neighboring glomeruli and send a few branches to other glomeruli nearby, while other mPNs innervate many glomeruli more or less evenly. The morphological characteristics of mPNs strongly suggest that they may play important roles in encoding odor blends.

A reasonable speculation on the response characteristics of mPNs is that they may display differential responses to an odor blend versus the blend constituents presented separately. This assumes that separate ORC populations, each of which encodes a constituent of a blend, send their axonal terminals to different glomeruli innervated by the mPN in question. If the responses of the mPN signal the presence of an odor blend, one should observe a blend-selective response of the mPN. The insect MGC offers an opportunity for studying this question because each glomerulus of the MGC exclusively receives input from ORCs that are tuned to one of the pheromone components. Although limited, some physiological data support the idea

that mPNs encode pheromone blends instead of the blend components. For example, in *A. segetum*, a type of mPN that innervates three subcompartments of the MGC showed no response to any of the four components of the female sex pheromones of this species but was strongly activated by the blend of these four components (Hansson et al. 1994). Similar results were reported in a later study on the same species (Wu et al. 1996) and also in another moth species, *Heliothis virescens* (Christensen et al. 1995). In an antennal transplant experiment that used *H. virescens* and *H. zea*, mPNs innervating multiple subcompartments of MGC responded to the two behaviorally attractive pheromone components as well as to the binary mixture of these components. However, these neurons responded more strongly to the blend and also followed a higher rate of repetitive blend pulses than that of single-component pulses (Vickers et al. 2005). The results from this experiment also indicate that the ORC input dictates the response specificity of the MGC PNs, including both uPNs and mPNs. The physiological responses of mPNs outside the MGC are rarely studied in great detail. An mPN, however, was reported in *Spodoptera littoralis* that innervates several glomeruli in the lateral region of the AL and that responded to two green leaf volatiles, *E*-2-hexenal and octanol (Anton and Hansson 1994). Unlike the cases in the MGC, however, it remains to be seen if these responses were mediated by one or more types of ORCs, thus making it difficult to assess the role of such particular mPNs in blend coding. These results emphasize the need for more systematical studies on the functional roles of the mPNs that innervate the sexually isomorphic glomeruli of the AL.

An important consideration in evaluating various coding strategies concerns the temporal structure of the stimulus itself (Vickers et al. 2001). Odor plumes released by an advertising female moth have an intermittent structure due to eddies and turbulence in the moving air stream. This structure has been shown to have dramatic effects upon the behavioral performance of male moths (Baker 1990; Girling and Carde 2007), presumably due to its advantage in keeping a signal's integrity against noisy chemical background (Liu and Haynes 1992) as well as in preventing sensory adaptation to the signal (Baker et al. 2003). As a consequence of the natural stimulus dynamics, further accentuated by the movement of the male through the plume, pheromone-laden filaments arrive at the antennal surface intermittently. The ability of AL PNs to accurately follow these pulses has important consequences for the behavior of these insects. Existing evidence demonstrates that this ability is enhanced by odor blends (Christensen and Hildebrand 1997; Heinbockel et al. 2004), suggesting that PNs' response dynamics could emerge as a new coding feature that results from the configural processing of odor blends in the AL. The inhibitory inputs to these PNs mediated by GABAergic local interneurons (LNs) are believed to account for the blend-associated response dynamics (Christensen and Hildebrand 1997; Heinbockel et al. 2004).

Owing to the fact that ORCs that express the same type of OR proteins converge onto a single glomerulus where they directly transmit information to uPNs, these AL neurons are expected to inherit the odor information transduced by the ORCs (Wang et al. 2003). The degree of configural processing of odor blends at the ORC level is limited; therefore, the responses of uPNs to odor blends are likely dictated by the effective components in the blend. In other words, uPN response may contain mainly information about single components. However, each glomerulus is a functional information-processing unit rather than merely a relay station. The involvement of LNs in glomerular processing will likely modify the output of uPNs (Olsen and Wilson 2008), either by enhancing or inhibiting uPNs' activity (see below for more details of uPNs receiving information laterally). The mPNs, on the other hand, may receive concurrent inputs from multiple ORC types, which may carry information about constituents of an odor blend. Depending on the arborization pattern of an mPN, none of the ORC types might be able to dictate the mPN's response. The final output of the mPN may be entirely determined by the balance between all concurrent ORC inputs and the result of local processing, thus rendering little similarity to the response evoked by single components alone (Hansson et al. 1994; Christensen et al. 1995; Vickers et al. 2005; Wu et al. 1996).

While the uPNs carry information about the identity of single components, they may still produce responses that contain novel features signaling the presence of an odor blend. Such features are likely the result of glomerular processing that is affected by the stimulation with odor blends. For instance, some uPNs that innervate the MGC of *M. sexta* AL followed stimulus dynamics with greater fidelity when stimulated with the pheromone blend compared to single components, probably because one of the components activated interglomerular inhibition that facilitated the process of repolarization and fast post-stimulus recovery (Christensen and Hildebrand 1997; Heinbockel et al. 2004). Furthermore, in addition to individual responses, uPNs may also collectively develop some group features to encode odor blends. For example, the spiking activities of MGC PNs in *M. sexta* are better synchronized to each other in the context of the pheromone blend (Lei et al. 2002). Interglomerular inhibition may contribute to such enhanced synchrony.

Evidence from Population Studies Are the principles of mixture processing revealed at the single-neuron level maintained at the glomerular level? Results obtained with

various optical imaging methods provide an approximate answer to this question. Because imaging data are usually collected over a large portion of OB/AL surface area, these methods not only allow assessment of the difference in activity level within individual glomeruli in response to odor blends and blend components but also facilitate a comparison of AL/OB-wide spatial patterns of glomerular activity evoked by odor blends with that by single components. The importance of the spatially distributed glomerular-activity pattern in encoding odors has been demonstrated by an increasing amount of evidence from various animal models (Vosshall 2001; Leon and Johnson 2003; Nikonov et al. 2005).

Application of voltage-sensitive dyes on the honeybee AL revealed that in some glomeruli the odor mixtures elicited transient responses, the magnitude of which was approximately the summation of the responses evoked by the individual components of that mixture, and in other glomeruli the mixture-evoked response was reduced; the responses in some glomeruli showed no differences for odor mixture and single components (Galizia et al. 2000). These data suggested that mixture interactions were a glomerulus-dependent phenomenon. An earlier calcium imaging study also in honeybee but using mixtures made from simpler odorants (e.g., citral, hexanol) showed that these mixtures suppressed glomerular responses as compared with the arithmetic summation of the component-evoked responses; and the more components included in a mixture, the stronger the suppression effect (Joerges et al. 1997). Although the validity of using arithmetic summation for studying mixture interactions is arguable (Tabor et al. 2004), mixture suppression was also evident in another calcium imaging study on the honeybee AL where the authors compared the mixture-evoked responses with the strongest response evoked by single components (Deisig et al. 2006). Results from a recent study that used the same technique on a moth AL, however, did not support the conclusion from the honeybee data. The mixture-evoked glomerular responses in *S. littoralis* AL were neither significantly suppressed nor enhanced as compared with the component-evoked responses, a result in favor of elemental processing of odor blends (Carlsson et al. 2007).

The spatial distribution of glomerular response patterns also show evidence of mixture interactions but dependent on odors as revealed by another calcium imaging study (Galizia et al. 1999). Antennal stimulation with a mixture of orange and clove oil odors on honeybees resulted in an activity pattern that was qualitatively different from the pattern predicted from the summation of actions of the individual odors. However, the pattern evoked by the mixture of clove oil and peppermint odors was nearly identical to the sum of the patterns produced by the two odors alone (Galizia et al. 1999). Similarly, two intrinsic imaging studies on mouse OB showed that mixture-evoked glomerular-activity pattern was a simple summation of the individual patterns evoked by the mixture components (Belluscio and Katz 2001; Lin et al. 2006). The most systematic approach along these lines so far was taken in a calcium imaging study in the honeybee, where two salient and two less salient odorants were used to make binary, ternary, and quaternary mixtures (Deisig et al. 2006). Then, the response similarity between mixture-evoked and single-component-evoked responses was quantified by calculating the Euclidean distances between them within a multi-dimensional space defined by all 24 glomeruli activated by the four single components and their mixtures. The results showed that the mixture-evoked response patterns were always more similar to the patterns evoked by salient odorants than to that by less salient (or weaker) odorants—a clear example of component dominance or elemental processing.

Taken together, an interesting picture emerges from these optophysiological studies. First, mixture suppression seems more prominent than mixture synergism (but see Carlsson et al. 2007). This phenomenon may be related to the action of the global and/or local inhibitory network in the insect AL (Lei et al. 2002; Sachse and Galizia 2002). GABAergic LNs are most likely activated upon odor stimulation. The more components included in an odor mixture the greater activation of the LN network and presumably the stronger suppressive effect (Deisig et al. 2006). Second, mixture representations seem to follow more closely the elemental rule of mixture processing. In other words, the response to a mixture may be predicted simply from the response to single components. This phenomenon may be related to the cellular sources that contribute to the calcium signal. Bath application of calcium dye may allow mainly observations on the response dynamics of ORCs (Galizia et al. 1998; Sachse and Galizia 2003) and possible contribution from glial cells (Galizia and Vetter 2004). Since there are limited mixture interactions at the ORC level, the glomerular responses to mixtures appear to be elemental as observed from the calcium dynamics of pre-synaptic terminals. However, this does not exclude the possibility that the glomerular output neurons may use configural principles to encode odor mixtures.

Circuit Mechanisms that Underlie Encoding of Odor Blends

What are the possible mechanisms that would allow glomerular output neurons not just to carry the information transmitted by ORCs converging onto a particular glomerulus but also information processed in other glomeruli? This is essentially equivalent to asking how a uPN

associated with a particular glomerulus can encode an odor mixture when the information concerning the blend is distributed beyond the glomerulus that the uPN innervates. The answer to this question must pertain to a process of lateral information transmission within the glomerular array, either through lateral excitatory or lateral inhibitory pathways.

Interglomerular Excitation and its Potential Role in Blend Coding Glomeruli in the OB/AL are interconnected by diverse types of interneurons, many of which are known to be GABAergic, thus serving as a substrate for lateral inhibition (Egger et al. 2003; Wilson and Laurent 2005; Vucinic et al. 2006). A recent report demonstrated the existence of cholinergic LNs in the AL of *Drosophila* (Shang et al. 2007), also providing an explanation for an earlier observation where PNs were shown to have a broader molecular receptor range than their cognate ORCs (Wilson et al. 2004). However, these results contradict another study in which the PNs faithfully transmitted information from their presynaptic ORCs (Wang et al. 2003). One of the functions of such interglomerular excitation, according to Shang et al. (2007), may be to boost PN signals and enhance their transmission to third-order neurons (mushroom body Kenyon cells) in a mechanism akin to stochastic resonance. We argue that, on the other hand, interglomerular excitation could be used by the AL circuits to combine information from a subset of glomeruli, which may participate in encoding an odor mixture. The heterogeneous local interneurons (hetero-LNs), described in the ALs of honeybee and moth (Christensen et al. 1993; Sun et al. 1993), could be a potential neural substrate for gathering information from a group of glomeruli and relaying it to a "core" unit, i.e., the glomerulus that is most densely innervated by the LN arbors. The uPNs innervating the "core" unit could theoretically be presented with all pieces of information about the mixture collected by other branches of the hetero-LN. To test this hypothesis, two key issues need to be resolved: (1) verification of the cholinergic properties of the hetero-LNs; (2) verification of the polarity of hetero-LN synapses, i.e., the dense arbors in the "core" glomerulus comprise mainly output synapses while the sparser arbors in neighboring glomeruli have only input synapses. These data are not yet available.

Interglomerular Inhibition and Its Potential Role in Blend Coding Except for the recently reported cholinergic LNs in the fly AL (Shang et al. 2007), LNs in insect AL and granule cells in vertebrate OB have been long considered as the major source of inhibition mediated by γ-aminobutyric acid (GABA) receptors in the AL/OB (Nowycky et al. 1981; Jahr and Nicoll 1982; Hoskins et al. 1986; Python and Stocker 2002; Wilson and Laurent 2005). Interglomerular inhibition plays important roles in enhancing contrast of odor representation (Yokoi et al. 1995; Sachse and Galizia 2002; Vucinic et al. 2006), shaping the response of glomerular output neurons (Wilson and Laurent 2005; Olsen and Wilson 2008) and strengthening intraglomerular synchrony (Lei et al. 2002). Additionally, such inhibitory mechanisms have also been proposed in a modeling study of the rat OB to underlie the behaviorally observed perceptual properties of complex odor stimuli, i.e., configural, elemental, or a mixture of the two odor interactions (Linster and Cleland 2004). In this model, interglomerular inhibition could be either removed or kept intact with comparisons made between the overlap of M/T cells' response patterns evoked by single components A and B, A (or B), and the binary mixture AB. The higher degree of overlap the mixtures, the more similar the responses. They found that with the interglomerular inhibition removed, the overlap between A- (or B-) and AB-evoked responses was positively correlated with the overlap between A- and B-evoked responses, but the overlap between AB and A (or B) was always greater than the overlap between A and B (Fig. 2). In other words, adding B (or A) to the mixture did not bring significant "new" features to the responses, as would be predicted by the principle of elemental processing of odor mixtures. In contrast, with the interglomerular inhibition intact, when the M/T cells' responses to the individual odors A and B were very similar, the M/T cells' responses to the binary mixture AB was dissimilar to either of the two individual odorant responses (Fig. 2). In other words, the mixture-evoked responses did not represent information for any single component, thus exhibiting characteristics of configural processing. The dissimilarity between mixture and component decreased as the dissimilarity of A and B increased until A and B evoked responses beyond the range of mutual inhibitory interactions, at which point interglomerular inhibition had no effect, resulting in elemental representations of odor mixtures (Linster and Cleland 2004; Fig. 2).

The significance of this work is that it provides a mechanistic explanation for conflicting behavioral or physiological observations where, in some instances, odor mixture evokes novel percepts or neural representations that are completely independent of that evoked by single components, while in other circumstances, the effect of odor mixture is a simple summation of the effects from single components. If two dissimilar odorants A′ and B′ evoke non-overlapping representations in OB, the mixture (A′B′) will produce a pattern of glomerular activity that is a linear summation of the individual patterns induced by A or B alone. However, when A and B become more similar, their corresponding glomerular responses become more

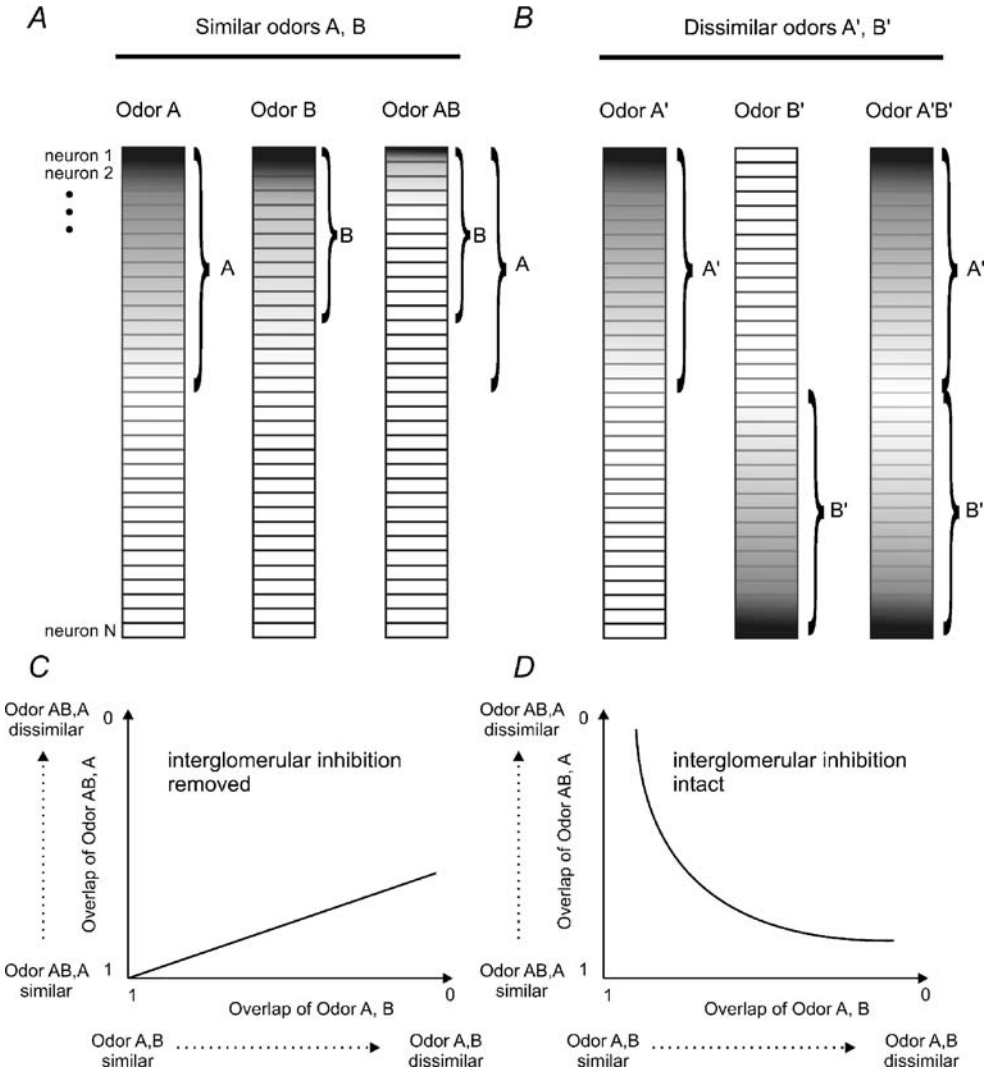

Fig. 2 A schematic representation of a conceptual model that explains how interglomerular inhibition in antennal lobe or olfactory bulb may enable configural and elemental processing of odor mixtures (adapted from Linster and Cleland 2004). **a** Two similar odors (*Odor A*, *B*) produce a highly overlapped representation on an array of olfactory neurons, i.e., almost the same set of neurons is activated by these two odors. The *shading levels* represent different magnitude of neural activation by these odors. Due to the interference of the interglomerular inhibitory circuits that are commonly activated by Odor A and B, the mixture of these two odors (*Odor AB*) produces an activation pattern that bears no resemblance to either A or B. Thus, the total representation for the mixture contains no information of its constituents—a main feature of configural processing. **b** In contrast, two dissimilar odors (*Odor A′*, *B′*) are represented by the array of olfactory neurons in distinctly different ways, with Odor A′ mainly encoded by the top 50% of neurons and Odor B′ by the bottom 50%. The interglomerular inhibitory circuits associated with the stimulation by A′ and B′ are non-overlapping, causing no interference to the total representation. The mixture of these two odors (*Odor A′B′*) additively activates the entire array of olfactory neurons. The neurons that encode Odor A′ are equally activated by Odor A′B′; thus, the total representation of Odor A′B′ contains the same amount of information on Odor A′ as it would if using Odor A′ alone—a main feature of elemental processing. The same is true for Odor B′. **c**, **d** In this model, after mathematically removing the interglomerular inhibition, the degree of overlap for neural representations of Odor A and B is positively correlated with that of Odor AB and A. However, when the interglomerular inhibition is kept intact, this relationship is reversed, meaning the more similar the Odor A and B, the less degree of overlap the representation for the mixture (Odor AB) and its constituent (Odor A)

overlapped; the mixture AB then will activate the interglomerular inhibitory circuits in such a way that the M/T cells activated by AB mixture will exert inhibitory effect on each other to various degrees, thus resulting in a final representation of the mixture that is not predictable from A- and B-evoked responses (Fig. 2). Moreover, the strength of interglomerular inhibition may be dependent on odors as well as the inhibitory neurons. Therefore, it is reasonable to expect that some odor mixtures will induce partially elemental and partially configural interactions. In a network with such inhibitory capability, one could observe all three types of mixture interactions: elemental, configural, and both.

Encoding Odor Blends at Later Stages of Central Processing

In vertebrates, odor information is transmitted from the OB to the olfactory cortex, which comprises five main areas: the anterior olfactory nucleus, the anterior and posterior piriform cortex (the largest cortical area that receives OB inputs), the olfactory tubercle, and parts of both the amygdala and entorhinal cortex. In insects, the anatomical organization of some of the olfactory centers downstream from the AL is similar to that of downstream olfactory brain areas in vertebrates. For instance, as in vertebrates, axons of glomerular output neurons project widely to second-order olfactory centers in the protocerebrum and overlap with the projections of output neurons from other glomeruli (Marin et al. 2002; Wong et al. 2002; Kanzaki et al. 2003; Tanaka et al. 2004; Zou et al. 2005; Komiyama and Luo 2006; Jefferis et al. 2007; Lin et al. 2007). It has been suggested that some protocerebral olfactory centers such as the MB and LP in insects are at the same hierarchical level as the mammalian piriform cortex (Davis 2004). Thus, in both vertebrates and insects, synthesis of olfactory stimulus features into "objects" (Hopfield 1991; Wilson 2003; Jefferis et al. 2007) could be produced by convergence of output axons from different glomeruli onto individual neurons in the piriform cortex or the MB and/or LP. How are odor mixtures and single components represented at this level of processing?

Mixture-Selective Responses in Higher-Order Neurons

Theoretically, cortical neurons may respond to combined afferental (M/T cells) inputs, reflecting a configural type of mixture interaction, thereby creating a novel cortical representation of odor mixtures. However, an electrophysiological study on rat olfactory cortex, particularly the anterior and posterior piriform cortex (aPCX and pPCX), did not report any evidence for such mixture-selective cortical neurons; instead, all neurons encountered showed responses to mixture and at least to one of the mixture components (Kadohisa and Wilson 2006). Interestingly, this study also showed that after training the animals to distinguish odor mixtures from their components, the aPCX neurons decorrelate their population response to mixtures with that to single components, indicating that the departure of mixture representation from component representation is an experience-dependent phenomenon at the population level. In insects, blend-selective responses at higher-order olfactory centers have been reported in male *M. sexta*, where some neurons arborizing in a third olfactory center, the lateral accessory lobe, exhibited weak or no response to single pheromone components but a remarkable long-lasting-excitation (LLE) response to the blend of two major pheromone components (Kanzaki et al. 1991). In a different moth species (*A. segetum*), about 15% of protocerebral neurons were blend selective, and about 45% were component selective (Lei et al. 2001). Blend-evoked LLE response was also observed in this study. However, in *Bombyx mori*, LLE could be evoked by a single pheromone component (Kanzaki and Shibuya 1992).

Spatial Segregation of Odor Representations at Second-Order Olfactory Centers

The common organization of the olfactory pathway up to the OB/AL level in vertebrates and insects has determined that information about odor mixtures is first segregated by ORCs then distributed among the glomerular output neurons. Psychophysical evidence, however, strongly suggest that olfaction is configural or synthetic (Laing and Francis 1989; Livermore and Laing 1996), indicating that the distributed OB/AL outputs are regrouped somewhere along the olfactory pathway. Where does the synthesis take place?

Examination of the connectivity pattern between the second-order (PNs) and the third-order (MB Kenyon cells and LH (Lateral Horn) interneurons) neurons in *Drosophila* revealed three projection zones in MB and LH that are formed by the terminals of PNs that receive information from particular subgroups of glomeruli in the AL, thus suggesting that the chemotopical representation produced at the AL persists in these two second-order olfactory centers (Tanaka et al. 2004). Indeed, a later study confirmed this hypothesis by revealing segregated zones for representations of fruit odors and putative pheromone compounds in the LH, with the former focusing on the posterior-dorsal and the latter on anterior-ventral portion of the LH (Jefferis et al. 2007). Electrophysiological evidence from channel catfish indicates that feeding cues and social cues are processed in distinct regions of the forebrain, a brain structure downstream from the OB (Nikonov et al. 2005). In *M. sexta*, it was long established that PNs that innervate MGC and other sexually isomorphic glomeruli project to different regions in the second-order olfactory center, namely, inferior lateral protocerebrum (ILPC) and LH, respectively (Homberg et al. 1988), and a similar segregation pattern has been reported in other insect species (Malun et al. 1993; Kanzaki et al. 2003; Kirschner et al. 2006). In *B. mori*, PNs that arborize in different subcompartments of the MGC project to different sub-regions of MB calyces and ILPC (Kanzaki et al. 2003).

Although it is clear that the output tracts from the AL project to distinct zones of MB and/or LH, is the information from AL further synthesized in these regions? Results from single-cell labeling studies on *Drosophila* demonstrate that MB Kenyon cells integrate information across all three projection zones of AL PNs distributed in

the calyces and send output to the lobe region of the MB. In LH, however, the third-order neurons only link segregated subgroups of PNs to specific brain areas (Tanaka et al. 2004). These data seem to suggest that configural processing of odor mixtures may be more likely occurring in the MB than in the LH, as the Kenyon cells are connected to many more types of PNs across all three calycal zones. Physiological data on this particular issue is limited at present, but some Kenyon cells recorded in locust showed mixture suppression and synergism when tested with two odorants (citral and geraniol) and their binary mixture (Broome et al. 2006).

Concluding Remarks and Future Directions

Blend coding is a complicated process that involves neural circuits at multiple synaptic levels along the olfactory pathway. Data discussed in this review strongly suggest that both elemental and configural processing of odor mixtures occur in the first stage of central processing as well as in the later stages. It is unclear at this point if this is because the olfactory systems are evolved to segregate information about a mixture and its constituents along the entire olfactory pathway, or because the central structure where the information about odor blends is synthesized is yet to be discovered. Data that address this issue will likely come from insect models because of their anatomical simplicity and experimental tractability. For example, a recent study of tracing olfactory neural pathway in the honeybee AL revealed that multiglomerular PNs terminate at a distinct area in the lateral protocerebrum (Kirschner et al. 2006), thus suggesting there may be specific pathways for transmitting information about odor blends.

Multiglomerular PNs are a particularly interesting population of neurons in the insect AL when considering the possible roles for mixture processing. Their anatomical features suggest that this population of neurons may function as hard-wired devices for encoding odor mixtures that are undoubtedly processed in multiple glomeruli. The uPNs, on the other hand, are highly efficient in transmitting information that is processed in single glomeruli. However, both lateral excitatory and inhibitory circuits in the AL may render these PNs capable of carrying information from other glomeruli, possibly related to odor mixtures. The difference in encoding odor mixtures by these two types of PNs may lie in the degree of plasticity. Experience may modify the strength of lateral interactions in the AL circuits, thereby affecting the coding efficiency for those odor-mixtures that activate the lateral circuits. The action of mPNs may represent a coding strategy for innately preferred odor blends such as sex pheromones or a particular food source. Another possibility is that the same odor mixture may be encoded by both types of PNs, thus rendering the olfactory system a capability to regulate its coding for innately preferred odor blends based on experience (Anderson et al. 2007). It is also possible that configural processing of odor mixtures may take place at the second-order olfactory centers (MB and/or LH) where the third-order neurons integrate information across multiple zones of the centers that receive parallel inputs from uPNs. Obviously, systematic, comparative studies on mPNs and uPNs are needed to understand fully their functional roles in encoding odor blends.

In summary, mixture interactions of odorants may appear to be elemental, configural, or both, depending on what level of processing and what type of cells are being observed. There may not be a universal rule on how odor mixtures are processed in the central olfactory system. Thus, a comparative approach to this question by using different animal models within an evolutionary context is essential.

References

ABEL, R., RYBAK, J., and MENZELS, R. 2001. Structure and response patterns of olfactory interneurons in the honeybee, *Apis mellifera*. *J. Comp. Neurol.* 437:363–383.

ALVARADO, M. C., and RUDY, J. W. 1992. Some properties of configural learning—an investigation of the transverse-patterning problem. *J. Exp. Psychol. Anim. Behav. Process.* 18:145–153.

ANDERSON, P., HANSSON, B. S., NILSSON, U., HAN, Q., SJOHOLM, M., SKALS, N., and ANTON, S. 2007. Increased behavioral and neuronal sensitivity to sex pheromone after brief odor experience in a moth. *Chem. Senses* 32:483–491.

ANTON, S., and HANSSON, B. S. 1994. Central processing of sex-pheromone, host odor, and oviposition deterrent information by interneurons in the antennal lobe of female *Spodoptera littoralis* (Lepidoptera, Nocituidae). *J. Comp. Neurol.* 350:199–214.

ANTON, S., and HANSSON, B. S. 1995. Sex-pheromone and plant-associated odor processing in antennal lobe interneurons of male *Spodoptera littoralis* (Lepidoptera, Noctuidae). *J. Comp. Physiol. A* 176:773–789.

ANTON, S., LÖFSDET, C., and HANSSON, B. S. 1997. Central nervous processing of sex pheromones in two strains of the European corn borer *Ostrinia nubilalis* (Lepidoptera: Pyralidae). *J. Exp. Biol.* 200:1073–1087.

BAKER, T. C. 1990. Upwind flight and casting flight: Complimentary phasic and tonic systems used for location of sex pheromone sources by male moths, pp. 18–25, in K. B. Døving (ed.). ISOT X Proceedings of Tenth International Symposium on Olfaction and TasteUniversity of Oslo, Oslo, Norway.

BAKER, T. C., HANSSON, B. S., LÖFSDET, C., and LÖFQVIST, J. 2003. Adaptation of male moth antennal neurons in a pheromone plume is associated with cessation of pheromone-mediated flight. *Chem. Senses* 14:439–448.

BELLUSCIO, L., and KATZ, L. C. 2001. Symmetry, stereotypy, and topography of odorant representations in mouse olfactory bulbs. *J. Neurosci.* 21:2113–2122.

BROOME, B. M., JAYARAMAN, V., and LAURENT, G. 2006. Encoding and decoding of overlapping odor sequences. *Neuron* 51:467–482.

CARLSSON, M. A., CHONG, K. Y., DANIELS, W., HANSSON, B. S., and PEARCE, T. C. 2007. Component information is preserved in glomerular responses to binary odor mixtures in the moth *Spodoptera littoralis*. *Chem. Senses* 32:433–443.

CARLSSON, M. A., KNUSEL, P., VERSCHURE, P. F. M., and HANSSON, B. S. 2005. Spatio-temporal Ca2+dynamics of moth olfactory projection neurons. *Eur. J. Neurosci.* 22:647–657.

CHRISTENSEN, T. A., and HILDEBRAND, J. G. 1997. Coincident stimulation with pheromone components improves temporal pattern resolution in central olfactory neurons. *J. Neurophysiol.* 77:775–781.

CHRISTENSEN, T. A., HILDEBRAND, J. G., TUMLINSON, J. H., and DOOLITTLE, R. E. 1989a. Sex pheromone blend of *Manduca sexta*: responses of central olfactory interneurons to antennal stimulation in male moths. *Arch. Insect Biochem. Physiol.* 10:281–289.

CHRISTENSEN, T. A., MUSTAPARTA, H., and HILDEBRAND, J. G. 1989b. Discrimination of sex-pheromone blends in the olfactory system of the moth. *Chem. Senses* 14:463–477.

CHRISTENSEN, T. A., MUSTAPARTA, H., and HILDEBRAND, J. G. 1991. Chemical communication in heliothine moths. II. Central processing of intra- and interspecific olfactory messages in the male corn earworm moth *Helicoverpa zea*. *J. Comp. Physiol. A* 169:259–274.

CHRISTENSEN, T. A., MUSTAPARTA, H., and HILDEBRAND, J. G. 1995. Chemical communication in heliothine moths VI. Parallel pathways for information processing in the macroglomerular complex of the male tobacco budworm moth *Heliothis virescens*. *J. Comp. Physiol. A* 177:545–557.

CHRISTENSEN, T. A., WALDROP, B. R., HARROW, I. D., and HILDEBRAND, J. G. 1993. Local interneurons and information-processing in the olfactory glomeruli of the moth *Manduca sexta*. *J. Comp. Physiol. A* 173:385–399.

DAVIS, R. L. 2004. Olfactory learning. *Neuron* 44:31–48.

DAVISON, I. G., and KATZ, L. C. 2007. Sparse and selective odor coding by mitral/tufted neurons in the main olfactory bulb. *J. Neurosci.* 27:2091–2101.

DEISIG, N., GIURFA, M., LACHNIT, H., and SANDOZ, J. C. 2006. Neural representation of olfactory mixtures in the honeybee antennal lobe. *Eur. J. Neurosci.* 24:1161–1174.

DUCHAMP-VIRET, P., DUCHAMP, A., and CHAPUT, M. A. 2000. Peripheral odor coding in the rat and frog: quality and intensity specification. *J. Neurosci.* 20:2383–2390.

EGGER, V., SVODOBA, K., and MAINEN, Z. F. 2003. Mechanisms of lateral inhibition in the olfactory bulb: efficiency and modulation of spike-evoked calcium influx into granule cells. *J. Neurosci.* 23:7551–7558.

ERICKSON, R. P., PRIOLO, C. V., WARWICK, Z. S., and SCHIFFMAN, S. S. 1990. Synthesis of tastes other than the primaries—implications for neural coding theories and the concept of suppression. *Chem. Senses* 15:495–504.

FONTA, C., SUN, X. J., and MASSON, C. 1993. Morphology and spatial-distribution of bee antennal lobe interneurons responsive to odors. *Chem. Senses* 18:101–119.

FRIED, H. U., FUSS, S. H., and KORSCHING, S. I. 2002. Selective imaging of presynaptic activity in the mouse olfactory bulb shows concentration and structure dependence of odor responses in identified glomeruli. *Proc. Natl. Acad. Sci. U. S. A.* 99:3222–3227.

FRIEDRICH, R. W., and KORSCHING, S. 1998. Chemotopic, combinatorial, and noncombinatorial odorant representations in the olfactory bulb revealed using a voltage-sensitive axon tracer. *J. Neurosci.* 18:9977–9988.

GALIZIA, C. G., and VETTER, R. 2004. Optical methods for analyzing odor-evoked activity in the insect brain, pp. 349–392, in T. A. Christensen (ed.). Advances in Insect Sensory NeuroscienceCRC, Boca Raton.

GALIZIA, C. G., NÄGLER, K., HÖLLDOBLER, B., and MENZEL, R. 1998. Odour coding is bilaterally symmetrical in the antennal lobes of honeybees (*Apis mellifera*). *Eur. J. Neurosci.* 10:2964–2974.

GALIZIA, C. G., SACHSE, S., RAPPERT, A., and MENZEL, R. 1999. The glomerular code for odor representation is species specific in the honeybee *Apis mellifera*. *Nat. Neurosci.* 2:473–478.

GALIZIA, C. G., KUTTNER, A., JOERGES, J., and MENZEL, R. 2000. Odour representation in honeybee olfactory glomeruli shows slow temporal dynamics: an optical recording study using a voltage-sensitive dye. *J. Insect Physiol.* 46:877–886.

GIRAUDET, P., BERTHOMMIER, F., and CHAPUT, M. 2002. Mitral cell temporal response patterns evoked by odor mixtures in the rat olfactory bulb. *J. Neurophysiol.* 88:829–838.

GIRLING, R. D., and CARDE, R. T. 2007. Analysis and manipulation of the structure of odor plumes from a piezo-electric release system and measurements of upwind flight of male almond moths, *Cadra cautella*, to pheromone plumes. *J. Chem. Ecol.* 33:1927–1945.

HALLEM, E. A., and CARLSON, J. R. 2006. Coding of odors by a receptor repertoire. *Cell* 125:143–160.

HANSSON, B. S., ANTON, S., and CHRISTENSEN, T. A. 1994. Structure and function of antennal lobe neurons in the male turnip moth, *Agrotis segetum* (Lepidoptera, Noctuidae). *J. Comp. Physiol. A* 175:547–562.

HANSSON, B. S., LARSSON, M. C., and LEAL, W. S. 1999. Green leaf volatile-detecting olfactory receptor neurons display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol.* 24:121–126.

HARTLIEB, E., ANTON, S., and HANSSON, B. S. 1997. Dose-dependent response characteristics of antennal lobe neurons in the male moth *Agrotis segetum* (Lepidoptera: Noctuidae). *J. Comp. Physiol. A* 181:469–476.

HEINBOCKEL, T., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 1999. Temporal tuning of odor responses in pheromone-responsive projection neurons in the brain of the sphinx moth *Manduca sexta*. *J. Comp. Neurol.* 409:1–12.

HEINBOCKEL, T., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2004. Representation of binary pheromone blends by glomerulus-specific olfactory projection neurons. *J. Comp. Physiol. A* V190:1023–1037.

HILLIER, N. K., and VICKERS, N. J. 2007. Physiology and antennal lobe projections of olfactory receptor neurons from sexually isomorphic sensilla on male Heliothis virescens. *J. Comp. Physiol. A* 193:649–663.

HILLIER, N. K., KLEINEIDAM, C., and VICKERS, N. J. 2006. Physiology and glomerular projections of olfactory receptor neurons on the antenna of female Heliothis virescens (Lepidoptera: Noctuidae) responsive to behaviorally relevant odors. *J. Comp. Physiol. A* 192:199–219.

HOMBERG, U., MONTAGUE, R. A., and HILDEBRAND, J. G. 1988. Anatomy of antenno-cerebral pathways in the brain of the sphinx moth *Manduca sexta*. *Cell Tissue Res.* 254:255–281.

HOPFIELD, J. J. 1991. Olfactory computation and object perception. *Proc. Natl. Acad. Sci. U. S. A.* 88:6462–6466.

HOSKINS, S. G., HOMBERG, U., KINGAN, T. G., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 1986. Immunocytochemistry of GABA in the antennal lobes of the sphinx moth *Manduca sexta*. *Cell Tissue Res.* 244:243–252.

JAHR, C. E., and NICOLL, R. A. 1982. An intracellular analysis of dendrodendritic inhibition in the turtle in vitro olfactory-bulb. *J. Physiol. Lond.* 326:213–234.

JEFFERIS, G. S. X. E., POTTER, C. J., CHAN, A. M., MARIN, E. C., ROHLFING, T., MAURER, C. R., and LUO, L. 2007. Comprehensive maps of *Drosophila* higher olfactory centers: spatially segregated fruit and pheromone representation. *Cell* 128:1187–1203.

JOERGES, J., KÜTTNER, A., GALIZIA, C. G., and MENZEL, R. 1997. Representations of odours and odour mixtures visualized in the honeybee brain. *Nature* 387:285–288.

KADOHISA, M., and WILSON, D. A. 2006. Separate encoding of identity and similarity of complex familiar odors in piriform cortex. *Proc. Natl. Acad. Sci. U. S. A.* 103:15206–15211.

KANG, J. S., and CAPRIO, J. 1995. Electrophysiological responses of single olfactory-bulb neurons to binary-mixtures of amino-acids in the channel catfish, *Ictalurus punctatus*. *J. Neurophysiol.* 74:1435–1443.

KANZAKI, R., ARBAS, E. A., and HILDEBRAND, J. G. 1991. Physiology and morphology of descending neurons in pheromone-processing olfactory pathways in the male moth *Manduca sexta*. *J. Comp. Physiol. A* 169:1–14.

KANZAKI, R., ARBAS, E. A., STRAUSFELD, N. J., and HILDEBRAND, J. G. 1989. Physiology and morphology of projection neurons in the antennal lobe of the male moth *Manduca sexta*. *J. Comp. Physiol. A* 165:427–453.

KANZAKI, R., and SHIBUYA, T. 1992. Long-lasting excitation of protocerebral bilateral neurons in the pheromone-processing pathways of the male moth *Bombyx mori*. *Brain Res.* 587: 211–215.

KANZAKI, R., SOO, K., SEKI, Y., and WADA, S. 2003. Projections to higher olfactory centers from subdivisions of the antennal lobe macroglomerular complex of the male silkmoth. *Chem. Senses* 28:113–130.

KAY, L. M., CRK, T., and THORNGATE, J. 2005. A redefinition of odor mixture quality. *Behav. Neurosci.* 119:726–733.

KAY, L. M., LOWRY, C. A., and JACOBS, H. A. 2003. Receptor contributions to configural and elemental odor mixture perception. *Behav. Neurosci.* 117:1108–1114.

KIRSCHNER, S., KLEINEIDAM, C. J., ZUBE, C., RYBAK, J., GRUNEWALD, B., and ROSSLER, W. 2006. Dual olfactory pathway in the honeybee, *Apis mellifera*. *J. Comp. Neurol.* 499:933–952.

KOMIYAMA, T., and LUO, L. 2006. Development of wiring specificity in the olfactory system. *Curr. Opin. Neurobiol.* 16:67–73.

KORSCHING, S. I. 2001. Odor maps in the brain: spatial aspects of odor representation in sensory surface and olfactory bulb. *Cell. Mol. Life Sci.* 58:520–530.

LAING, D. G., and FRANCIS, G. W. 1989. The capacity of humans to identify odors in mixtures. *Physiol. Behav.* 46:809–814.

LEI, H., ANTON, S., and HANSSON, B. S. 2001. Olfactory protocerebral pathways processing sex pheromone and plant odor information in the male moth *Agrotis segetum*. *J. Comp. Neurol.* 432:356–370.

LEI, H., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 2002. Local inhibition modulates odor-evoked synchronization of glomerulus-specific output neurons. *Nat. Neurosci.* 5:557–565.

LEON, M., and JOHNSON, B. A. 2003. Olfactory coding in the mammalian olfactory bulb. *Brain Res. Rev.* 42:23–32.

LIN, D. Y., SHEA, S. D., and KATZ, L. C. 2006. Representation of natural stimuli in the rodent main olfactory bulb. *Neuron* 50: 937–949.

LIN, H. -H., LAI, J. S. -Y., CHIN, A. -L., CHEN, Y. -C., and CHAING, A. -S. 2007. A map of olfactory representation in the *Drosophila* mushroom body. *Cell* 128:1205–1217.

LINSTER, C., and CLELAND, T. A. 2004. Configurational and elemental odor mixture perception can arise from local inhibition. *J. Comput. Neurosci.* 16:39–47.

LIU, Y. B., and HAYNES, K. F. 1992. Filamentous nature of pheromone plumes protects integrity of signal from background chemical noise in cabbage-looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 18:299–307.

LIVERMORE, A., and LAING, D. G. 1996. Influence of training and experience on the perception of multicomponent odor mixtures. *J. Exp. Psychol. Hum. Percept. Perform.* 22:267–277.

MA, M. H., and SHEPHERD, G. M. 2000. Functional mosaic organization of mouse olfactory receptor neurons. *Proc. Natl. Acad. Sci. U. S. A.* 97:12869–12874.

MALNIC, B., HIRONO, J., SATO, T., and BUCK, L. 1999. Combinatorial receptor codes for odors. *Cell* 96:713–723.

MALUN, D., WALDOW, U., KRAUS, D., and BOECKH, J. 1993. Connections between the deutocerebrum and the protocerebrum, and neuroanatomy of several classes of deutocerebral projection neurons in the brain of male *Periplaneta americana*. *J. Comp. Neurol.* 329:143–162.

MARIN, E. C., JEFFERIS, G. S. X. E., KOMIYAMA, T., ZHU, H., and LUO, L. 2002. Representation of the glomerular olfactory map in the *Drosophila* brain. *Cell* 109:243–255.

MEISTER, M., and BONHOEFFER, T. 2001. Tuning and topography in an odor map on the rat olfactory bulb. *J. Neurosci.* 21:1351–1360.

MOMBAERTS, P., WANG, F., DULAC, C., CHAO, S. K., NEMES, A., MENDELSOHN, M., EDMONDSON, J., and AXEL, R. 1996. Visualizing an olfactory sensory map. *Neuron* 87:675–686.

NIKONOV, A. A., FINGER, T. E., and CAPRIO, J. 2005. Beyond the olfactory bulb: an odotopic map in the forebrain. *Proc. Natl. Acad. Sci. U. S. A.* 102:18688–18693.

NOWYCKY, M. C., MORI, K., and SHEPHERD, G. M. 1981. GABAergic mechanisms of dendrodendritic synapses in isolated turtle olfactory bulb. *J. Neurophysiol.* 46:639–648.

OLSEN, S. R., and WILSON, R. I. 2008. Lateral presynaptic inhibition mediates gain control in an olfactory circuit. *Nature* 452:956–960.

PYTHON, F., and STOCKER, R. F. 2002. Immunoreactivity against choline acetyltransferase, gamma-aminobutyric acid, histamine, octopamine, and serotonin in the larval chemosensory system of *Drosophila melanogaster*. *J. Comp. Neurol.* 453:157–167.

RESSLER, K. J., SULLIVAN, S. L., and BUCK, L. B. 1994. Information coding in the olfactory system—evidence for a stereotyped and highly organized epitope map in the olfactory-bulb. *Cell* 79:1245–1255.

RO, H., MULLER, D., and MUSTAPARTA, H. 2007. Anatomical organization of antennal lobe projection neurons in the moth *Heliothis virescens*. *J. Comp. Neurol.* 500:658–675.

ROSTELIEN, T., BORG-KARLSON, A. K., and MUSTAPARTA, H. 2000. Selective receptor neuron responses to E-b-ocimene, b-myrcene, E,E-a-farnesene and homo-farnese in the moth *Heliothis virescens*, identified by gas chromatography linked to electrophysiology. *J. Comp. Physiol. A* 186:833–847.

RUBIN, B. D., and KATZ, L. C. 1999. Optical imaging of odorant representations in the mammalian olfactory bulb. *Neuron* 23:499–511.

SACHSE, S., and GALIZIA, C. G. 2003. The coding of odour-intensity in the honeybee antennal lobe: local computation optimizes odour representation. *Eur. J. Neurosci.* 18:2119–2132.

SACHSE, S., and GALIZIA, G. 2002. Role of inhibition for temporal and spatial odor representation in olfactory output neurons: a calcium imaging study. *J. Neurophysiol.* 87:1106–1117.

SHANG, Y., CLARIDGE-CHANG, A., SJULSON, L., PYPAERT, M., and MIESENBOCK, G. 2007. Excitatory local circuits and their implications for olfactory processing in the fly antennal lobe. *Cell* 128:601–612.

SHIELDS, V. D. C., and HILDEBRAND, J. G. 2001a. Responses of a population of antennal olfactory receptor cells in the female moth *Manduca sexta* to plant-associated volatile organic compounds. *J. Comp. Physiol. A* 186:1135–1151.

SHIELDS, V. D. C., and HILDEBRAND, J. G. 2001b. Recent advances in insect olfaction, specifically regarding the morphology and sensory physiology of antennal sensilla of the female sphinx moth *Manduca sexta*. *Microsc. Res. Tech.* 55:307–329.

SKIRI, H. T., GALIZIA, C. G., and MUSTAPARTA, H. 2004. Representation of primary plant odorants in the antennal lobe of the moth

Heliothis virescens using calcium imaging. *Chem. Senses* 29:253–267.

STOCKER, R. F., LIENHARD, M. C., BORST, A., and FISCHBACH, K. F. 1990. Neural architecture of the antennal lobe in *Drosophila melanogaster*. *Cell Tissue Res* 262:9–34.

SUN, X. J., FONTA, C., and MASSON, C. 1993. Odor quality processing by bee antennal lobe interneurons. *Chem. Senses* 18:355–377.

TABOR, R., YAKSI, E., WEISLOGEL, J. M., and FRIEDRICH, R. W. 2004. Processing of odor mixtures in the zebrafish olfactory bulb. *J. Neurosci.* 24:6611–6620.

TAKANASHI, T., ISHIKAWA, Y., ANDERSON, P., HUANG, Y., LÖFSTEDT, C., TATSUKI, S., and HANSSON, B. S. 2006. Unusual response characteristics of pheromone-specific olfactory receptor neurons in the Asian corn borer moth, *Ostrinia furnacalis*. *J. Exp. Biol.* 209:4946–4956.

TANAKA, N. T., AWASAKI, T., SHIMADA, T., and ITO, K. 2004. Integration of chemosensory pathways in the *Drosophila* second-order olfactory centers. *Curr. Biol.* 14:449–457.

TOUHARA, K. 2002. Odor discrimination by G protein-coupled olfactory receptors. *Microsc. Res. Tech.* 58:135–141.

UCHIDA, N., TAKAHASHI, Y. K., TANIFUJI, M., and MORI, K. 2000. Odor maps in the mammalian olfactory bulb: domain organization and odorant structural features. *Nat. Neurosci.* 3:1035–1043.

VICKERS, N. J., CHRISTENSEN, T. A., BAKER, T. C., and HILDEBRAND, J. G. 2001. Odour-plume dynamics influence the brain's olfactory code. *Nature* 410:466–470.

VICKERS, N. J., POOLE, K., and LINN, C. E. 2005. Plasticity in central olfactory processing and pheromone blend discrimination following interspecies antennal imaginal disc transplantation. *J. Comp. Neurol.* 491:141–156.

VOSSHALL, L. B. 2001. The molecular logic of olfaction in *Drosophila*. *Chem. Senses* 26:207–213.

VOSSHALL, L. B., WONG, A. M., and AXEL, R. 2000. An olfactory sensory map in the fly brain. *Cell* 102:147–159.

VUCINIC, D., COHEN, L. B., and KOSMIDIS, E. K. 2006. Interglomerular center-surround inhibition shapes odorant-evoked input to the mouse olfactory bulb in vivo. *J. Neurophysiol.* 95:1881–1887.

WACHOWIAK, M., and ACHE, B. W. 1994. Morphology and physiology of multiglomerular olfactory projection neurons in the spiny lobster. *J. Comp. Physiol. A* 175:35–48.

WANG, J. W., WONG, A. M., FLORES, J., VOSSHALL, L. B., and AXEL, R. 2003. Two-photon calcium imaging reveals an odor-evoked map of activity in the fly brain. *Cell* 112:271–282.

WESTBROOK, R. F., and CHARNOCK, D. J. 1985. Learning about a complex flavor in the rat—some evidence against a multiple, directional association model. *Aust. J. Psychol.* 37:41–49.

WILSON, D. A. 2003. Rapid, experience-induced enhancement in odorant discrimination by anterior piriform cortex neurons. *J. Neurophysiol.* 90:65–72.

WILSON, R. I., TURNER, G. C., and LAURENT, G. 2004. Transformation of olfactory representations in the *Drosophila* antennal lobe. *Science* 303:366–370.

WILSON, R. I., and LAURENT, G. 2005. Role of GABAergic inhibition in shaping odor-evoked spatiotemporal patterns in the *Drosophila* antennal lobe. *J. Neurosci.* 25:9069–9079.

WILTROUT, C., DOGRA, S., and LINSTER, C. 2003. Configurational and nonconfigurational interactions between odorants in binary mixtures. *Behav. Neurosci.* 117:236–245.

WONG, A. M., WANG, J. W., and AXEL, R. 2002. Spatial representation of the glomerular map in the *Drosophila* protocerebrum. *Cell* 109:229–241.

WU, W., ANTON, S., LÖFSDET, C., and HANSSON, B. S. 1996. Discrimination among pheromone component blends by interneurons in male antennal lobes of two populations of the turnip moth, *Agrotis segetum*. Proc. Natl. *Acad. Sci. U. S. A.* 93:8022–8027.

YAO, C. A., IGNELL, R., and CARLSON, J. R. 2005. Chemosensory coding by neurons in the coeloconic sensilla of the *Drosophila* antenna. *J. Neurosci.* 25:8359–8367.

YOKOI, M., MORI, K., and NAKANISHI, S. 1995. Refinement of odor molecule tuning by dendrodendritic synaptic inhibition in the olfactory-bulb. *Proc. Natl. Acad. Sci. U. S. A.* 92:3371–3375.

ZOU, Z. H., LI, F. S., and BUCK, L. B. 2005. Odor maps in the olfactory cortex. *Proc. Natl. Acad. Sci. U. S. A.* 102:7724–7729.

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REVIEW ARTICLE

Neural Computations with Mammalian Infochemicals

A. Gelperin

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Abstract The mammalian olfactory system is the most sensitive and discriminating molecular recognition system known, able to detect a few dozen critical molecules in the face of strong and variable background odorants. The set of information-containing volatile molecules used to transmit information within and between mammalian species shows both great molecular and informational diversity. Chemosensory neuroscientists that apply traditional reductionist methods to the analysis of information processing and computational principles in the olfactory system find great value in understanding the ecological and ethological context in which mammalian olfactory communication occurs. This review highlights a subset of the molecular armamentarium and information transmissions relevant to understanding the uses of olfactory communication by mammals in an ecological context.

Keywords Olfaction · Information processing · Odor learning · Pheromones · Scent marking · Olfactory bulb · Piriform cortex · Chemical signaling · Olfactory models · Olfactory computation · Active sampling · Receptor mapping · Neural information processing

Abbreviations

2DG	2-deoxyglucose
AOB	accessory olfactory bulb
CA1	cornu ammonis field 1
CA3	cornu ammonis field 3
c-FOS	cellular-Finkel osteogenic sarcoma
Egr-1	early growth response protein 1
fMRI	functional magnetic resonance imaging
HLA	human lymphocyte antigen
kDa	kiloDalton
MHC	major histocompatibility complex
MOB	main olfactory bulb
MOE	main olfactory epithelium
M/T	mitral/tufted
MTMT	(methylthio)methanethiol
ORN	olfactory receptor neuron
VNO	vomeronasal organ

Dedicated to Tom Eisner, for his inspiring example of how to do science well and joyfully.

A. Gelperin (✉)
Monell Chemical Senses Center,
Philadelphia, PA, USA
e-mail: agelperin@monell.org

Introduction

There is a variety of direct ecological implications that arise from basic properties of mammalian odor information-processing systems. This is because chemical communication via odorants is a critical sensory channel for many mammalian species and is used to transmit and receive information relating to many factors of ecological significance, including the identity of predators, prey, territory, gender, reproductive status, social status, food safety, disease burden, and nutritional status. Information transmission via infochemicals, a generic term for chemicals that convey information to conspecifics or members of other species, has direct and dramatic effects on the structure of food webs in both terrestrial and aquatic environments (Vos et al. 2006) and is widespread among vertebrate species (Sbarbati and Osculati 2006). Mammalian social odor processing recently has been reviewed (Brennan and Kendrick 2006), so I focus on computational issues that arise when odorant stimuli and response systems of direct ecological relevance are considered.

To understand the possible functional implications of chemical communication among mammalian species, we

must first consider the special characteristics of the multiple channels of sensory input and chemical information processing available to receive and decode chemical messages. The mammalian olfactory system has four distinct arrays of receptors (see below), with partially overlapping functions. The spatial and temporal dispersion of information carried by odorants due to turbulent flow (Murlis et al. 1992; Vickers 2000; Balkovsky and Shraiman 2002; Vergassola et al. 2007) places special constraints on olfactory receptor arrays and odorant information-processing circuits, which must balance the conflicting requirements for maximum sensitivity to transient signals carried by turbulent flow *versus* generation of false-positive messages due to noise fluctuations in receptors maintained at high sensitivity. Odorant information processing by mammalian brains is also notable for allowing access of chemical sensory information to cortical circuits that bypass the thalamic gating applied to visual, auditory, and tactile stimuli (Shipley and Ennis 1995; Shepherd et al. 2004; Kay and Sherman 2007), which is one reason that olfactory cues can elicit immediate and emotion-laden memories (Chu and Downes 2004; Jellinek 2004). Olfactory information processing often involves reliable and robust memory formation (Wilson and Stevenson 2006) so that decision making about an odorant cue on subsequent presentations is conditioned by the consequences and contingencies of the prior presentations of the odorant. Odorant receptors are also used for multiple functions, for example to guide the chemotactic behavior of mammalian sperm (Vanderhaeghen et al. 1993; Spehr et al. 2003, 2006), with direct implications for reproductive fitness. Odorant receptors also respond to stimuli in the gut lumen to trigger serotonin release from enterochromaffin cells (Braun et al. 2007). These aspects of mammalian olfaction and others with direct ecological implications are explored in more detail below.

Functional Organization of the Olfactory System

Odorant Receptor Genes

The functional dissection of mammalian olfaction was given great impetus by the discovery of a large gene family that codes for putative olfactory receptor proteins (Buck and Axel 1991), recognized by the Nobel award in 2004 (Axel 2005; Buck 2005). Subsequent genetic studies of olfactory receptor genes (Zhang and Firestein 2002) have clarified the evolutionary processes that lead to the diversification or decimation of receptor arrays in diverse species (Bargmann 2006), including rodents, humans, and nonhuman primates (Young et al. 2002; Young and Trask 2002; Gilad et al. 2003; Hoppe et al. 2006). For example, loss of olfactory receptor genes in some primates coincides with the appearance of full trichromatic vision (Gilad et al. 2004). Human–mouse receptor gene comparisons have made testable predictions about the nature of the odorant-binding site used when the odorant receptor protein is in its natural habitat, the membrane of the olfactory cilium (Man et al. 2004; Abaffy et al. 2007). Odorant receptor genes can be classified into two types: fish-like class I genes and mammalian-specific class II genes (Hirota et al. 2007).

The ability to genetically manipulate properties of the olfactory sensor array in rodents has clarified aspects of the transduction process in olfaction (Restrepo et al. 1996; Kini and Firestein 2001; Rawson and Gomez 2002), including the role of cyclic nucleotide-gated ion channels (Kelliher et al. 2003; Lin et al. 2004). Genetic tools have also been employed to examine aspects of the central organization of olfactory circuits (Zou et al. 2005; Zou and Buck 2006) including the generation of transgenic mice that express a channelrhodopsin fusion protein that allows localized light application to the olfactory bulb in anesthetized mice to activate mitral cells while recording the effects of mitral cell activity in the piriform cortex (Arenkiel et al. 2007). A glutamate receptor gated by light pulses has also been demonstrated (Szobota et al. 2007), which is of direct relevance to the analysis of the multiple types of glutamate receptors in olfactory information-processing circuits (Lowe 2003; Davila et al. 2007; Ma and Lowe 2007).

Multiple Olfactory Receptor Arrays

The olfactory system receives input from four distinct groups of sensory receptors, including those located in the main olfactory epithelium (MOE) and vomeronasal organ (VNO; Fig. 1), septal organ, and the Grueneberg ganglion (Breer et al. 2006; Storan and Key 2006). The hydrodynamics of air flow within the tortuous passages of the nasal cavity (Zhao et al. 2006) combines with the chemical nature of the olfactory mucus (Kurtz et al. 2004) or other fluids containing the cilia of the olfactory sensory neurons, in combination with the water solubility of the odorant molecule (Mozell and Jagodowicz 1974; Kent et al. 1996, 2003), to determine which types of odorant molecules interact most effectively with each anatomically distinct group of sensory receptors. The MOE responds to volatile odorants and some pheromones while the VNO responds to pheromones and some volatile odorants (Halpern and Martinez-Marcos 2003; Dulac and Wagner 2006; Spehr et al. 2006; Lin et al. 2007). The cilia of sensory neurons in the VNO are located in a fluid-filled trench (Keverne 1999; Leinders-Zufall et al. 2004) while the cilia of sensory neurons in the MOE are embedded in a 30-μm-thick mucus layer (Getchell et al. 1984). These physical and chemical considerations suggest that chemical signals that are conveyed by physical contact of the recipient animal's

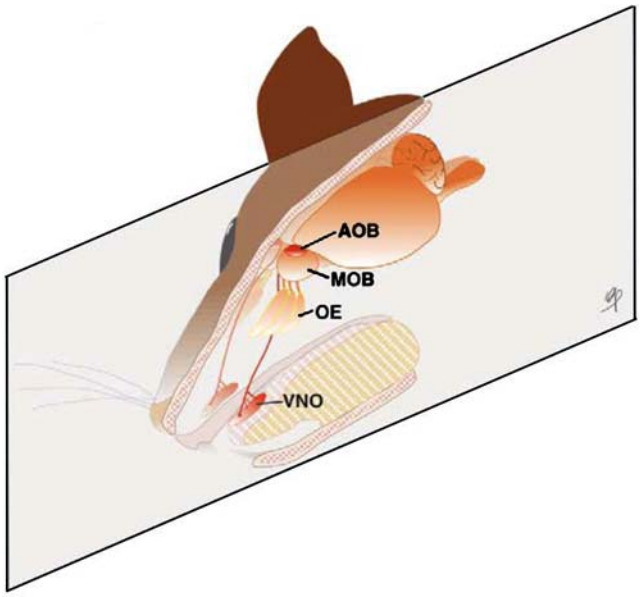

Fig. 1 Two major components of the rodent olfactory system are shown, the olfactory epithelium (*OE*) connected to the main olfactory bulb (*MOB*) and the vomeronasal organ (*VNO*) connected to the accessory olfactory bulb (*AOB*). This figure is taken from part C of Fig. 1 from Baxi et al. (2006)

nose with the body surface of the animal emitting the chemosignal are likely to involve the VNO, although not exclusively the VNO (Wysocki et al. 1980). Some VNO sensory neurons are remarkably sensitive to components of conspecific urine (Holy et al. 2000; Leinders-Zufall et al. 2000), as are some sensory neurons in the MOE, as inferred from selective responses of mouse mitral cells to (methylthio)methanethiol (MTMT), an infochemical present only in male mouse urine (Lin et al. 2005).

Central Neural Circuitry for Olfactory Processing

The major central sites of olfactory information processing are shown in Fig. 2, which also indicates the extent of feedback connections in addition to multiple feed forward pathways. Immunohistochemistry for cellular-Finkel osteogenic sarcoma (c-Fos) and early growth response protein 1 implicates several areas of the brain in individual recognition in hamsters, particularly the anterior piriform (olfactory) cortex, the CA1 and CA3 regions of the anterior

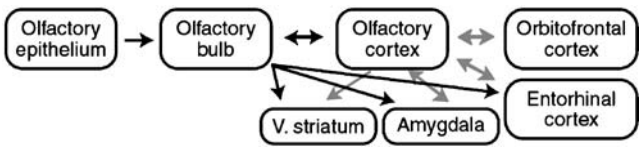

Fig. 2 Diagram to show the major central connections of the output of the main olfactory bulb. Note the prevalence of *arrows* indicating feedback as well as feed forward connections. This figure is taken from part A of Fig. 1 from Wilson and Mainen (2006)

dorsal hippocampus, anterior and posterior dentate gyrus, and perirhinal cortex (Lai et al. 2005). There are correlates of olfactory recognition memory in orbitofrontal cortex (Ramus and Eichenbaum 2000), but the orbitofrontal cortex is involved in much more general computations (Murray et al. 2007). Field potential recordings in central olfactory structures have been used to track the results of olfactory processing during behavior (Chabaud et al. 2000; Martin et al. 2006). The dynamics of these central oscillations is dependent on the behavioral task being performed during the recording (Kay 2003; Gelperin 2006; Rinberg and Gelperin 2006). For example, gamma oscillatory power (65–85 Hz) in the rat olfactory bulb during a two-alternative choice task is modulated in the intact system according to task demands with dramatic increases in gamma power during the discrimination of molecularly similar odorants in contrast to dissimilar odorants (Beshel et al. 2007). Recordings in young rats question the computational necessity of coherent network oscillations for olfactory recognition (Fletcher et al. 2005). The tracking of the progress and results of olfactory information processing in the mammalian brain is greatly facilitated by the development of an isolated guinea pig brain preparation retaining the olfactory epithelium that allows access to olfactory areas during natural odor stimulation (Ishikawa et al. 2007). Initial results with this preparation in which both odor-evoked local field potentials and unit activity were recorded found neural response patterns consistent with previous results obtained in recordings from both brain slices and anesthetized preparations. Depending on the state of anesthesia of the isolated guinea pig brain *in vitro*, results can be compared directly to those obtained in the awake head-fixed rodent preparation (Verhagen et al. 2007) and the awake behaving mouse (Rinberg et al. 2006).

Odorant Processing and Neurogenesis

On the time scale of days and longer, olfactory experience can influence odorant information processing by altering the actual cellular composition of the processing circuitry. Neurogenesis in the adult brain provides new cells in the main olfactory bulb (MOB) and in the hippocampus (Lois and Alvarez-Buylla 1994; Carleton et al. 2003; Gould 2007), both of which are involved in the processing and storage of olfactory memories (Olariu et al. 2005). Infochemical interactions among conspecifics, by using both pheromones and nonpheromones, can have effects on the dynamics of neurogenesis, a process that continually populates the MOB with new interneurons (Lledo et al. 2006; Gheusi and Lledo 2007; Mizrahi 2007) and sensory input fibers (Graziadei and Graziadei-Monti 1979; Leung et al. 2007). The turnover of juxtaglomerular neurons, an important category of local inhibitory interneurons in the

glomerular layer of the MOB (De Marchis et al. 2007), is approximately 3% per month (Mizrahi et al. 2006). The survival rate of some elements of olfactory circuitry, for example granule cells (Petreanu and Alvarez-Buylla 2002), is modulated by olfactory experience (Alonso et al. 2006; Mandairon et al. 2006a,b). Recently generated adult-born olfactory granule neurons and older pre-existing granule neurons undergo contrasting experience-dependent modifications *in vivo* (Magavi et al. 2005). Different stem cells that originate in the subventricular zone have different embryonic origins and generate different neuronal progeny in the adult olfactory bulb (Young et al. 2007). The pheromones of dominant but not subordinate male mice stimulate neurogenesis in both the MOB and the hippocampus of female mice. The blocking of this pheromone-induced neurogenesis eliminates the preference of females for dominant males (Mak et al. 2007). This completes the feedback loop from odorant-induced brain plasticity to brain preference for conspecific odorants that trigger the plasticity. Any realistic model of mammalian mating system dynamics must include these newly demonstrated feedback loops.

Types of Olfactory Information

Chemistry of Infosignals

The diverse sources of chemical infosignals, such as glands (Kimoto et al. 2005), excretia (Vendruscolo et al. 2006), breath (Galef et al. 1988), body (Beauchamp and Yamazaki 2003), and tears (Haga et al. 2007), result in both lipid-soluble and water-soluble components, which tend to be absorbed into the olfactory mucosa at different rates during the transit of the inspired air over the sensory surface of the MOE (Mozell and Jagodowicz 1973). This has direct consequences for the regions of the MOB that will be activated by the infosignal (Johnson et al. 2007a,b; Johnson and Leon 2007) and hence for the central representation of the chemosignal. The influence of relative solubility of components of the chemosignal is also dependent on flow rate of the inspired air during sniffing and the periodicity of sniffing (Scott 2006; Scott et al. 2006; Schaefer and Margrie 2007), which determine the strength and timing of the sensory input to the mitral/tufted (M/T) cells of the olfactory bulb (Buonviso et al. 2006; Roux et al. 2006). Modeling of nasal airflow (Zhao and Dalton 2007) is essential to understanding the effects of flow rate on differential absorption.

Predator Signals

The analysis of the chemical nature and neural processing of olfactory signals from predators has focused on odors produced by carnivores and omnivores and the processing of distinctive predator odors by rodents, particularly rats, mice, and voles (Dielenberg and McGregor 2001; Apfelbach et al. 2005; Fendt et al. 2005). Sources of predator odors include urine, feces, and anal gland secretions, as well as skin and fur (Kats and Dill 1998). Predator odors that produce strong behavioral responses in rodents are 2,4,5-trimethylthiazoline (TMT), a component of fox feces (Rosen et al. 2006; Staples and McGregor 2006), 2-propylthietane, a synthetic weasel odor (Heale and Vanderwolf 1994), and mustelan, derived from the anal glands of mink (Schildknecht et al. 1976). The chemical complexity of the chemosignals in male and female ferret urine has been clarified recently by the application of a range of chemical analysis techniques that have identified 30 volatile compounds in urine and 26 volatile compounds in anal gland secretions (Zhang et al. 2005). Based on the findings of Lin et al. (2006), it is possible that only a small subset of these identified components actually carry information to the recipient.

Are predator odors processed in a distinctive way by the olfactory bulb and olfactory cortex? Early data indicated that weasel and fox odors selectively activated beta oscillation (15–30 Hz) in the rat dentate gyrus (Heale et al. 1994), an indicator of coherent activity in the central circuit. More recent investigations indicate that beta-band oscillations of local field potential in the olfactory bulb are not specific to predator odors but rather depend on the concentration (vapor pressure) of the odorant stimulus (Lowry and Kay 2007). Direct analysis of the glomerular activation pattern in ferrets set up by chemosignals from conspecifics has been obtained by analyzing patterns of c-Fos immunoreactivity of juxtraglomerular cells in the MOB (Woodley and Baum 2004). Considerably less activation of ventral glomeruli was seen when volatile male odors were used as compared to direct physical contact with a male (Batterton et al. 2006). It will be instructive to compare the topology of the activation of glomeruli in the olfactory bulb set up by predator odors with the mapping of monomolecular odorant activation patterns in the MOB of rats (Johnson and Leon 2007). The mouse glomerular map is different from the rat glomerular map (Soucy et al. 2007), so the published activation maps for the rat MOB based on 2-deoxyglucose uptake patterns are of limited utility for studies of mouse MOB activation patterns.

Recent work has shown that genetic ablation of olfactory receptor neurons (ORNs) in specific regions of the olfactory epithelium can yield a mouse able to detect a predator odor, e.g., fox odor (TMT), but no longer exhibiting the normal fear responses (freezing, avoidance) to the source of the predator odor (Kobayakawa et al. 2007). One interpretation is that some sets of glomeruli receiving input from receptors activated by a predator odor lead to the activation

of the amygdala and generation of fear responses, while other glomeruli receiving input for receptors activated by the same predator odor are processed differently to allow odorant recognition in the absence of fear or avoidance responses. These observations on altered predator responses in mice with reduced populations of ORNs may provide part of the mechanism for alterations in aversion to predator odors (urine) in rats caused by the infection with *Toxoplasma*, although other mechanisms are possible, as parasite cysts are found in the rat's amygdalar structures (Vyas et al. 2007a). Since the effects of *Toxoplasma* infection are dose dependent with an inverted U-shaped curve (Vyas et al. 2007b), the fitness costs associated with the change in host odor processing suggest that the effect is optimized rather than maximized.

Sexual Signals

The placing of sexual infomolecules in their ecological setting indicates the complexity of the analytical task facing the olfactory system. For example, female meadow voles (*Microtus pennslyvanicus*) can identify clearly the individual male whose scent mark is on top of another male scent mark (Johnston et al. 1997). The olfactory system, particularly the VNO (Lepri and Wysocki 1987), must be able to differentiate and identify the stronger scent blend that belongs to the male with the topmost scent mark in the face of distracting components of very similar nature from the male that deposited the bottommost scent mark. Meadow voles may take longer to process a complex odorant blend of two scent marks as compared to a single scent mark, by analogy with recent findings concerning odorant-sampling patterns in mice. To maintain accuracy of odorant discrimination, mice must take longer samples of odor mixtures that are hard to analyze (54% odor A plus 46% odor B vs. 46% odor A plus 54% odor B) than odor mixtures that are easy to analyze (70% odor A plus 30% odor B vs. 30% odor A plus 70% odor B; Rinberg et al. 2006; Slotnick 2007a). When given the opportunity, mice will take a single sniff of an odorant and suffer reduced accuracy of identification on a hard odor discrimination (Uchida and Mainen 2003; Uchida et al. 2006). Proteins in urine scent marks, particularly lipocalins, slow the release of semiochemicals (infochemicals) such as 2-*sec*-butyl-4, 5-dihydrothiazole and 2,3-dehydro-exo-brevicomin, used by male mice to indicate their state of aggression (Hurst et al. 1998; Robertson et al. 2007; Stopkova et al. 2007). The sex and reproductive status of both the sender and receiver influence responses to scent marks, as shown clearly in recent work that measures responses to isolated scent marks by the ring-tailed lemur (*Lemur catta*), a socially complex species with a functional VNO (Knapp et al. 2006; Scordato et al. 2007).

A large body of work has associated the reception of sexual signals with the VNO (Wysocki 1979; Beauchamp et al. 1982; Boehm et al. 2005; Boehm 2006; Brennan and Kendrick 2006; Kelliher et al. 2006). VNO stimulation by components of male mouse urine can trigger ovulation (More 2006). Genetic manipulations of laboratory mice have shown the behavioral consequences of genetically eliminating function in a large class of VNO sensory neurons. The elimination of the cation channel transient receptor potential 2 normally expressed in VNO sensory neurons showed that this channel is necessary for normal direction of aggressive behavior and selection of sexual partners by male mice (Del Punta et al. 2002; Leypold et al. 2002; Stowers et al. 2002). Olfactory sensory neurons in the VNO project directly to the accessory olfactory bulb (AOB). Single-unit recordings from mitral cells in the AOB obtained while the instrumented mouse explored an anesthetized conspecific demonstrated remarkably strong and prolonged activation of AOB mitral cells during the exploration of the head and anogenital areas of the anesthetized conspecific (Luo et al. 2003). Some neurons in the AOB showed selective activation depending on the sex and strain of the anesthetized conspecific emitting the social signals. The VNO also plays an important role in processing nonpheromonal signals (Restrepo et al. 2004; Baxi et al. 2006) and contains ORNs that express odorant receptor genes in common with the MOE (Levai et al. 2006). The findings of a more general role for the VNO were presaged by results that show that the vascular pumping mechanism of the VNO was activated in hamsters during novel stimulation accompanied by arousal, not just in situations where the VNO is known to be important (Meredith 1994). The MOE and MOB are also involved in reception and processing of sexual signals. By using functional magnetic resonance imaging to quantitate odorant responses in the MOB and AOB, it was found that 2-heptanone, a known mouse pheromone, produced strong signals in the MOB as well as the AOB, while urine odor, containing both pheromone and nonpheromone odors, produced clear signals in limited regions of the MOB and large regions of the AOB (Xu et al. 2005). Genetic analysis has provided extremely useful insights into pheromone signaling (Dulac and Wagner 2006), particularly the spatially selective projection pattern used by VNO neurons to connect with the AOB (Wagner et al. 2006). The use of pheromones for mammalian communication, including human communication, has recently been reviewed (Wysocki and Preti 2004, 2008).

The remarkable variability of the role of the VNO in rodent social communication is highlighted by studies in the naked mole rat (*Heterocephalus glaber*), a eusocial species with a small VNO that shows no postnatal growth in volume, thus implying a minor role of the VNO in

maintaining the social structure of the colony. This is in contrast with other rodent species (Weiler et al. 1999; Smith et al. 2007) with quite different mating strategies (Maico et al. 2003). The VNO undergoes continual turnover of its sensory receptors during adult life (Martinez-Marcos et al. 2005), as found in the MOE, which may be modulated by exposure to species-specific infochemicals.

Individual Identity Signals

One goal of olfactory information transmission for sexual selection is to control and optimize the diversity of alleles in the major histocompatibility complex (MHC) genes (the MHC haplotype) that regulate important functions of the immune system (Boyse et al. 1987; Penn 2002; Knapp 2005; Restrepo et al. 2006). Receptors activated by the complex blend of MHC-related urinary chemosignals (Willse et al. 2005) are located in both the VNO and MOE (Wysocki et al. 2004). Volatile and nonvolatile signals coded by MHC genes are complementary determinants of the rodent odortype (Restrepo et al. 2006). MHC class I peptides have been identified as compounds that elicit the pregnancy block effect (Bruce 1959) via the VNO (Leinders-Zufall et al. 2004; Thompson et al. 2007). The protein products of the MHC haplotype plus the genetic background in which the MHC genes reside combine to produce a unique individual odor signature or odortype (Penn et al. 2007), by which an individual can be recognized by conspecifics (Willse et al. 2006) or members of other species (Gilbert et al. 1986).

Measurement of event-related evoked potentials set up by human lymphocyte antigen-associated odorants in humans is a promising technique for studying neural processing of odortype-related chemosignals (Pause et al. 1998, 2006). Olfactory event-related potentials can have a reliability equivalent to that of visual and auditory event-related potentials (Lorig 2000; Thesen and Murphy 2002). The use of functional magnetic resonance imaging (Ogawa et al. 1990) during olfactory stimulation allows initial localization of brain modules participating in the processing of olfactory stimuli in humans (Winston et al. 2005) and in mice (Schafer et al. 2006). Work on humans has highlighted the activation of the amygdala and orbital prefrontal cortex (Dolan 2007) by odorant stimuli.

Health Status Signals

The unique odortype of an individual can signal its state of health, including nutritional status, parasite load, and active disease processes. This is to be expected, as any process that alters body chemistry is likely to produce alterations in body (Thom and Hurst 2004) and breath odor (Whittle et al. 2007). For example, mice that harbor the mammary tumor virus have an altered body odor that can be decoded by conspecifics, even in the absence of tumors (Yamazaki et al. 2002). Female mice avoid the body and urine odors of infected males, an effect that is blocked selectively by genetic lesions of the oxytocin gene (Kavaliers et al. 2005a, b). Female mice can discriminate the chemical cues from malaria-infected male mice *versus* healthy male mice (Barthelemy et al. 2005). The influence of MHC genes on mate choice is often interpreted as a mechanism to augment disease resistance of the offspring. The avoidance by females of parasitized males is taken as evidence for the "contagion indicator" hypothesis of parasite-mediated sexual selection (Hamilton and Zuk 1982; Able 1996). There are tradeoffs in the allocation of resources between sexual signals and the immune system (Peters 2007).

Nutritional state is another general indicator of health status that is reflected in the odor signals emitted by an individual. The state of food deprivation directly affects the interest of meadow voles (*Microtus pennsylvanicus*) in interacting with conspecifics of the opposite sex, as measured by their responses to scent marks (Pierce et al. 2007). Recent evidence suggests that leptin, a 16-kDa hormone that plays a key role in regulating energy intake and energy expenditure, acting through leptin receptors, modulates olfactory-mediated preingestive behavior (Getchell et al. 2006), as measured using a buried food paradigm. Leptin receptors are also found in sustentacular cells and sensory neurons in the MOE (Baly et al. 2007). Thus, there is both a central and peripheral mechanism for a direct link between the regulation of nutritional state and olfactory responsiveness and central processing (Aime et al. 2007). Anther peptide involved in appetite and energy regulation, orexin (Sakurai et al. 1998), also has direct effects on M/T cell activity (Hardy et al. 2005) and olfactory perception (Gorojankina et al. 2007; Julliard et al. 2007).

Computational Issues in Olfaction

Sparse Coding

An important issue in computational olfaction is how to code the spatial and temporal patterns set up by natural odorants, particularly complex blends of multiple components, so that similar but behaviorally distinct odorant blends can be discriminated (Jortner et al. 2007; Stopfer 2007). Sparse coding, in which a small number of distributed neurons represent stimulus features (Olshausen and Field 2004), is a general feature of sensory systems (Willmore and Tolhurst 2001) and offers multiple computational advantages, such as increased storage capacity in memory, energy conservation, and perhaps ease of readout.

There is evidence that the network of M/T cells in the mammalian olfactory bulb uses a sparse representation for monomolecular odorants (Kay and Laurent 1999; Rinberg et al. 2006; Davison and Katz 2007). M/T cells have a high spontaneous rate of action potential production (25–30 Hz). After a mouse learns that the delivery of an odorant predicts the delivery of a water reward, M/T cells increase their activity prior to delivery of an odorant. Responses of M/T cells in the awake behaving rodent cannot be predicted from their responses in the anesthetized animal (Rinberg et al. 2006), so issues of coding of natural odors of ecological relevance must be addressed with *in vivo* recording, by using either multisite electrical (Luo et al. 2003; Martin et al. 2004; Lehmkuhle et al. 2006; Calu et al. 2007) or optical (Cinelli et al. 1995; Singer et al. 2007; Verhagen et al. 2007) recording, during the execution of odorant-guided behavior (Mainen 2006; Rinberg and Gelperin 2006).

An issue central to any consideration of the spatial and temporal aspects of odorant coding in the MOB (Schaefer and Margrie 2007) and in higher centers such as the piriform cortex (Rennaker et al. 2007) is how the array of olfactory sensory receptors in the MOE maps onto the array of glomeruli in the MOB. In rodents, each ORN expresses only one of the set of 1,000 olfactory receptor genes, and all receptors that express a given receptor gene project to two of the 2,000 glomeruli in each MOB. Since even a monomolecular odorant excites a number of different types of olfactory sensory neurons (Malnic et al. 1999; Kajiya et al. 2001) and since each receptor is activated by several related odorants (Araneda et al. 2000), most natural odorants elicit a spatial pattern of glomerular activation on the bulbar surface. If the odorant is a complex blend, then the spatial pattern is more complex (Lin et al. 2006). The relationship between odorant receptor activation, glomerular activation patterns, and perception in awake animals remains to be clarified. It is interesting to note that alterations in some single odorant receptor genes can make subtle contributions to the odorant percept (Keller and Vosshall 2007).

Olfactory Feature Detectors

The canonical view of olfactory coding is that any given odorant stimulates a set of different types of olfactory receptors and thence a set of olfactory glomeruli, with significant temporal evolution of the firing patterns of the activated sensory neurons and M/T cells during even short bouts of odorant sampling (sniffing). A variety of measurements of glomerular activation patterns support this view of a distributed representation (Xu et al. 2000; Chen and Shepherd 2005; Mori et al. 2006; Wachowiak and Shipley 2006; Youngentob et al. 2006). An alternative view is that some receptors and glomeruli have very narrow chemical tuning curves, perhaps to mediate hard-wired behavioral responses. In flies, some narrowly tuned ORNs connect with narrowly tuned projection neurons to trigger a reliable behavioral response, while other narrowly tuned receptors connect to broadly tuned projection neurons involved in combinatorial coding (Schlief and Wilson 2007). In mice, some complex natural odorants with direct relevance to social communication, such as the odor of urine, contain single components with potent effects. Of the hundreds of compounds present in mouse urine, single mitral cells can have extremely narrowly tuned response spectra, such that single M/T cells can be tuned to a single urine component, such as MTMT, present only in male mouse urine (Lin et al. 2005). The addition of synthetic MTMT to mouse urine makes it more attractive to female mice. It will be important to determine if the same pattern of selective responsiveness is present in mitral cells recorded in the awake mouse. Clearly, the broad-tuning and narrow-tuning mechanisms are complementary. Some mitral cells may have specific response spectra while others are broadly tuned during neural decoding of the same complex natural stimulus. Our understanding of glomerular and, by inference, M/T cell processing of odorant input has been significantly enhanced by the recent demonstration, by using localized microinjections of a retrograde-specific pseudorabies virus into the rat MOB (Willhite et al. 2006), of a striking columnar organization extending across all layers of the olfactory bulb from the glomerular layer to the deep granule cell layer.

Separating Target from Background

A general problem in odor target recognition is how to separate (segment) an olfactory target odorant of interest from the odor background. Part of the target recognition mechanism involves adaptation to steady-state stimuli, also known as background subtraction. There are mechanisms at the receptor level (Reisert 2005; Goyert et al. 2007), bulbar level (Fletcher and Wilson 2003), and cortical level (Kadohisa and Wilson 2006; Wilson et al. 2006; Linster et al. 2007) to achieve the adaptation of neural activity to a constant background stimulus while maintaining sensitivity to a novel stimulus. Cortical contributions were clarified in a recent study showing that the infusion of a glutamate receptor antagonist bilaterally into the anterior piriform cortex can reduce the habituation of exploratory behavior to a novel odor or the odor of a conspecific (Yadon and Wilson 2005).

Olfactory enrichment can increase the ability of rodents to discriminate components of a mixture (Mandairon et al. 2006c). Whether a blend of odors will be perceived as a unitary odor object (configural perception) or as a mixture of components (elemental perception) is dependent on past experience as well as the nature of the components (Kay

et al. 2003, 2005). If the animal always experiences the components in a constant proportion and never experiences a component in isolation, the perception is more likely to be configural (Hopfield and Gelperin 1989). These factors bear directly on how an odorant representation is stored and on what aspects of the odorant stimulus are available for association with other cues and consequences. When a meadow vole samples the odor of a scent mark, all the components are present in fixed proportions. If the components of the scent mark volatilize at different rates, then an older scent mark will appear to have different proportions of the components due to selective depletion of odor components from the source. This might provide a means to distinguish the top (fresh) scent mark from the bottom (depleted) scent mark (Ferkin and Pierce 2007). The central representation may achieve a signal independent of absolute concentration by using a relational representation based on relative levels of activity in glomerular components (Chalansonnet and Chaput 1998; Cleland et al. 2007).

Fault Tolerance in Olfaction

As might be anticipated in a sensory system of such critical importance, the olfactory system has considerable redundancy in its sensor arrays, as evidenced by the ability to maintain sensitivity and discrimination ability in the face of massive destruction of the sensory sheet (Slotnick 2007a; Slotnick et al. 2007). Similarly, the distributed representation of odorants in the olfactory bulb allows the central circuitry to retain significant function in the face of extensive central lesions (Slotnick et al. 2004; McBride and Slotnick 2006). These findings (Bisulco and Slotnick 2003; Slotnick and Bisulco 2003) have been interpreted as challenges to a model of localized and fixed components of the glomerular spatial coding of odorants (Johnson and Leon 2007). From a system design point of view, it is remarkable that rats with one MOB removed completely and only 20% of the remaining bulb available retain significant olfactory ability (Lu and Slotnick 1998). Given the accessibility of the MOB to pathogens traveling up the olfactory nerve (Mori et al. 2005), it may be adaptive to have sufficient redundancy in MOB processing circuits that olfactory function survives bacterial or viral assault.

Network Models in Olfaction

As studies of olfactory information processing and olfactory biophysics progress, structural models that incorporate essential elements of neuronal circuitry and response dynamics have appeared (Meredith 1992; Hopfield 1999; Laurent et al. 2001; Brody and Hopfield 2003; Davison et al. 2003; Bathellier et al. 2006; de Souza and Antunes 2007). These models have used receptor array and M/T cell activation functions taken from experimental measurements and have made suggestions about the critical role of local inhibition in shaping network dynamics (Cleland and Linster 2005). A model that uses glomeruli with only two states of activity, on or off, can account for several aspects of observed behavior in olfactory psychophysics, including effects of olfactory bulb lesions (Koulakov et al. 2007). When these models have evolved to process multicomponent mixtures with very brief sampling times, they may suggest solutions to olfactory processing problems of direct relevance to ecological interactions via infochemicals. A model that incorporates unsupervised learning and adaptation shows how new neurons incorporated into the circuitry of the MOB can optimize odor processing under changing conditions (Cecchi et al. 2001; Chambers and Conroy 2007).

Stimulus Sampling Strategies

The responses of M/T cells show a temporal structure in the odor-specific patterns of their onset latencies (Schaefer and Margrie 2007), so there are both spatial and temporal aspects of the central representation of odorants. In some tasks, rodents make a decision about an odorant in a single sniff (Uchida and Mainen 2003; Abraham et al. 2004; Kepecs et al. 2006; Slotnick 2007b), as do humans (Laing 1986; Mainland and Sobel 2006), who are better at scent tracking (Novotny et al. 2007) and olfactory acuity (Shepherd 2004) than previously believed. For example, the smelling of a single component of male sweat, androstadienone (4,16-androstaien-3-one), can alter salivary levels of cortisol in women (Wyart et al. 2007). Humans have been suggested to be an excellent animal model for the study of system-level olfaction (Zelano and Sobel 2005).

The reliance on input from a single sniff in making some forms of olfactory recognition may relate to the pattern of odor dispersal by turbulent flow in which at some distance from the source, the odorant is present in packets that may only allow sampling with a single sniff (Murlis et al. 2000; Miller et al. 2007). An understanding of these issues is critical for design of robots equipped with electronic olfactory systems that actively sample odor plumes (Settles 2005; Vergassola et al. 2007).

Conclusions

The mammalian olfactory system is an extremely sensitive analytical device, able to respond to small numbers of ligands (34) bound to receptor proteins in the ciliary membranes of olfactory receptor cells (Menini et al. 1995;

Bhandawat et al. 2005). The analysis of the spatiotemporal pattern of activity from receptors activated by chemically diverse natural chemosignals is able to extract weak known signals from a background of strong time-varying signals, segment components of odor objects, and perform concentration-invariant recognition and discrimination (Wilson and Mainen 2006). A variety of reliable and robust learning mechanisms, associative and nonassociative, operate on the central representations of odors to bias future responses based on previous exposures. This exquisite analytical system operates during chemical communication events with conspecifics, predators, and prey to detect and decode chemosignals relevant to ecological interactions of every type. The task for future studies of ecologically relevant central processing of olfactory signals is to use stimuli of clear relevance to ecological information transmission and study processing events in the awake behaving animal while relevant stimuli are being processed.

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References

ABAFFY, T., MALHOTRA, A., and LUETJE, C. W. 2007. The molecular basis for ligand specificity in a mouse olfactory receptor: a network of functionally important residues. *J. Biol. Chem.* 282:1216–1224.

ABLE, D. J. 1996. The contagion indicator hypothesis for parasite-mediated sexual selection. *Proc. Natl. Acad. Sci. USA* 93: 2229–2233.

ABRAHAM, N. M., SPORS, H., CARLETON, A., MARGRIE, T. W., KUNER, T., and SCHAEFER, A. T. 2004. Maintaining accuracy at the expense of speed: stimulus similarity defines odor discrimination time in mice. *Neuron* 44:865–876.

AIME, P., DUCHAMP-VIRET, P., CHAPUT, M. A., SAVIGNER, A., MAHFOUZ, M., and JULLIARD, A. K. 2007. Fasting increases and satiation decreases olfactory detection for a neutral odor in rats. *Behav. Brain Res.* 179:258–264.

ALONSO, M., VIOLLET, C., GABELLEC, M. M., MEAS-YEDID, V., OLIVO-MARIN, J. C., and LLEDO, P. M. 2006. Olfactory discrimination learning increases the survival of adult-born neurons in the olfactory bulb. *J. Neurosci.* 26:10508–10513.

APFELBACH, R., BLANCHARD, C. D., BLANCHARD, R. J., HAYES, R. A., and MCGREGOR, I. S. 2005. The effects of predator odors in mammalian prey species: a review of field and laboratory studies. *Neurosci. Biobehav. Rev.* 29:1123–1144.

ARANEDA, R. C., KINI, A. D., and FIRESTEIN, S. 2000. The molecular receptive range of an odorant receptor. *Nat. Neurosci.* 3: 1248–1255.

ARENKIEL, B. R., PECA, J., DAVISON, I. G., FELICIANO, C., DEISSEROTH, K., AUGUSTINE, G. J., EHLERS, M. D., and FENG, G. 2007. In vivo light-induced activation of neural circuitry in transgenic mice expressing channelrhodopsin-2. *Neuron* 54:205–218.

AXEL, R. 2005. Scents and sensibility: a molecular logic of olfactory perception (Nobel lecture). *Angew. Chem. Int. Ed. Engl.* 44: 6110–6127.

BALKOVSKY, E., and SHRAIMAN, B. I. 2002. Olfactory search at high Reynolds number. *Proc. Natl. Acad. Sci. USA* 99:12583–12588.

BALY, C., AIOUN, J., BADONNEL, K., LACROIX, M. C., DURIEUX, D., SCHLEGEL, C., SALESSE, R., and CAILLOL, M. 2007. Leptin and its receptors are present in the rat olfactory mucosa and modulated by the nutritional status. *Brain Res.* 1129:130–141.

BARGMANN, C. I. 2006. Comparative chemosensation from receptors to ecology. *Nature* 444:295–301.

BARTHELEMY, M., GABRION, C., and PETIT, G. 2005. Does chronic malaria modify the odours of its male mouse host? *Can. J. Zool.* 83:1079–1086.

BATHELLIER, B., LAGIER, S., FAURE, P., and LLEDO, P. M. 2006. Circuit properties generating gamma oscillations in a network model of the olfactory bulb. *J Neurophysiol.* 95:2678–2691.

BATTERTON, M. N., ROBARTS, D., WOODLEY, S. K., and BAUM, M. J. 2006. Comparison of odor and mating-induced glomerular activation in the main olfactory bulb of estrous female ferrets. *Neurosci. Lett.* 400:224–229.

BAXI, K. N., DORRIES, K. M., and EISTHEN, H. L. 2006. Is the vomeronasal system really specialized for detecting pheromones? *Trends Neurosci.* 29:1–7.

BEAUCHAMP, G. K., and YAMAZAKI, K. 2003. Chemical signaling in mice. *Biochem. Soc. Trans.* 31:147–151.

BEAUCHAMP, G. K., MARTIN, I. G., WYSOCKI, C. J., and WELLINGTON, J. L. 1982. Chemoinvestigatory and sexual behavior of male guinea pigs following vomeronasal organ removal. *Physiol. Behav.* 29:329–336.

BESHEL, J., KOPELL, N., and KAY, L. M. 2007. Olfactory bulb gamma oscillations are enhanced with task demands. *J. Neurosci.* 27: 8358–8365.

BHANDAWAT, V., REISERT, J., and YAU, K. W. 2005. Elementary response of olfactory receptor neurons to odorants. *Science* 308: 1931–1934.

BISULCO, S., and SLOTNICK, B. 2003. Olfactory discrimination of short chain fatty acids in rats with large bilateral lesions of the olfactory bulbs. *Chem. Senses* 28:361–370.

BOEHM, U. 2006. The vomeronasal system in mice: from the nose to the hypothalamus—and back!. *Semin. Cell Dev. Biol.* 17:471–479.

BOEHM, U., ZOU, Z., and BUCK, L. B. 2005. Feedback loops link odor and pheromone signaling with reproduction. *Cell* 123:683–695.

BOYSE, E. A., BEAUCHAMP, G., and YAMAZAKI, K. 1987. The genetics of body scent. *Trends Genet.* 3:97–102.

BRAUN, T., VOLAND, P., KUNZ, L., PRINZ, C., and GRATZL, M. 2007. Enterochromaffin cells of the human gut: sensors for spices and odorants. *Gastroenterology* 132:1890–1901.

BREER, H., FLEISCHER, J., and STROTMANN, J. 2006. The sense of smell: multiple olfactory subsystems. *Cell Mol. Life Sci.* 63:1465–1475.

BRENNAN, P. A., and KENDRICK, K. M. 2006. Mammalian social odours: attraction and individual recognition. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 361:2061–2078.

BRODY, C. D., and HOPFIELD, J. J. 2003. Simple networks for spike-timing-based computation, with application to olfactory processing. *Neuron* 37:843–852.

BRUCE, H. M. 1959. An exteroceptive block to pregnancy in the mouse. *Nature* 184:105.

BUCK, L. B. 2005. Unraveling the sense of smell (Nobel lecture). *Angew. Chem. Int. Ed. Engl.* 44:6128–6140.

BUCK, L., and AXEL, R. 1991. A novel multigene family may encode odorant receptors: a molecular basis for odor recognition. *Cell* 65:175–187.

BUONVISO, N., AMAT, C., and LITAUDON, P. 2006. Respiratory modulation of olfactory neurons in the rodent brain. *Chem. Senses* 31:145–154.

CALU, D. J., ROESCH, M. R., STALNAKER, T. A., and SCHOENBAUM, G. 2007. Associative encoding in posterior piriform cortex during odor discrimination and reversal learning. *Cereb. Cortex* 17: 1342–1349.

CARLETON, A., PETREANU, L. T., LANSFORD, R., ALVAREZ-BUYLLA, A., and LLEDO, P. M. 2003. Becoming a new neuron in the adult olfactory bulb. *Nat. Neurosci.* 6:507–518.

CECCHI, G. A., PETREANU, L. T., ALVAREZ-BUYLLA, A., and MAGNASCO, M. O. 2001. Unsupervised learning and adaptation in a model of adult neurogenesis. *J Comput. Neurosci.* 11: 175–182.

CHABAUD, P., RAVEL, N., WILSON, D. A., MOULY, A. M., VIGOUROUX, M., FARGET, V., and GERVAIS, R. 2000. Exposure to behaviourally relevant odour reveals differential characteristics in rat central olfactory pathways as studied through oscillatory activities. *Chem. Senses* 25:561–573.

CHALANSONNET, M., and CHAPUT, M. A. 1998. Olfactory bulb output cell temporal response patterns to increasing odor concentrations in freely breathing rats. *Chem. Senses* 23:1–9.

CHAMBERS, R. A., and CONROY, S. K. 2007. Network modeling of adult neurogenesis: shifting rates of neuronal turnover optimally gears network learning according to novelty gradient. *J. Cogn. Neurosci.* 19:1–12.

CHEN, W. R., and SHEPHERD, G. M. 2005. The olfactory glomerulus: a cortical module with specific functions. *J. Neurocytol.* 34: 353–360.

CHU, S., and DOWNES, J. J. 2004. Proust reinterpreted: can Proust's account of odour-cued autobiographical memory recall really be investigated? A reply to Jellinek. *Chem. Senses* 29:459–461.

CINELLI, A. R., HAMILTON, K. A., and KAUER, J. S. 1995. Salamander olfactory bulb neuronal activity observed by video rate, voltage-sensitive dye imaging. III. Spatial and temporal properties of responses evoked by odorant stimulation. *J. Neurophysiol.* 73: 2053–2071.

CLELAND, T. A., and LINSTER, C. 2005. Computation in the olfactory system. *Chem. Senses* 30:801–813.

CLELAND, T. A., JOHNSON, B. A., LEON, M., and LINSTER, C. 2007. Relational representation in the olfactory system. *Proc. Natl. Acad. Sci. USA* 104:1953–1958.

DAVILA, N. G., HOUPT, T. A., and TROMBLEY, P. Q. 2007. Expression and function of kainate receptors in the rat olfactory bulb. *Synapse* 61:320–334.

DAVISON, I. G., and KATZ, L. C. 2007. Sparse and selective odor coding by mitral/tufted neurons in the main olfactory bulb. *J. Neurosci.* 27:2091–2101.

DAVISON, A. P., FENG, J. F., and BROWN, D. 2003. Dendrodendritic inhibition and simulated odor responses in a detailed olfactory bulb network model. *J. Neurophysiol.* 90:1921–1935.

DE MARCHIS, S., BOVETTI, S., CARLETTI, B., HSIEH, Y. C., GARZOTTO, D., PERETTO, P., FASOLO, A., PUCHE, A. C., and ROSSI, F. 2007. Generation of distinct types of periglomerular olfactory bulb interneurons during development and in adult mice: implication for intrinsic properties of the subventricular zone progenitor population. *J. Neurosci.* 27:657–664.

DE SOUZA, F. M. S., and ANTUNES, G. 2007. Biophysics of olfaction. *Rep. Prog. Phys* 70:451–491.

DEL PUNTA, K., LEINDERS-ZUFALL, T., RODRIGUEZ, I., JUKAM, D., WYSOCKI, C. J., OGAWA, S., ZUFALL, F., and MOMBAERTS, P. 2002. Deficient pheromone responses in mice lacking a cluster of vomeronasal receptor genes. *Nature* 419:70–74.

DIELENBERG, R. A., and MCGREGOR, I. S. 2001. Defensive behavior in rats towards predatory odors: a review. *Neurosci. Biobehav. Rev.* 25:597–609.

DOLAN, R. J. 2007. The human amygdala and orbital prefrontal cortex in behavioural regulation. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* 362:787–799.

DULAC, C., and WAGNER, S. 2006. Genetic analysis of brain circuits underlying pheromone signaling. *Annu. Rev. Genet.* 40:449–467.

FENDT, M., ENDRES, T., LOWRY, C. A., APFELBACH, R., and MCGREGOR, I. S. 2005. TMT-induced autonomic and behavioral changes and the neural basis of its processing. *Neurosci. Biobehav. Rev.* 29:1145–1156.

FERKIN, M. H., and PIERCE, A. A. 2007. Perspectives on over-marking: is it good to be on top? *J. Ethol.* 25:107–116.

FLETCHER, M. L., and WILSON, D. A. 2003. Olfactory bulb mitral cell-tufted cell plasticity: odorant-specific tuning reflects previous odorant exposure. *J. Neurosci.* 23:6946–6955.

FLETCHER, M. L., SMITH, A. M., BEST, A. R., and WILSON, D. A. 2005. High-frequency oscillations are not necessary for simple olfactory discriminations in young rats. *J. Neurosci.* 25:792–798.

GALEF, B. G. JR, MASON, J. R., PRETI, G., and BEAN, N. J. 1988. Carbon disulfide: a semiochemical mediating socially-induced diet choice in rats. *Physiol. Behav.* 42:119–124.

GELPERIN, A. 2006. Olfactory computations and network oscillations. *J. Neurosci.* 26:1663–1668.

GETCHELL, T. V., MARGOLIS, F. L., and GETCHELL, M. L. 1984. Perireceptor and receptor events in vertebrate olfaction. *Prog. Neurobiol.* 23:317–345.

GETCHELL, T. V., KWONG, K., SAUNDERS, C. P., STROMBERG, A. J., and GETCHELL, M. L. 2006. Leptin regulates olfactory-mediated behavior in ob/ob mice. *Physiol. Behav.* 87:848–856.

GHEUSI, G., and LLEDO, P. M. 2007. Control of early events in olfactory processing by adult neurogenesis. *Chem. Senses* 32: 397–409.

GILAD, Y., BUSTAMANTE, C. D., LANCET, D., and PAABO, S. 2003. Natural selection on the olfactory receptor gene family in humans and chimpanzees. *Am. J. Hum. Genet.* 73:489–501.

GILAD, Y., WIEBE, V., PRZEWORSKI, M., LANCET, D., and PAABO, S. 2004. Loss of olfactory receptor genes coincides with the acquisition of full trichromatic vision in primates. *PLoS Biol.* 2:E5.

GILBERT, A. N., YAMAZAKI, K., BEAUCHAMP, G. K., and THOMAS, L. 1986. Olfactory discrimination of mouse strains (*Mus musculus*) and major histocompatibility types by humans (*Homo sapiens*). *J. Comp. Psych.* 100:262–265.

GOROJANKINA, T., GREBERT, D., SALESSE, R., TANFIN, Z., and CAILLOL, M. 2007. Study of orexins signal transduction pathways in rat olfactory mucosa and in olfactory sensory neurons-derived cell line Odora: multiple orexin signalling pathways. *Regul. Pept.* 141:73–85.

GOULD, E. 2007. How widespread is adult neurogenesis in mammals? *Nat. Rev. Neurosci.* 8:481–488.

GOYERT, H. F., FRANK, M. E., GENT, J. F., and HETTINGER, T. P. 2007. Characteristic component odors emerge from mixtures after selective adaptation. *Brain Res. Bull.* 72:1–9.

GRAZIADEI, P. P. C., and GRAZIADEI-MONTI, G. A. 1979. Neurogenesis and neuron regeneration in the olfactory system of mammals. I. Morphological aspects of differentiation and structural organization of the olfactory sensory neurons. *J. Neurocytol.* 8:1–18.

HAGA, S., KIMOTO, H., and TOUHARA, K. 2007. Molecular characterization of vomeronasal sensory neurons responding to a male-specific peptide in tear fluid: sexual communication in mice. *Pure Appl. Chem.* 79:775–783.

HALPERN, M., and MARTINEZ-MARCOS, A. 2003. Structure and function of the vomeronasal system: an update. *Prog. Neurobiol.* 70:245–318.

HAMILTON, W. D., and ZUK, M. 1982. Heritable true fitness and bright birds: a role for parasites? *Science* 218:384–387.

HARDY, A. B., AIOUN, J., BALY, C., JULLIARD, K. A., CAILLOL, M., SALESSE, R., and DUCHAMP-VIRET, P. 2005. Orexin A modulates mitral cell activity in the rat olfactory bulb: patch-clamp study on slices and imunocytochemical localization of orexin receptors. *Endocrinology* 146:4042–4053.

HEALE, V. R., and VANDERWOLF, C. H. 1994. Toluene and weasel (2-propylthierane) odors suppress feeding in the rat. *J. Chem. Ecol.* 20:2953–2958.

HEALE, V. R., VANDERWOLF, C. H., and KAVALIERS, M. 1994. Components of weasel and fox odors elicit fast wave bursts in the dentate gyrus of rats. *Behav. Brain Res.* 63:159–165.

HIROTA, J., OMURA, M., and MOMBAERTS, P. 2007. Differential impact of Lhx2 deficiency on expression of class I and class II odorant receptor genes in mouse. *Mol. Cell Neurosci.* 34:679–688.

HOLY, T. E., DULAC, C., and MEISTER, M. 2000. Responses of vomeronasal neurons to natural stimuli. *Science* 289:1569–1572.

HOPFIELD, J. J. 1999. Odor space and olfactory processing: collective algorithms and neural implementation. *Proc. Natl. Acad. Sci. USA* 96:12506–12511.

HOPFIELD, J. F., and GELPERIN, A. 1989. Differential conditioning to a compound stimulus and its components in the terrestrial mollusc *Limax maximus*. *Behav. Neurosci.* 103:274–293.

HOPPE, R., LAMBERT, T. D., SAMOLLOW, P. B., BREER, H., and STROTMANN, J. 2006. Evolution of the "OR37" subfamily of olfactory receptors: a cross-species comparison. *J. Mol. Evol.* 62:460–472.

HURST, J. L., ROBERTSON, D. H. L., TOLLADAY, U., and BEYNON, R. J. 1998. Proteins in urine scent marks of male house mice extend the longevity of olfactory signals. *Anim. Behav.* 55: 1289–1297.

ISHIKAWA, T., SATO, T., SHIMIZU, A., TSUTSUI, K., DE CURTIS, M., and IIJIMA, T. 2007. Odor-driven activity in the olfactory cortex of an in vitro isolated guinea pig whole brain with olfactory epithelium. *J. Neurophysiol.* 97:670–679.

JELLINEK, J. S. 2004. Proust remembered: has Proust's account of odor-cued autobiographical memory recall really been investigated? *Chem. Senses* 29:455–458 (author reply 459–461).

JOHNSON, B. A., and LEON, M. 2007. Chemotopic odorant coding in a mammalian olfactory system. *J. Comp. Neurol.* 503:1–34.

JOHNSTON, R. E., SOROKIN, E. S., and FERKIN, M. H. 1997. Female voles discriminate males' over-marks and prefer top-scent males. *Anim. Behav.* 54:679–690.

JOHNSON, B. A., ARGUELLO, S., and LEON, M. 2007a. Odorants with multiple oxygen-containing functional groups and other odorants with high water solubility preferentially activate posterior olfactory bulb glomeruli. *J. Comp. Neurol.* 502:468–482.

JOHNSON, B. A., ONG, J., LEE, K., HO, S. L., ARGUELLO, S., and LEON, M. 2007b. Effects of double and triple bonds on the spatial representations of odorants in the rat olfactory bulb. *J. Comp. Neurol.* 500:720–733.

JORTNER, R. A., FARIVAR, S. S., and LAURENT, G. 2007. A simple connectivity scheme for sparse coding in an olfactory system. *J. Neurosci.* 27:1659–1669.

JULLIARD, A. K., CHAPUT, M. A., APELBAUM, A., AIME, P., MAHFOUZ, M., and DUCHAMP-VIRET, P. 2007. Changes in rat olfactory detection performance induced by orexin and leptin mimicking fasting and satiation. *Behav. Brain Res.* 183:123–129.

KADOHISA, M., and WILSON, D. A. 2006. Olfactory cortical adaptation facilitates detection of odors against background. *J. Neurophysiol.* 95:1888–1896.

KAJIYA, K., INAKI, K., TANAKA, M., HAGA, T., KATAOKA, H., and TOUHARA, K. 2001. Molecular bases of odor discrimination: reconstitution of olfactory receptors that recognize overlapping sets of odorants. *J. Neurosci.* 21:6018–6025.

KATS, L. B., and DILL, L. M. 1998. The scent of death: chemosensory assessment of predation risk by prey animals. *Ecoscience* 5:361–394.

KAVALIERS, M., CHOLERIS, E., and PFAFF, D. W. 2005a. Genes, odours and the recognition of parasitized individuals by rodents. *Trends Parasitol.* 21:423–429.

KAVALIERS, M., CHOLERIS, E., and PFAFF, D. W. 2005b. Recognition and avoidance of the odors of parasitized conspecifics and predators: differential genomic correlates. *Neurosci. Biobehav. Rev.* 29:1347–1359.

KAY, L. M. 2003. Two species of gamma oscillations in the olfactory bulb: dependence on behavioral state and synaptic interactions. *J. Integr. Neurosci.* 2:31–44.

KAY, L. M., and LAURENT, G. 1999. Odor- and context-dependent modulation of mitral cell activity in behaving rats. *Nature Neurosci.* 2:1003–1009.

KAY, L. M., and SHERMAN, S. M. 2007. An argument for an olfactory thalamus. *Trends Neurosci.* 30:47–53.

KAY, L. M., LOWRY, C. A., and JACOBS, H. A. 2003. Receptor contributions to configural and elemental odor mixture perception. *Behav. Neurosci.* 117:1108–1114.

KAY, L. M., CRK, T., and THORNGATE, J. 2005. A redefinition of odor mixture quality. *Behav. Neurosci.* 119:726–733.

KELLER, A., and VOSSHALL, L. B. 2007. Influence of odorant receptor repertoire on odor perception in humans and fruit flies. *Proc. Natl. Acad. Sci. USA* 104:5614–5619.

KELLIHER, K. R., SPEHR, M., LI, X. H., ZUFALL, F., and LEINDERS-ZUFALL, T. 2006. Pheromonal recognition memory induced by TRPC2-independent vomeronasal sensing. *Eur. J. Neurosci.* 23:3385–3390.

KELLIHER, K. R., ZIESMANN, J., MUNGER, S. D., REED, R. R., and ZUFALL, F. 2003. Importance of the CNGA4 channel gene for odor discrimination and adaptation in behaving mice. *Proc. Natl. Acad. Aci. USA* 100:4299–4304.

KENT, P. F., MOZELL, M. M., MURPHY, S. J., and HORNUNG, D. E. 1996. The interaction of imposed and inherent olfactory mucosal activity patterns and their composite representation in a mammalian species using voltage-sensitive dyes. *J. Neurosci.* 16:345–353.

KENT, P. F., MOZELL, M. M., YOUNGENTOB, S. L., and YURCO, P. 2003. Mucosal activity patterns as a basis for olfactory discrimination: comparing behavior and optical recordings. *Brain Res.* 981:1–11.

KEPECS, A., UCHIDA, N., and MAINEN, Z. F. 2006. The sniff as a unit of olfactory processing. *Chem. Senses* 31:167–179.

KEVERNE, E. B. 1999. The vomeronasal organ. *Science* 286:716–720.

KIMOTO, H., HAGA, S., SATO, K., and TOUHARA, K. 2005. Sex-specific peptides from exocrine glands stimulate mouse vomeronasal sensory neurons. *Nature* 437:898–901.

KINI, A. D., and FIRESTEIN, S. 2001. The molecular basis of olfaction. *Chimia* 55:453–459.

KNAPP, L. A. 2005. The ABCs of MHC. *Evol. Anthropol.* 14:28–37.

KNAPP, L. A., ROBSON, J., and WATERHOUSE, J. S. 2006. Olfactory signals and the MHC: a review and a case study in *Lemur catta*. *Am. J. Primatol.* 68:568–584.

KOBAYAKAWA, K., KOBAYAKAWA, R., MATSUMOTO, H., OKA, Y., IMAI, T., IKAWA, M., OKABE, M., IKEDA, T., ITOHARA, S., KIKUSUI, T. et al. 2007. Innate versus learned odour processing in the mouse olfactory bulb. *Nature* 450:503–508.

KOULAKOV, A., RINBERG, D., and GELPERIN, A. 2007. Olfactory coding with all-or-nothing glomeruli. *J. Neurophysiol.* 98: 3134–3142.

KURTZ, D. B., ZHAO, K., HORNUNG, D. E., and SCHERER, P. 2004. Experimental and numerical determination of odorant solubility in nasal and olfactory mucosa. *Chem. Senses* 29:763–773.

LAI, W. S., RAMIRO, L. L., YU, H. A., and JOHNSTON, R. E. 2005. Recognition of familiar individuals in golden hamsters: a new method and functional neuroanatomy. *J. Neurosci.* 25: 11239–11247.

LAING, D. G. 1986. Identification of single dissimilar odors is achieved by humans with a single sniff. *Physiol. Behav.* 37:163–170.

LAURENT, G., STOPFER, M., FRIEDRICH, R. W., RABINOVICH, M. I., VOLKOVSKII, A., and ABARBANEL, H. D. 2001. Odor encoding as an active, dynamical process: experiments, computation, and theory. *Annu. Rev. Neurosci.* 24:263–297.

LEHMKUHLE, M. J., NORMANN, R. A., and MAYNARD, E. M. 2006. Trial-by-trial discrimination of three enantiomer pairs by neural ensembles in mammalian olfactory bulb. *J. Neurophysiol.* 95:1369–1379.

LEINDERS-ZUFALL, T., LANE, A. P., PUCHE, A. C., MA, W., NOVOTNY, M. V., SHIPLEY, M. T., and ZUFALL, F. 2000. Ultrasensitive pheromone detection by mammalian vomeronasal neurons. *Nature* 405:792–796.

LEINDERS-ZUFALL, T., BRENNAN, P., WIDMAYER, P., CHANDRAMANI, P. S., MAUL-PAVICIC, A., JAGER, M., LI, X. H., BREER, H., ZUFALL, F., and BOEHM, T. 2004. MHC class I peptides as chemosensory signals in the vomeronasal organ. *Science* 306: 1033–1037.

LEPRI, J. J., and WYSOCKI, C. J. 1987. Removal of the vomeronasal organ disrupts the activation of reproduction in female voles. *Physiol. Behav.* 40:349–355.

LEUNG, C. T., COULOMBE, P. A., and REED, R. R. 2007. Contribution of olfactory neural stem cells to tissue maintenance and regeneration. *Nat. Neurosci.* 10:720–726.

LEVAI, O., FEISTEL, T., BREER, H., and STROTMANN, J. 2006. Cells in the vomeronasal organ express odorant receptors but project to the accessory olfactory bulb. *J. Comp. Neurol.* 498: 476–490.

LEYPOLD, B. G., YU, C. R., LEINDERS-ZUFALL, T., KIM, M. M., ZUFALL, F., and AXEL, R. 2002. Altered sexual and social behaviors in trp2 mutant mice. *Proc. Natl. Acad. Sci. USA* 99:6376–6381.

LIN, W., ARELLANO, J., SLOTNICK, B., and RESTREPO, D. 2004. Odors detected by mice deficient in cyclic nucleotide-gated channel subunit A2 stimulate the main olfactory system. *J. Neurosci.* 24:3703–3710.

LIN, D. Y., ZHANG, S. Z., BLOCK, E., and KATZ, L. C. 2005. Encoding social signals in the mouse main olfactory bulb. *Nature* 434:470–477.

LIN, D. Y., SHEA, S. D., and KATZ, L. C. 2006. Representation of natural stimuli in the rodent main olfactory bulb. *Neuron* 50: 937–949.

LIN, W., MARGOLSKEE, R., DONNERT, G., HELL, S. W., and RESTREPO, D. 2007. Olfactory neurons expressing transient receptor potential channel M5 (TRPM5) are involved in sensing semiochemicals. *Proc. Natl. Acad. Sci. USA* 104: 2471–2476.

LINSTER, C., HENRY, L., KADOHISA, M., and WILSON, D. A. 2007. Synaptic adaptation and odor-background segmentation. *Neurobiol. Learn. Mem.* 87:352–360.

LLEDO, P. M., ALONSO, M., and GRUBB, M. S. 2006. Adult neurogenesis and functional plasticity in neuronal circuits. *Nat. Rev. Neurosci.* 7:179–193.

LOIS, C., and ALVAREZ-BUYLLA, A. 1994. Long-distance neuronal migration in the adult mammalian brain. *Science* 264:1145–1148.

LORIG, T. S. 2000. The application of electroencephalographic techniques to the study of human olfaction: a review and tutorial. *Int. J. Psychophysiol.* 36:91–104.

LOWE, G. 2003. Flash photolysis reveals a diversity of ionotropic glutamate receptors on the mitral cell somatodendritic membrane. *J. Neurophysiol.* 90:1737–1746.

LOWRY, C. A., and KAY, L. M. 2007. Chemical factors determine olfactory system Beta oscillations in waking rats. *J. Neurophysiol.* 98:394–404.

LU, X. C. M., and SLOTNICK, B. M. 1998. Olfaction in rats with extensive lesions of the olfactory bulbs: implications for odor coding. *Neuroscience* 84:849–866.

LUO, M., FEE, M. S., and KATZ, L. C. 2003. Encoding pheromonal signals in the accessory olfactory bulb of behaving mice. *Science* 299:1196–1201.

MA, J., and LOWE, G. 2007. Calcium permeable AMPA receptors and autoreceptors in external tufted cells of rat olfactory bulb. *Neuroscience* 144:1094–1108.

MAGAVI, S. S. P., MITCHELL, B. D., SZENTIRMAI, O., CARTER, B. S., and MACKLIS, J. D. 2005. Adult-born and preexisting olfactory granule neurons undergo distinct experience-dependent modifications of their olfactory responses in vivo. *J. Neurosci.* 25: 10729–10739.

MAICO, L. M., BURROWS, A. M., MOONEY, M. P., SIEGEL, M. I., BHATNAGAR, K. P., and SMITH, T. D. 2003. Size of the vomeronasal organ in wild Microtus with different mating strategies. *Acta Biol. Hung.* 54:263–273.

MAINEN, Z. F. 2006. Behavioral analysis of olfactory coding and computation in rodents. *Curr. Opin. Neurobiol.* 16:429–434.

MAINLAND, J., and SOBEL, N. 2006. The sniff is part of the olfactory percept. *Chem. Senses* 31:181–196.

MAK, G. K., ENWERE, E. K., GREGG, C., PAKARAINEN, T., POUTANEN, M., HUHTANIEMI, I., and WEISS, S. 2007. Male pheromone-stimulated neurogenesis in the adult female brain: possible role in mating behavior. *Nat. Neurosci.* 10:1003–1011.

MALNIC, B., HIRONO, J., SATO, T., and BUCK, L. 1999. Combinatorial receptor codes for odors. *Cell* 96:713–723.

MAN, O., GILAD, Y., and LANCET, D. 2004. Prediction of the odorant binding site of olfactory receptor proteins by human-mouse comparisons. *Protein Sci.* 13:240–254.

MANDAIRON, N., SACQUET, J., GARCIA, S., RAVEL, N., JOURDAN, F., and DIDIER, A. 2006a. Neurogenic correlates of an olfactory discrimination task in the adult olfactory bulb. *Eur. J. Neurosci.* 24:3578–3588.

MANDAIRON, N., SACQUET, J., JOURDAN, F., and DIDIER, A. 2006b. Long-term fate and distribution of newborn cells in the adult mouse olfactory bulb: influences of olfactory deprivation. *Neuroscience* 141:443–451.

MANDAIRON, N., STACK, C., and LINSTER, C. 2006c. Olfactory enrichment improves the recognition of individual components in mixtures. *Physiol. Behav.* 89:379–384.

MARTIN, C., GERVAIS, R., CHABAUD, P., MESSAOUDI, B., and RAVEL, N. 2004. Learning-induced modulation of oscillatory activities in the mammalian olfactory system: the role of the centrifugal fibres. *J. Physiol. Paris* 98:467–478.

MARTIN, C., GERVAIS, R., MESSAOUDI, B., and RAVEL, N. 2006. Learning-induced oscillatory activities correlated to odour recognition: a network activity. *Eur. J. Neurosci.* 23:1801–1810.

MARTINEZ-MARCOS, A., JIA, C., QUAN, W., and HALPERN, M. 2005. Neurogenesis, migration, and apoptosis in the vomeronasal epithelium of adult mice. *J. Neurobiol.* 63:173–187.

MCBRIDE, K., and SLOTNICK, B. 2006. Discrimination between the enantiomers of carvone and of terpinen-4-ol odorants in normal rats and those with lesions of the olfactory bulbs. *J. Neurosci.* 26:9892–9901.

MENINI, A., PICCO, C., and FIRESTEIN, S. 1995. Quantal-like current fluctuations induced by odorants in olfactory receptor cells. *Nature* 373:435–437.

MEREDITH, M. 1992. Neural circuit computation—complex patterns in the olfactory bulb. *Brain Res. Bull.* 29:111–117.

MEREDITH, M. 1994. Chronic recording of vomeronasal pump activation in awake behaving hamsters. *Physiol. Behav.* 56:345–354.

MILLER, G. L., LOUDON, C., and FREED, S. 2007. Position around a tree: consequences for pheromone detection. *J. Chem. Ecol.* 33:541–554.

MIZRAHI, A. 2007. Dendritic development and plasticity of adult-born neurons in the mouse olfactory bulb. *Nat. Neurosci.* 10:444–452.

MIZRAHI, A., LU, J., IRVING, R., FENG, G., and KATZ, L. C. 2006. In vivo imaging of juxtaglomerular neuron turnover in the mouse olfactory bulb. *Proc. Natl. Acad. Sci. USA* 103:1912–1917.

MORE, L. 2006. Mouse major urinary proteins trigger ovulation via the vomeronasal organ. *Chem. Senses* 31:393–401.

MORI, I., NISHIYAMA, Y., YOKOCHI, T., and KIMURA, Y. 2005. Olfactory transmission of neurotropic viruses. *J. Neurovirol.* 11:129–137.

MORI, K., TAKAHASHI, Y. K., IGARASHI, K. M., and YAMAGUCHI, M. 2006. Maps of odorant molecular features in the mammalian olfactory bulb. *Physiol. Rev.* 86:409–433.

MOZELL, M. M., and JAGODOWICZ, M. 1973. Chromatographic separation of odorants by the nose: retention times measured across in vivo olfactory mucosa. *Science* 181:1247–1249.

MOZELL, M. M., and JAGODOWICZ, M. 1974. Mechanisms underlying the analysis of odorant quality at the level of the olfactory mucosa. I. Spatiotemporal sorption patterns. *Ann. NY Acad. Sci.* 237:76–90.

MURLIS, J., ELKINTON, J. S., and CARDE, R. T. 1992. Odor plumes and how insects use them. *Ann. Rev. Entomol.* 37:505–532.

MURLIS, J., WILLIS, M. A., and CARDE, R. T. 2000. Spatial and temporal structures of pheromone plumes in fields and forests. *Physiol. Entomol.* 25:211–222.

MURRAY, E. A., O'DOHERTY, J. P., and SCHOENBAUM, G. 2007. What we know and do not know about the functions of the orbitofrontal cortex after 20 years of cross-species studies. *J. Neurosci.* 27:8166–8169.

NOVOTNY, M. V., SOINI, H. A., KOYAMA, S., WIESLER, D., BRUCE, K. E., and PENN, D. J. 2007. Chemical identification of MHC-influenced volatile compounds in mouse urine. I. Quantitative proportions of major chemosignals. *J. Chem. Ecol.* 33:417–434.

OGAWA, S., LEE, T. M., NAYAK, A. S., and GLYNN, P. 1990. Oxygenation-sensitive contrast in magnetic resonance image of rodent brain at high magnetic fields. *Magn. Reson. Med.* 14: 68–78.

OLARIU, A., CLEAVER, K. M., SHORE, L. E., BREWER, M. D., and CAMERON, H. A. 2005. A natural form of learning can increase and decrease the survival of new neurons in the dentate gyrus. *Hippocampus* 15:750–762.

OLSHAUSEN, B. A., and FIELD, D. J. 2004. Sparse coding of sensory inputs. *Curr. Opin. Neurobiol.* 14:481–487.

PAUSE, B. M., KRAUEL, K., SOJKA, B., and FERSTL, R. 1998. Body odor evoked potentials: a new method to study the chemosensory perception of self and non-self in humans. *Genetica* 104:285–294.

PAUSE, B. M., KRAUEL, K., SCHRADER, C., SOJKA, B., WESTPHAL, E., MULLER-RUCHHOLTZ, W., and FERSTL, R. 2006. The human brain is a detector of chemosensorily transmitted HLA-class I-similarity in same- and opposite-sex relations. *Proc. Biol. Sci.* 273:471–478.

PENN, D. J. 2002. The scent of genetic compatibility: sexual selection and the major histocompatibility complex. *Ethology* 108:1–21.

PENN, D. J., OBERZAUCHER, E., GRAMMER, K., FISCHER, G., SOINI, H. A., WIESLER, D., NOVOTNY, M. V., DIXON, S. J., XU, Y., and BRERETON, R. G. 2007. Individual and gender fingerprints in human body odour. *J. R. Soc. Interface* 4:331–340.

PETERS, A. 2007. Testosterone and carotenoids: an integrated view of trade-offs between immunity and sexual signalling. *Bioessays* 29:427–430.

PETREANU, L., and ALVAREZ-BUYLLA, A. 2002. Maturation and death of adult-born olfactory bulb granule neurons: role of olfaction. *J. Neurosci.* 22:6106–6113.

PIERCE, A. A., VAUGHN, A. A., and FERKIN, M. H. 2007. Food deprivation suppresses a preference for the top-scent mark of an over-mark in meadow voles (*Microtus pennsylvanicus*). *Ethology* 113:480–486.

RAMUS, S. J., and EICHENBAUM, H. 2000. Neural correlates of olfactory recognition memory in the rat orbitofrontal cortex. *J. Neurosci.* 20:8199–8208.

RAWSON, N. E., and GOMEZ, G. 2002. Cell and molecular biology of human olfaction. *Microsc. Res. Tech.* 58:142–151.

REISERT, J. 2005. Signal transduction in vertebrate olfactory receptor cells. *Lect. Notes Math.* 1867:155–171.

RENNAKER, R. L., CHEN, C. F., RUYLE, A. M., SLOAN, A. M., and WILSON, D. A. 2007. Spatial and temporal distribution of odorant-evoked activity in the piriform cortex. *J. Neurosci.* 27:1534–1542.

RESTREPO, D., TEETER, J. H., and SCHILD, D. 1996. Second messenger signaling in olfactory transduction. *J. Neurobiol.* 30:37–48.

RESTREPO, D., ARELLANO, J., OLIVA, A. M., SCHAFER, M. L., and LIN, W. 2004. Emerging views on the distinct but related roles of the main and accessory olfactory systems in responsiveness to chemosensory signals in mice. *Horm. Behav.* 46:247–256.

RESTREPO, D., LIN, W., SALCEDO, E., YAMAZAKI, K., and BEAUCHAMP, G. 2006. Odortypes and MHC peptides: complementary chemosignals of MHC haplotype? *Trends Neurosci.* 29:604–609.

RINBERG, D., and GELPERIN, A. 2006. Olfactory neuronal dynamics in behaving animals. *Semin. Cell Dev. Biol.* 17:454–461.

RINBERG, D., KOULAKOV, A., and GELPERIN, A. 2006. Sparse odor coding in the behaving mouse. *J. Neurosci.* 26:8857–8865.

ROBERTSON, D. H., HURST, J. L., SEARLE, J. B., GUNDUZ, I., and BEYNON, R. J. 2007. Characterization and comparison of major urinary proteins from the house mouse, *Mus musculus domesticus*, and the aboriginal mouse, *Mus macedonicus*. *J. Chem. Ecol.* 33:613–630.

ROSEN, J. B., WEST, E. A., and DONLEY, M. P. 2006. Not all rat strains are equal: differential unconditioned fear responses to the synthetic fox odor 2,4,5-trimethylthiazoline in three outbred rat strains. *Behav. Neurosci.* 120:290–297.

ROUX, S. G., GARCIA, S., BERTRAND, B., CENIER, T., VIGOUROUX, M., BUONVISO, N., and LITAUDON, P. 2006. Respiratory cycle as time basis: an improved method for averaging olfactory neural events. *J. Neurosci. Methods* 152:173–178.

SAKURAI, T., AMEMIYA, A., ISHII, M., MATSUZAKI, I., CHEMELLI, R. M., TANAKA, H., WILLIAMS, S. C., RICHARDSON, J. A., KOZLOWSKI, G. P., WILSON, S. et al. 1998. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* 92: 573–585.

SBARBATI, A., and OSCULATI, F. 2006. Allelochemical communication in vertebrates: kairomones, allomones and synomones. *Cells Tissues Organs* 183:206–219.

SCHAEFER, A. T., and MARGRIE, T. W. 2007. Spatiotemporal representations in the olfactory system. *Trends Neurosci.* 30: 92–100.

SCHAFER, J. R., KIDA, I., XU, F., ROTHMAN, D. L., and HYDER, F. 2006. Reproducibility of odor maps by fMRI in rodents. *Neuroimage* 31:1238–1246.

SCHILDKNECHT, H., WILZ, I., ENZMANN, F., GRUND, N., and ZIEGLER, M. 1976. Mustelan, the malodorous substance from the anal gland of

the mink (*Mustela vison*) and the polecat (*Mustela putorius*). *Angew. Chem. Int. Ed. Engl.* 15:242–243.

SCHLIEF, M. L., and WILSON, R. I. 2007. Olfactory processing and behavior downstream from highly selective receptor neurons. *Nat. Neurosci.* 10:623–630.

SCORDATO, E. S., DUBAY, G., and DREA, C. M. 2007. Chemical composition of scent marks in the ringtailed lemur (*Lemur catta*): glandular differences, seasonal variation, and individual signatures. *Chem. Senses* 32:493–504.

SCOTT, J. W. 2006. Sniffing and spatiotemporal coding in olfaction. *Chem. Senses* 31:119–130.

SCOTT, J. W., ACEVEDO, H. P., and SHERRILL, L. 2006. Effects of concentration and sniff flow rate on the rat electroolfactogram. *Chem. Senses* 31:581–593.

SETTLES, G. S. 2005. Sniffers: fluid-dynamic sampling for olfactory trace detection in nature and homeland security—the 2004 Freeman scholar lecture. *J. Fluids Eng.* 127:189–218.

SHEPHERD, G. M. 2004. The human sense of smell: are we better than we think? *PLoS Biol.* 2:572–575.

SHEPHERD, G. M., CHEN, W. R., and GREER, C. A. 2004. Olfactory bulb, pp. 133–169, in G. M. Shepherd (ed.). The Synaptic Organization of the Brain, 5th edn. Oxford University Press, New York.

SHIPLEY, M. T., and ENNIS, M. 1995. Functional organization of olfactory system. *J. Neurobiol.* 30:123–176.

SINGER, B. H., KIM, S., and ZOCHOWSKI, M. 2007. Binaral interaction and centrifugal input enhances spatial contrast in olfactory bulb activation. *Eur. J. Neurosci.* 25:576–586.

SLOTNICK, B. 2007a. Olfactory performance of rats after selective deafferentation of the olfactory bulb by 3-methyl indole. *Chem. Senses* 32:173–181.

SLOTNICK, B. 2007b. Response accuracy and odor sampling time in mice trained to discriminate between enantiomers of carvone and those of terpinen-4-ol. *Chem. Senses* 32:721–725.

SLOTNICK, B., and BISULCO, S. 2003. Detection and discrimination of carvone entantiomers in rats with olfactory bulb lesions. *Neuroscience* 121:451–457.

SLOTNICK, B., COCKERHAM, R., and PICKETT, E. 2004. Olfaction in olfactory bulbectomized rats. *J. Neurosci.* 24:9195–9200.

SLOTNICK, B., SANGUINO, A., HUSBAND, S., MARQUINO, G., and SILBERBERG, A. 2007. Olfaction and olfactory epithelium in mice treated with zinc gluconate. *Laryngoscope* 117:743–749.

SMITH, T. D., BHATNAGAR, K. P., DENNIS, J. C., MORRISON, E. E., and PARK, T. J. 2007. Growth-deficient vomeronasal organs in the naked mole-rat (*Heterocephalus glaber*). *Brain Res.* 1132: 78–83.

SOUCY, E. R., ALBEANU, D. F., MURTHY, V. N., and MEISTER, M. 2007. Comparing glomerular maps in the olfactory bulbs of mice and rats, in Cosyne 2007 Abstracts, p. 55, Salt Lake City, UT.

SPEHR, M., GISSELMANN, G., POPLAWSKI, A., RIFFELL, J. A., WETZEL, C. H., ZIMMER, R. K., and HATT, H. 2003. Identification of a testicular odorant receptor mediating human sperm chemotaxis. *Science* 299:2054–2058.

SPEHR, M., SCHWANE, K., RIFFELL, J. A., ZIMMER, R. K., and HATT, H. 2006. Odorant receptors and olfactory-like signaling mechanisms in mammalian sperm. *Mol. Cell Endocrinol.* 250:128–136.

STAPLES, L. G., and MCGREGOR, I. S. 2006. Defensive responses of Wistar and Sprague–Dawley rats to cat odour and TMT. *Behav. Brain Res.* 172:351–354.

STOPFER, M. 2007. Olfactory processing: massive convergence onto sparse codes. *Curr. Biol.* 17:R363–R364.

STOPKOVA, R., STOPKA, P., JANOTOVA, K., and JEDELSKY, P. L. 2007. Species-specific expression of major urinary proteins in the house mice (*Mus musculus musculus* and *Mus musculus domesticus*). *J. Chem. Ecol.* 33:861–869.

STORAN, M. J., and KEY, B. 2006. Septal organ of Gruneberg is part of the olfactory system. *J. Comp. Neurol.* 494:834–844.

STOWERS, L., HOLY, T. E., MEISTER, M., DULAC, C., and KOENTGES, G. 2002. Loss of sex discrimination and male-male aggression in mice deficient for TRP2. *Science* 295:1493–1500.

SZOBOTA, S., GOROSTIZA, P., DEL BENE, F., WYART, C., FORTIN, D. L., KOLSTAD, K. D., TULYATHAN, O., VOLGRAF, M., NUMANO, R., AARON, H. L. et al. 2007. Remote control of neuronal activity with a light-gated glutamate receptor. *Neuron* 54:535–545.

THESEN, T., and MURPHY, C. 2002. Reliability analysis of event-related brain potentials to olfactory stimuli. *Psychophysiology* 39:733–738.

THOM, M. D., and HURST, J. L. 2004. Individual recognition by scent. *Ann. Zool. Fenn.* 41:765–787.

THOMPSON, R. N., MCMILLON, R., NAPIER, A., and WEKESA, K. S. 2007. Pregnancy block by MHC class I peptides is mediated via the production of inositol 1,4,5-trisphosphate in the mouse vomeronasal organ. *J. Exp. Biol.* 210:1406–1412.

UCHIDA, N., and MAINEN, Z. F. 2003. Speed and accuracy of olfactory discrimination in the rat. *Nat. Neurosci.* 6:1224–1229.

UCHIDA, N., KEPECS, A., and MAINEN, Z. F. 2006. Seeing at a glance, smelling in a whiff: rapid forms of perceptual decision making. *Nat. Rev. Neurosci.* 7:485–491.

VANDERHAEGHEN, P., SCHURMANS, S., VASSART, G., and PARMENTIER, M. 1993. Olfactory receptors are displayed on dog mature sperm cells. *J. Cell Biol.* 123:1441–1452.

VENDRUSCOLO, L. F., VENDRUSCOLO, J. C., TERENINA-RIGALDIE, E., RABA, F., RAMOS, A., TAKAHASHI, R. N., and MORMEDE, P. 2006. Genetic influences on behavioral and neuroendocrine responses to predator-odor stress in rats. *Neurosci. Lett.* 409:89–94.

VERGASSOLA, M., VILLERMAUX, E., and SHRAIMAN, B. I. 2007. 'Infotaxis' as a strategy for searching without gradients. *Nature* 445:406–409.

VERHAGEN, J. V., WESSON, D. W., NETOFF, T. I., WHITE, J. A., and WACHOWIAK, M. 2007. Sniffing controls an adaptive filter of sensory input to the olfactory bulb. *Nat. Neurosci.* 10:631–639.

VICKERS, N. J. 2000. Mechanisms of animal navigation in odor plumes. *Biol. Bull.* 198:203–212.

VOS, M., VET, L. E. M., WACKERS, F. L., MIDDELBURG, J. J., VAN DER PUTTEN, W. H., MOOIJ, W. M., HEIP, C. H. R., and VAN DONK, E. 2006. Infochemicals structure marine, terrestrial and freshwater food webs: implications for ecological informatics. *Ecol. Inform.* 1:23–32.

VYAS, A., KIM, S. K., GIACOMINI, N., BOOTHROYD, J. C., and SAPOLSKY, R. M. 2007a. Behavioral changes induced by *Toxoplasma* infection of rodents are highly specific to aversion of cat odors. *Proc. Natl. Acad. Sci. USA* 104:6442–6447.

VYAS, A., KIM, S. K., and SAPOLSKY, R. M. 2007b. The effects of toxoplasma infection on rodent behavior are dependent on dose of the stimulus. *Neuroscience* 148:342–348.

WACHOWIAK, M., and SHIPLEY, M. T. 2006. Coding and synaptic processing of sensory information in the glomerular layer of the olfactory bulb. *Semin. Cell Dev. Biol.* 17:411–423.

WAGNER, S., GRESSER, A. L., TORELLO, A. T., and DULAC, C. 2006. A multireceptor genetic approach uncovers an ordered integration of VNO sensory inputs in the accessory olfactory bulb. *Neuron* 50:697–709.

WEILER, E., MCCULLOCH, M. A., and FARBMAN, A. I. 1999. Proliferation in the vomeronasal organ of the rat during postnatal development. *Eur. J. Neurosci.* 11:700–711.

WHITTLE, C. L., FAKHARZADEH, S., EADES, J., and PRETI, G. 2007. Human breath odors and their use in diagnosis. *Ann. NY Acad. Sci.* 1098:252–266.

WILLHITE, D. C., NGUYEN, K. T., MASURKAR, A. V., GREER, C. A., SHEPHERD, G. M., and CHEN, W. R. 2006. Viral tracing identifies

distributed columnar organization in the olfactory bulb. *Proc. Natl. Acad. Sci. USA* 103:12592–12597.

WILLMORE, B., and TOLHURST, D. J. 2001. Characterizing the sparseness of neural codes. *Network* 12:255–270.

WILLSE, A., BELCHER, A. M., PRETI, G., WAHL, J. H., THRESHER, M., YANG, P., YAMAZAKI, K., and BEAUCHAMP, G. K. 2005. Identification of major histocompatibility complex-regulated body odorants by statistical analysis of a comparative gas chromatography/mass spectrometry experiment. *Anal. Chem.* 77:2348–2361.

WILLSE, A., KWAK, J., YAMAZAKI, K., PRETI, G., WAHL, J. H., and BEAUCHAMP, G. K. 2006. Individual odortypes: interaction of MHC and background genes. *Immunogenetics* 58:967–982.

WILSON, R. I., and MAINEN, Z. F. 2006. Early events in olfactory processing. *Annu. Rev. Neurosci.* 29:163–201.

WILSON, D. A., and STEVENSON, R. J. 2006. Learning to Smell. p. 309. Johns Hopkins University Press, Baltimore, MD.

WILSON, D. A., KADOHISA, M., and FLETCHER, M. L. 2006. Cortical contributions to olfaction: plasticity and perception. *Semin. Cell Dev. Biol.* 17:462–470.

WINSTON, J. S., GOTTFRIED, J. A., KILNER, J. M., and DOLAN, R. J. 2005. Integrated neural representations of odor intensity and affective valence in human amygdala. *J. Neurosci.* 25:8903–8907.

WOODLEY, S. K., and BAUM, M. J. 2004. Differential activation of glomeruli in the ferret's main olfactory bulb by anal scent gland odours from males and females: an early step in mate identification. *Eur. J. Neurosci.* 20:1025–1032.

WYART, C., WEBSTER, W. W., CHEN, J. H., WILSON, S. R., MCCLARY, A., KHAN, R. M., and SOBEL, N. 2007. Smelling a single component of male sweat alters levels of cortisol in women. *J. Neurosci.* 27:1261–1265.

WYSOCKI, C. J. 1979. Neurobehavioral evidence for the involvement of the vomeronasal system in mammalian reproduction. *Neurosci. Biobehav. Rev.* 3:301–341.

WYSOCKI, C. J., and PRETI, G. 2004. Facts, fallacies, fears, and frustrations with human pheromones. *Anat. Rec.* 281A:1201–1211.

WYSOCKI, C. J., and PRETI, G. 2008. Pheromones: mammalian pheromones, in Encyclopedia of Neuroscience.

WYSOCKI, C. J., WELLINGTON, J. L., and BEAUCHAMP, G. K. 1980. Access of urinary nonvolatiles to the mammalian vomeronasal organ. *Science* 207:781–783.

WYSOCKI, C. J., YAMAZAKI, K., CURRAN, M., WYSOCKI, L. M., and BEAUCHAMP, G. K. 2004. Mice (*Mus musculus*) lacking a vomeronasal organ can discriminate MHC-determined odortypes. *Horm. Behav.* 46:241–246.

XU, F., GREER, C. A., and SHEPHERD, G. M. 2000. Odor maps in the olfactory bulb. *J. Comp. Neurol.* 422:489–495.

XU, F. Q., SCHAEFER, M., KIDA, I., SCHAFER, J., LIU, N., ROTHMAN, D. L., HYDER, F., RESTREPO, D., and SHEPHERD, G. M. 2005. Simultaneous activation of mouse main and accessory olfactory bulbs by odors or pheromones. *J. Comp. Neurol.* 489:491–500.

YADON, C. A., and WILSON, D. A. 2005. The role of metabotropic glutamate receptors and cortical adaptation in habituation of odor-guided behavior. *Learn. Mem.* 12:601–605.

YAMAZAKI, K., BOYSE, E. A., BARD, J., CURRAN, M., KIM, D., ROSS, S. R., and BEAUCHAMP, G. K. 2002. Presence of mouse mammary tumor virus specifically alters the body odor of mice. *Proc. Natl. Acad. Sci. USA* 99:5612–5615.

YOUNG, J. M., and TRASK, B. J. 2002. The sense of smell: genomics of vertebrate odorant receptors. *Hum. Mol. Genet.* 11:1153–1160.

YOUNG, J. M., FRIEDMAN, C., WILLIAMS, E. M., ROSS, J. A., TONNES-PRIDDY, L., and TRASK, B. J. 2002. Different evolutionary processes shaped the mouse and human olfactory receptor gene families. *Hum. Mol. Genet.* 11:535–546.

YOUNG, K. M., FOGARTY, M., KESSARIS, N., and RICHARDSON, W. D. 2007. Subventricular zone stem cells are heterogeneous with respect to their embryonic origins and neurogenic fates in the adult olfactory bulb. *J. Neurosci.* 27:8286–8296.

YOUNGENTOB, S. L., JOHNSON, B. A., LEON, M., SHEEHE, P. R., and KENT, P. F. 2006. Predicting odorant quality perceptions from multidimensional scaling of olfactory bulb glomerular activity patterns. *Behav. Neurosci.* 120:1337–1345.

ZELANO, C., and SOBEL, N. 2005. Humans as an animal model for systems-level organization of olfaction. *Neuron* 48:431–454.

ZHANG, X., and FIRESTEIN, S. 2002. The olfactory gene superfamily in the mouse. *Nature Neurosci.* 5:124–133.

ZHANG, J. X., SOINI, H. A., BRUCE, K. E., WIESLER, D., WOODLEY, S. K., BAUM, M. J., and NOVOTNY, M. V. 2005. Putative chemosignals of the ferret (*Mustela furo*) associated with individual and gender recognition. *Chem. Senses* 30:727–737.

ZHAO, K., and DALTON, P. 2007. The way the wind blows: implications of modeling nasal airflow. *Curr. Allergy Asthma Rep.* 7:117–125.

ZHAO, K., DALTON, P., YANG, G. C., and SCHERER, P. W. 2006. Numerical modeling of turbulent and laminar airflow and odorant transport during sniffing in the human and rat nose. *Chem. Senses* 31:107–118.

ZOU, Z., and BUCK, L. B. 2006. Combinatorial effects of odorant mixes in olfactory cortex. *Science* 311:1477–1481.

ZOU, Z., LI, F., and BUCK, L. B. 2005. Odor maps in the olfactory cortex. *Proc. Natl Acad. Sci. USA* 102:7724–7729.

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Precise Manipulation Through a Modeling Study

Editorial Preface (Papers by M.E. Puente, G.G. Kennedy, F. Gould, and K. Magori)

Marcel Dicke

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Organisms are characterized by a suite of characteristics that collectively constitute a phenotype. Interactions with the biotic and abiotic environment result in modifications of the phenotype, and consequently in modifications of the interactions of the individual with community members. Chemical ecologists have a keen interest in the information component of phenotypes. They unravel the mechanisms that underlie phenotypic changes in terms of the emission of information-conveying chemicals and the ecological consequences of such phenotypic changes. For instance, feeding by a pierid caterpillar on a brassicaceous plant results in the production of a complex bouquet of volatiles that include fatty-acid-derived compounds, terpenoids, and glucosinolate-derived compounds (Geervliet et al. 1997). The induced volatiles can evoke a range of behavioral responses. They result not only in the attraction of parasitic wasps that attack the caterpillars (Scascighini et al. 2005), but also in the attraction of *Plutella xylostella* moths for whom the *Pieris*-infested plant provides enemy-free space (Shiojiri et al. 2002).

Induced phenotypic changes allow organisms to adjust to local conditions. However, this requires that an individual perceives environmental changes and responds to them at a speed that results in timely phenotypic change. The expression of phenotypic plasticity is usually thought to be an indication of costs related to the expressed characteristics, either in terms of energy or in terms of ecology (Agrawal 2001). The ecology of herbivore-induced plant volatiles (HIPV) has shown a rapid development since the late 1980s (Dicke and Sabelis 1988; Turlings et al. 1990). In the past decade, molecular genetic tools have been embraced by students in this field (Van Poecke and Dicke 2002; Kessler et al. 2004; Halitschke et al. 2008). In doing so, certain characteristics could be eliminated from or enhanced in plants with a chirurgic precision at the level of individual genes. This approach has allowed us to study individuals that had been modified in the expression of individual genes, usually in a digital method where the gene of interest was knocked out or silenced or where the expression of the gene of interest was enhanced. These methods have revolutionized the field of insect–plant ecology (Dicke et al. 2004; Kessler et al. 2004). They allow the investigation of the effects of individual characteristics on species interactions as well as community dynamics.

Apart from molecular genetical tools, mathematical modeling is another powerful tool that allows researchers to investigate the effects of individual characteristics on species interactions. If sufficient knowledge about a system has been gathered to develop an adequate model, the model parameters may be modified to assess their effects on species interactions. Within the field of chemical ecology, modeling has not been a tool that has been exploited frequently (but see e.g., Vos et al. 2001; Kobayashi et al. 2006). Analytical tools have mainly dominated the field. The following two papers by Puente and colleagues have taken a detailed modeling approach to the study of herbivore-induced parasitoid attraction. They especially address the temporal and spatial aspects of plant–parasitoid interactions through HIPV. Timing of HIPV emission relative to herbivore presence is likely to be important, as it determines the reliability of the cues. The induction dynamics of volatile emission vary with the type of volatiles and their biosynthesis. However, after herbivores have been eliminated, continuation of the emission is no

M. Dicke (✉)
Laboratory of Entomology, Wageningen University,
P.O. Box, 8031, 6700 EH Wageningen, The Netherlands
e-mail: marcel.dicke@wur.nl

longer needed for the plant, and if the emission continues, this is likely to devaluate the volatiles as a signal to carnivorous arthropods. In the research field of HIPV, more attention has been paid to the induction of volatiles (Scascighini et al. 2005; Maffei et al. 2007) than to the relaxation of their production and emission (Fatouros et al. 2005). To investigate the effects of genetic variation in parasitoids, fewer tools are available at present. Although this may change with the rapid development of molecular genetic tools for parasitoids, at present a modeling approach is one of the few options.

In their papers, Puente and colleagues take a behavioral ecological approach to the role of HIPV in plant–parasitoid interactions. In doing so they modify characteristics of the emission dynamics as well as the response characteristics of parasitoids. This results in an interesting approach that yields new insight into the role of HIPV in the foraging success of parasitoids and in the success with which plants improve the effectiveness of herbivore attack by parasitoids. In this manner, they can modify the dynamics of HIPV signaling in ways that molecular geneticists can only dream of. For instance, Puente et al. (2008a, b) can investigate the effect of the time interval between herbivore attack and HIPV emission by comparing five plant types that differ in the induction delay in a linear manner. Their approach results in new hypotheses that can be investigated through an experimental approach. This approach is a valuable, yet not often employed, tool to chemical ecologists that complements analytical, molecular genetic, and behavioral approaches.

References

AGRAWAL, A. A. 2001. Phenotypic plasticity in the interactions and evolution of species. *Science* 294:321–326.

DICKE, M. and SABELIS, M. W. 1988. How plants obtain predatory mites as bodyguards. *Neth J. Zool.* 38:148–165.

DICKE, M., VAN LOON, J. J. A., and DE JONG, P. W. 2004. Ecogenomics benefits community ecology. *Science* 305:618–619.

FATOUROS, N. E., VAN LOON, J. J. A., HORDIJK, K. A., SMID, H. M., and DICKE, M. 2005. Herbivore-induced plant volatiles mediate in-flight host discrimination by parasitoids. *J. Chem. Ecol.* 31:2033–2047.

GEERVLIET, J. B. F., POSTHUMUS, M. A., VET, L. E. M., and DICKE, M. 1997. Comparative analysis of headspace volatiles from different caterpillar-infested and uninfested food plants of Pieris species. *J. Chem. Ecol.* 23:2935–2954.

HALITSCHKE, R., STENBERG, J. A., KESSLER, D., KESSLER, A., and BALDWIN, I. T. 2008. Shared signals—'alarm calls' from plants increase apparency to herbivores and their enemies in nature. *Ecol. Lett.* 11:24–34.

KESSLER A., HALITSCHKE R., and BALDWIN I. T. 2004. Silencing the jasmonate cascade: induced plant defenses and insect populations. *Science* 305:665–668.

KOBAYASHI Y., YAMAMURA N., and SABELIS M. W. 2006. Evolution of talking plants in a tritrophic context: Conditions for uninfested plants to attract predators prior to herbivore attack. *J. Theor. Biol.* 243:361–374.

MAFFEI M. E., MITHOFER A., and BOLAND W. 2007. Before gene expression: early events in plant–insect interaction. *Trends Plant Sci.* 12:310–316.

PUENTE M., KENNEDY G. G., and GOULD F. 2008a. The impact of herbivore-induced plant volatiles on parasitoid foraging success: a general deterministic model. *J. Chem. Ecol.* 34, DOI 10.1007/s10886-008-9471-x

PUENTE M., MAGORI K., KENNEDY G. G., and GOULD F. 2008b. Impact of herbivore-induced plant volatiles on parasitoid foraging success: a spatial simulation of the *Cotesia rubecula*, *Pieris rapae*, and *Brassica oleracea* system. *J. Chem. Ecol.* 34, DOI 10.1007/s10886-008-9472-9

SCASCIGHINI N., MATTIACCI L., D'ALESSANDRO M., HERN A., ROTT A. S., and DORN S. 2005. New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction. *Chemoecology* 15:97–104.

SHIOJIRI K., TAKABAYASHI J., YANO S., and TAKAFUJI A. 2002. Oviposition preferences of herbivores are affected by tritrophic interaction webs. *Ecol Lett.* 5:186–192.

TURLINGS T. C. J., TUMLINSON J. H., and LEWIS W. J. 1990. Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. *Science* 250:1251–1253.

VAN POECKE R. M. P. and DICKE M. 2002. Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway. *J. Exp. Bot.* 53:1793–1799.

VOS M., MORENO BERROCAL S., Karamaouna F., HEMERIK L. and VET L. E. M. 2001. Plant-mediated indirect effects and the persistence of parasitoid-herbivore communities. *Ecol. Lett.* 4:38–45.

J Chem Ecol (2008) 34:945–958
DOI 10.1007/s10886-008-9471-x

The Impact of Herbivore-Induced Plant Volatiles on Parasitoid Foraging Success: A General Deterministic Model

Molly E. Puente · George G. Kennedy · Fred Gould

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Abstract Parasitoids respond to volatiles that plants produce when injured by herbivores. A considerable body of literature addresses the chemical pathways of herbivore-induced volatile production. However, there is almost no theory or data on how timing of volatile release in relationship to host availability for parasitization impacts the utility of these cues to parasitoids and on the extent that this volatile release timing might increase or decrease the percent of herbivores that become parasitized. This kind of information is critical in judging the benefits that might accrue from a breeding program aimed at enhancing herbivore-responsive volatile production. We developed a general model to begin examining this issue by using available parameters from two tritrophic systems. The model uses herbivore oviposition, development, and mortality rates, linked to a range of plant volatile induction and cessation periods for calculating the proportion of plants in a field that are (1) not producing volatiles but occupied by suitable herbivore hosts, (2) producing volatiles and occupied by suitable herbivore hosts, (3) producing volatiles but not occupied by suitable herbivore hosts, and (4) not producing volatiles and not occupied by suitable herbivore hosts. The impact of the plant volatiles on parasitoid foraging success is then determined by comparing the expected number of hosts parasitized when the parasitoid focuses solely on the volatile-producing plants to when it forages randomly among all plants. Under some conditions, parasitoids can attack three times more herbivores if they focus on volatile-producing plants. However, when we simulate plants that take several days to cease volatile production after pupation or death of the herbivore, parasitization rate does not increase when parasitoids use volatiles as cues. The utility of the volatile cues is consistently greater when a smaller proportion of plants is occupied by herbivores, indicating that their usefulness may be reduced to zero in fields saturated with volatiles.

Keywords Herbivore-induced plant volatiles · Tritrophic interactions · Parasitoid behavior · Deterministic model · Signal utility · *Heliothis virescens* · *Pieris rapae*

M. E. Puente · G. G. Kennedy · F. Gould (✉)
Department of Entomology, North Carolina State University, Campus Box 7634, Raleigh, NC 27695, USA
e-mail: fred_gould@ncsu.edu

Introduction

Plant volatiles induced by herbivory have been documented in numerous systems (Karban and Baldwin 1997). There are many examples of parasitoids orienting to herbivore-induced plant volatiles as a means of finding hosts, both in the field (e.g., DeMoraes et al. 1998; Oppenheim and Gould 2002) and in wind tunnels (e.g., Kaiser and Carde 1991; DeMoraes and Lewis 1999). One hypothesis about the evolution of this plant/parasitoid interaction suggests that plants evolve volatile production because those genotypes that produce herbivore-induced volatiles are more successful in attracting predators/parasitoids and therefore have higher fitness (Janssen et al. 2002). In contrast, a simpler hypothesis holds that plants produce herbivore-induced volatiles as a by-product of plant biochemistry and that parasitoids happen to have receptors that enable detection of these volatiles (Agrawal and Karban 1999). The value of attraction is hypothesized to be determined by the relative reliability and detectability of the plant signal (Vet et al. 1991). While host cues are under

selection to be as undetectable as possible, plant cues are not under such selection. Parasitoids that are attracted to these cues are rewarded when these cues are a reliable indicator of presence of a suitable host.

However, even though plant cues are not directly selected against, they may not always provide a reliable signal to parasitoids. One potential source of misleading signals occurs when plants produce similar volatiles in response to a number of herbivore species, only some of which are hosts of a specific parasitoid (Rose et al. 1998; van Poecke et al. 2003). Another occurs when parasitoids respond to plants when the inducing herbivore is either too old to be attacked (Mattiacci and Dicke 1995; Brodeur and Vet 1995) or has left the plant (Mattiacci et al. 2001). The aim of this paper is to examine the extent to which variation in timing of volatile emission impacts utility of these cues for parasitoids.

In addition to natural sources of temporally misleading signals, crop breeding for over-expression of volatiles may produce situations where parasitoids gain less from response to the volatile. There has been considerable interest in breeding plants to produce increased quantity and quality of volatiles (Bottrell et al. 1998; Degenhardt et al. 2003; Lou et al. 2006). It is also possible that properly timed, increased volatile quantity could improve the efficiency of biological control agents by allowing parasitoids to target their foraging only to damaged plants with little cost to the plants. Initial investigations show the metabolic costs to the plant may not be significant enough to rule out breeding for constitutive volatile production. Several papers that propose this strategy mention that plant signals should be synchronized with herbivore presence to avoid a "calling wolf" scenario (Bottrell et al. 1998; Degenhardt et al. 2003); however, none specifies precisely how plant signals need to be synchronized. While a number of studies examine the chemical pathways plants use to produce signals (e.g., Kessler and Baldwin 2002), there are no published reports that we could find elucidating the regulation of specific genes that turn off volatile production when herbivory ceases.

As demonstrated by Holling (1959), predator and parasitoid foraging success is limited by three parameters: handling time, total available foraging time, and host encounter rate. Predators and parasitoids can take advantage of many visual, olfactory, and auditory cues in their environment to optimize host encounter rate. If herbivore-induced volatiles from plants can be used by the parasitoid to optimize these three parameters, then response to the volatiles can increase parasitoid foraging success. On the other hand, if parasitoid response to the volatiles increases handling time, decreases the available foraging time, or decreases the host encounter rate, response to induced volatiles could become less valuable.

The number of systems in which parasitoids are attracted to herbivore-induced plant volatile signals indicates that there must be some advantage to this attraction behavior; however, the few studies that quantify the fitness advantage parasitoids gain by following volatiles tend to focus on patch abandonment and modulating giving-up time (Vos and Hemerik 2003) rather than parasitoid attraction to individual plants in a larger environmental context. One purpose of this paper is to use modeling to examine the extent to which both the herbivore's life history and the temporal pattern of the plant's response to herbivory determine the benefit to the parasitoid of responding to herbivore-induced plant volatiles. Modeling has been instrumental in identifying other key aspects of parasitoid–host interactions, such as the value of refuges and asynchrony in parasitoid–host population dynamics (Takagi 1999).

The structure of the model is based on an extension of the Holling predation equation (Holling 1959). Life history and behavioral traits of two specific tritrophic systems that are potential candidates for genetic modification of volatile signal production were used in setting default parameters in our model. The first consists of tobacco, *Heliothis virescens*, and *Cardiochiles nigriceps*. De Moraes et al. (2001) found that female moths avoided ovipositing on plants emitting herbivore-induced volatiles, so for this system, we examined the consequences for parasitoid wasps if moths limit their oviposition to uninfested plants. The second system consists of *Brassica oleracea*, *Pieris* spp., and *Cotesia glomerata*. Females of the parasitoid *C. glomerata* are attracted to plant volatiles induced by all instars of *P. brassicae* (Mattiacci and Dicke 1995); however, parasitoid larvae suffer greater mortality due to increased encapsulation if laid in third or later host instar caterpillars. Geographically isolated populations of *C. glomerata* vary in their foraging behavior and host range (Vos and Vet 2004). Given the general nature of the model developed here, we did not address intraspecific variation, and we used data from congeneric species when needed. We used our model to examine the consequences of narrowing the window of available host instars that a parasitoid could attack. While parameter values from these two tritrophic systems were used as starting points, we also explored a wider set of parameter values to gain a broader understanding of the impacts of signal–host synchronization.

Overall, we expected that signals more closely synchronized with host presence would be more relevant to foraging parasitoids, so that plants in a field that could respond to herbivory in 1 day would produce more relevant signals than plants that took 5 days. However, before analysis with this model, we did not know whether the cost of delaying a signal from 1 to 5 days would be substantial or hardly noticeable. Similarly, while we expected herbivore density to impact the relevance of the volatiles to

parasitoids, we did not know whether this would be a major or a minor effect. Our use of general characteristics of two systems, with varying herbivore life histories, allowed us to identify what variables were more sensitive to initial conditions when predicting the relevance of volatiles to parasitoids. A companion paper describes a more detailed stochastic and spatial model of the interactions between *Cotesia rubecula* and *P. rapae* (Puente et al. 2008, following paper).

Methods and Materials

Incorporating Plant Volatiles into Holling's Equation The fitness of a solitary parasitoid can be correlated directly with the number of hosts successfully attacked, as each host can lead to one offspring. Therefore, the relevance of herbivore-induced plant signals can be defined as a ratio of the number of hosts attacked if a parasitoid follows signals, compared to if it randomly forages with respect to plant signals.

$$\mathrm{Rel} = \frac{N_{\mathrm{ASig}}}{N_{\mathrm{ARan}}} \tag{1}$$

where *Rel* is the signal relevance to the parasitoid, N_{ASig} is the number of hosts attacked by parasitoids that focus only on plants that are producing herbivore-induced volatile signals, and N_{ARan} is the number of hosts attacked by randomly searching parasitoids. If *Rel* is equal to 1, either foraging method yields the same fitness; if $Rel>1$, then a parasitoid is more efficient by responding to plant signals, and if $Rel<1$, then the parasitoid is more efficient when it ignores plant signals.

The predation equation developed by Holling (1959) provides a way of predicting the number of prey (or hosts in this case) attacked, given the predator's (or parasitoid's) behavior and the host density.

$$N_{\mathrm{A}} = \frac{T_{\mathrm{t}} \times a \times x}{1 + a \times b \times x} \tag{2}$$

where T_{t} is the total time available for foraging, a is the "instantaneous rate of discovery," b is the handling time for a single oviposition, and x is the density of hosts. If we substitute this into the previous equation, we get

$$Rel = \left(\frac{T_t \times a_{Sig} \times x_{Sig}}{1 + a_{Sig} \times b_{Sig} \times x_{Sig}}\right) \Big/ \left(\frac{T_t \times a_{Ran} \times x_{Ran}}{1 + a_{Ran} \times b_{Ran} \times x_{Ran}}\right) \tag{3}$$

The definitions of the variables in the Holling equation and the units of measure are system-specific. In our model, we can assume that the induction phenomenon occurs systemically throughout a plant (e.g., Mattiacci et al. 2001) and that it is constrained to a single plant. The density of hosts, x, in the Holling model is traditionally provided in hosts per square meter, but because induction is occurring at the level of plants, not meters, we assume one plant per square meter and thus give our density measurements in "hosts/plant."

The total time available T_{t} is the amount of time the parasitoid remains foraging in the relevant environment. We assume that T_{t} is for a single day and that the parasitoid forages only in the field of interest for that day, thus giving us a daily attack rate. To keep all time units equal, T_{t} is measured in seconds.

The instantaneous rate of discovery a, also known as the area of discovery, is traditionally given in the units of area per unit of time (the lower case "a" should not be confused with upper case "A" in N_{A}, which is the total attack rate). We are interested in how many plants the parasitoid can visit rather than the area that can be covered; therefore, we make a few assumptions about the area of discovery. We assume that the parasitoid forages by visiting the nearest neighboring plant or, in the case of following volatile signals, the nearest neighboring signaling plant. To calculate the area of discovery, a, for randomly foraging parasitoids, we take the parasitoid flight speed (in meters per second) and multiply by the density of plants (plants per meter). Multiplying T_{t} by a gives the maximum number of plants a parasitoid can visit during the total foraging time. Multiplying that result by x gives the maximum number of hosts the parasitoid could possibly encounter if no time were required to find and handle the host once the parasitoid arrived at a plant.

Because a fraction of that total time is spent in handling hosts, the actual number of the hosts attacked is less than the maximal number of plants that the parasitoid could encounter. The more plants encountered, the more time that must be invested in both searching for and handling hosts. The handling time, b, is the amount of time the parasitoid spends from the time it encounters a host to the time it stops handling the host. Because this model follows just a single host per plant, the parasitoid is assumed to leave the plant immediately after handling the host. However, in cases where multiple larvae could be encountered on a single plant, b, the term we add to describe on-plant searching, could be modified with functions to describe continued on-plant searching behavior. The total time spent in handling hosts is $a \times b \times x$. The actual number of plants a parasitoid can visit is then $a \times \mathrm{x} \times$the time available for searching, which is only a fraction of the total time available for foraging. This fraction can be calculated as $T_{\mathrm{t}}/(1\, a \times b \times x)$.

However, this assumes that handling time is fixed for each encounter. While the time it takes for a parasitoid to sting a found host may be equal regardless of the plant's state, the amount of handling time a parasitoid searches a plant before finding (or giving up on) a host may depend on

how the parasitoid perceives the plant state. For example, when encountering herbivore-induced plant volatile cues, some parasitoids search plants longer before leaving (Nealis 1990; Horikoshi et al. 1997; Sato and Ohsaki 2004). By prolonging a parasitoid's giving-up time, herbivore-induced plant volatiles can impact the time budget for foraging parasitoids. To account for a difference in giving-up time, as part of the overall handling time, we modified the Holling equation as follows:

$$N_A = (T_t \times a \times x)/(1 + a \times b \times x + a \times c \times E + a \times c/2 \times O) \quad (4)$$

Where c is the giving-up time per plant, E is the proportion of plants that are empty of hosts, and O is the proportion of plants in the field that are occupied by a host. The total time a parasitoid must devote to searching if no herbivore is present is $a \times c \times E$, which is the probability of a parasitoid landing on an empty plant ($a \times E$) multiplied by the rate of giving-up time per arresting plant. If an herbivore is present (O), we assume that the searching time ceases as soon as the host is found. If we assume that the probability that the host is found per unit time is the same from the instant that a parasitoid lands up to the moment the search time expires, then the average time spent will be half the giving-up time. Therefore, the time spent searching occupied plants is $a \times c/2 \times O$.

Given these definitions of the variables, we can ask how a parasitoid's decision to follow or ignore induced plant volatiles can change the values of these variables. There have been many hypothesized mechanisms through which herbivore-induced plant volatiles can influence parasitoid foraging. For example, parasitoids may change their turning radius (Kareiva and Odell 1987), alter their flight speed (Norlund 1981), or change their total time budget to spend more time feeding per day (Siekmann et al. 2004). While our model is flexible enough to examine these hypotheses, in this paper, we will explore only the impact of parasitoids bypassing non-signaling plants on signal relevance; for further exploration of the model, see Puente (2007).

By identifying the preferred host–plant complex out of a mixed background, parasitoids can bypass uninformative plants. When the parasitoid bypasses non-signaling plants, we assume it maintains the same flight speed and handling time but restricts its environment to signaling plants only. In natural settings, a parasitoid may also pick up on other cues (such as herbivore frass) that may induce it to investigate non-signaling plants but that is beyond the scope of this model. The parasitoid may, therefore, encounter a different density of hosts if it preferentially forages on signaling plants rather than foraging randomly. The impact this has on the signal relevance depends on the underlying host density and the plant signal reliability.

At any point in time, a plant could be in one of four possible qualitative states. Individual plants are either emitting volatiles that could act as signals (S) or not emitting (N) and are either occupied (O) by a suitable herbivore host or empty (E) of a suitable host; thus, a plant's state can be NO, NE, SO, or SE. While a single plant does not retain its state for an entire season, a field may reach an equilibrium distribution of plants in those four categories.

A parasitoid responding to plant signals perceives the host density as the number of SO plants (n_{SO}) divided by the sum of all signaling plants ($n_{SO}\, n_{SE}$), while a parasitoid randomly foraging perceives the host density as the sum of all occupied plants ($n_{SO}\, n_{NO}$) divided by the total number of plants in the field. Mathematically,

$$x_{Sig} = n_{SO}/(n_{SO} + n_{SE}) \quad (5a)$$

$$x_{Ran} = (n_{SO} + n_{NO})/(n_{SO} + n_{NO} + n_{SE} + n_{NE}) \quad (5b)$$

All other variables being held constant, if ($x_{Sig} > x_{Ran}$), signal relevance will be greater than one, and if ($x_{Sig} < x_{Ran}$), signal relevance will be less than one. However, because density is not linearly correlated with signal relevance (x appears in both the numerator and the denominator), the magnitude of this advantage in terms of number of hosts attacked depends on the actual values of n_{SO}, n_{NO}, n_{SE}, and n_{NE}.

Additionally, these four states impact the values of E and O in Eq. 4, so if we include arrestment in our equations, we can make the following substitutions:

$$E_{Sig} = n_{SE}/(n_{SO} + n_{SE}) \quad (6a)$$

$$E_{Ran} = (n_{SE} + n_{NE})/(n_{SO} + n_{NO} + n_{SE} + n_{NE}) \quad (6b)$$

Additionally, we can substitute Eq. 5a and 5b for O in both the signaling and randomly foraging equations.

When we have finished making these substitutions, we have the following equations:

$$N_{ASig} = \frac{T_t \times a_{Sig} \times \left(\frac{n_{SO}}{n_{SO}+n_{SE}}\right)}{1 + a_{Sig} \times b_{Sig} \times \left(\frac{n_{SO}}{n_{SO}+n_{SE}}\right) + a_{Sig} \times c_{Sig} \times \left(\frac{n_{SE}}{n_{SO}+n_{SE}}\right) + a_{Sig} \times \frac{c_{Sig}}{2} \times \left(\frac{n_{SO}}{n_{SO}+n_{SE}}\right)} \quad (7)$$

$$N_{\mathrm{ARan}} = \frac{T_t \times a_{\mathrm{Ran}} \times \left(\frac{n_{\mathrm{SO}}+n_{\mathrm{NO}}}{n_{\mathrm{SO}}+n_{\mathrm{SE}}+n_{\mathrm{NO}}+n_{\mathrm{NE}}}\right)}{1 + a_{\mathrm{Ran}} * b_{\mathrm{Ran}} * \left(\frac{n_{\mathrm{SO}}+n_{\mathrm{NO}}}{n_{\mathrm{SO}}+n_{\mathrm{SE}}+n_{\mathrm{NO}}+n_{\mathrm{NE}}}\right) + a_{\mathrm{Ran}} \times c_{\mathrm{Ran}} \times \left(\frac{n_{\mathrm{SE}}+n_{\mathrm{NE}}}{n_{\mathrm{SO}}+n_{\mathrm{SE}}+n_{\mathrm{NO}}+n_{\mathrm{NE}}}\right) + a_{\mathrm{Ran}} \times \frac{c_{\mathrm{Ran}}}{2} \times \left(\frac{n_{\mathrm{SO}}+n_{\mathrm{NO}}}{n_{\mathrm{SO}}+n_{\mathrm{SE}}+n_{\mathrm{NO}}+n_{\mathrm{NE}}}\right)} \quad (8)$$

Finally, when parasitoids bypass non-signaling plants, we assume that the parasitoid's flight speed does not change, but the distance between perceived nearest neighbors increases. The area of discovery for parasitoids following signals must be adjusted to account for the increase in distance:

$$a_{\mathrm{Sig}} = a_{\mathrm{Ran}} \times \sqrt{\frac{n_{\mathrm{SO}} + n_{\mathrm{SE}}}{n_{\mathrm{SO}} + n_{\mathrm{SE}} + n_{\mathrm{NO}} + n_{\mathrm{NE}}}} \quad (9)$$

If we assume that our field is square, then the number of plants in each row is the square root of the total number of plants in the field (the sum of the number of plants in the four states). Likewise, if we assume the signaling plants are distributed equally between the rows, then the number of signaling plants per row is the square root of the total number of signaling plants (the sum of the number of plants in SO and SE states). If we assume that the parasitoid forages along a straight path, then the density of signaling plants encountered is the square root of the density of signaling plants in the field.

To understand what distribution of plant states may occur in natural populations, we use an age–class transition model for two herbivore examples. Figure 1 demonstrates transition of plants between the different states.

The model begins with all of the plants neither occupied nor induced (NE) and runs for 100 time steps. Each time step represents a single day. A set fraction of plants ranging from 0.1 to 0.9 are newly "occupied" by herbivores at each time step. For a lepidopteran herbivore, the occupation rate is equivalent to the rate of eggs hatching in a single day, which is equivalent to a daily oviposition rate, assuming there is no significant egg mortality. Occupation rate is expected to correlate with the proportion of plants infested. As plants become occupied, they move either from NE to NO or from SE to SO.

A plant remains occupied as long as a suitable larval host is feeding. Pupation, mortality, and dispersal are all potential ways for plants to be abandoned by larvae, and the probability of these events depends on the age of the larva. The maximum number of days a plant can remain infested by a single larva and thus the maximum number of infestation age classes is the development time for the herbivore larva. When larvae pupate, the plants on which they resided are moved into the SE class unless a plant is simultaneously occupied by a younger larva. When a parasitoid cannot attack an herbivore because it has matured beyond its vulnerable instars, the plant is considered "empty," similar to what happens when the herbivore pupates; however, because the herbivore continues to feed, the plant continues to produce volatile signals. Because these unsuitable hosts are classified as empty, a parasitoid encountering these hosts spends no handling time (b) in the current model, but it would be appropriate to add handling time of unsuitable hosts in specific systems.

Because herbivore mortality rate depends on larval age, the model includes different infestation age classes for each day of larval occupancy. At each time step, each age class is multiplied by the appropriate mortality rate, which accounts for density-independent mortality sources for that larval age class such as weather and diffuse predation. The fraction of plants with dying larvae are moved into the SE state if they were on a signaling plant and moved into the NE plant state for non-signaling plants, while the remaining plants are advanced to the next infestation age class. For the systems

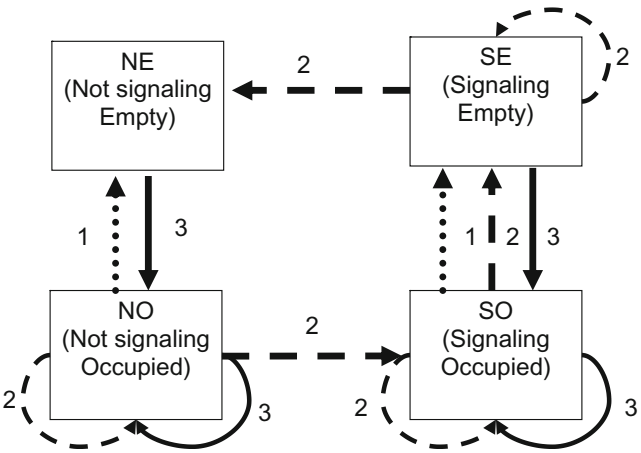

Fig. 1 Flow diagram describing transitions between the four plant states, with each plant state represented by a box. Age classes of herbivores are modeled within each of the occupied (O) states, and relaxation rate-time classes are modeled within the signaling empty (SE) state (not shown). Each time step consists of the following three sequential operations: (1) Occupied plants are multiplied by the mortality rate, and the fraction of plants with dying larvae move to one of the two empty states (dotted line). (2) Larvae on occupied plants are matured by one time step (*dashed line*). Plants with larvae that become older than the induction time move from the not signaling occupied (NO) state to signaling occupied (SO) state, and plants with larvae that become pupae move from SO to the signaling empty (SE) state. Plants in SE long enough to turn off the signal moved from SE to the not signaling empty (NE) state. (3) All age classes and states are multiplied by the occupation rate (*solid line*) and the fraction of plants receiving new larvae have the larval age class set to 1

explored here, dispersal of larvae to other plants was not a major factor, so dispersal was left out, but when expanded to other systems, herbivore migration would both cause occupied plants to become empty and empty plants to become occupied.

When a plant is reinfested, it is classified by its youngest larva, i.e., placed in the first infestation age class, but remains in the "signaling" state if it was previously signaling. This allows for plants to remain signaling for longer than the time of a single larva's development when multiple larvae reside on a plant. However, this introduces a potential bias to the model. In the situation in which the youngest larva dies before the others, the plant would be moved into the "empty" category before the plant was in fact abandoned. This bias is unavoidable because the model cannot follow the fate of individual larvae on each plant.

Although volatile production is probably a continuous function in real plants, we modeled it as a discrete binary function. This means that we assumed that the parasitoids have a perception threshold for volatile concentrations; a plant was "signaling" if it was producing enough volatiles to be perceived by the parasitoid and "not signaling" if the concentration was below the parasitoid's perception threshold. In addition to describing induction as an "on–off" function, we assumed that the concentration of volatiles only changed at the beginning of a daily time step. The induction delay at which plants move from N to S after the onset of herbivory is set at values ranging from 1–5 d for this model. The relaxation delay at which plants move from S to N after the cessation of herbivory is also fixed at values ranging from 1–5 d.

This model can allow us to generate the proportion of plants in each state over time, and we can take these distributions and substitute them into Eqs. 7 and 8. Because we are primarily concerned with the impact of herbivore population density on signal relevance, we hold a, b, and T_t constant, using parameters from the literature for the two-system examples we provide. For more information on the assumptions made in this model, see Puente (2007).

Model Parameters We used life history data on *H. virescens* and *P. rapae* for setting parameter values in the model. *H. virescens* is a generalist in the taxon Noctuidae and attacks many important crop plants including cotton and tobacco (Neunzig 1969). It has many well-known parasitoids and predators, both specialists and generalists. *C. nigriceps*, a parasitoid that attacks all stages of *H. virescens* larvae (Lewis and Vinson 1971), is preferentially attracted to *H. virescens* on certain host plants and can distinguish *H. virescens* infestations from infestations by closely related *Helicoverpa zea* (DeMoraes et al. 1998). In field studies, *C. nigriceps* preferred hosts on tobacco rather than on cotton, regardless of the dominant host plant available (DeMoraes and Lewis 1999; Tillman and Mullinix 2003). While difference in volatile production may explain how parasitoids can distinguish between cotton and tobacco, it does not explain why *C. nigriceps* prefers tobacco. Understanding the temporal dynamics of induction may help us understand host preferences.

Daily mortality rates for *H. virescens* were calculated for each larval stage by using data from Johnson and Gould (1992). Because *H. virescens* varies widely in its survival rates, a low and a high mortality scenario are examined (see Table 2). *H. virescens* adults avoid ovipositing on plants that are already occupied by larvae or eggs (DeMoraes et al. 2001). Under the special case that adult herbivores avoid ovipositing on already-infested plants ("limited oviposition" condition), only plants in the NE state are multiplied by the occupation rate. When this assumption is lifted to allow the default multiple ovipositions on a plant ("multiple oviposition" condition), all plant age classes are multiplied by the occupation rate. Both oviposition scenarios are considered for this herbivore.

Parameter values for *C. nigriceps* were obtained from Tillman and Mullinix (2003) and are summarized on Table 1. The parasitoids are typically active between 0900 and 1500 hours, and spend about half of that time engaged in host foraging behaviors, which translates to a T_t of 3 hr or 10,800 sec. On the host plant tobacco, parasitoids spend 11.6 sec hovering and 11.7 sec searching around a plant for a total of 23.3 sec searching per plant, giving an estimate of

Table 1 Variables in the model

Parameter	*Heliothis virescens*	*Pieris rapae*
	Cardiochiles nigriceps	*Cotesia glomerata*
Tt (sec)	10,800[a]	14,400[b]
a (plant/sec)	0.043[a]	0.33[c]
b (sec/hosts)	20.5[a]	13.1[d]
c (sec/plant)	128.7[a]	73.5[d]
Occupation rate (new larvae/total plants/day)	0.1–0.9	0.1–0.9
Induction delay (days)	1–5	1–5
Relaxation delay (days)	1–5	1–5
Oviposition[e]	Limited and multiple	Multiple
Host attack stage (instar)	5	1–5

[a] Tillman and Mullinix 2003
[b] Kaiser and Cardé 1991
[c] Kaiser et al. 1994
[d] Sato and Ohsaki 2004
[e] 'Oviposition' refers to whether the host limits oviposition to plants that are neither occupied nor signaling (Limited) or will place multiple larvae on one plant (Multiple).

0.043 plants per second for *a*. The handling time for oviposition and preening (*b*) was 20.5 sec per host. Finally, the time spent in what Tillman and Mullinix (2003) refer to as "agony search," a measure of the giving-up-time estimate for *c*, was estimated to be 128.7 sec for wasps on tobacco.

The *P. rapae* tritrophic system has been extensively studied both because it is tractable and because it has economic relevance for many crops. *P. rapae* is a butterfly in the taxon Pieridae that specializes on brassicacean plants, including crops such as cabbage, broccoli, and Brussels sprouts, and the experimental model system, *Arabidopsis thaliana* (Courtney 1986). A major larval parasitoid for *P. rapae* in some geographic areas (Vos and Vet 2004), *Cotesia glomerata*, is restricted to surviving in only the early instars of *P. rapae* but cannot distinguish the age of the larvae based on plant volatiles alone (Mattiacci and Dicke 1995). The temporal pattern of herbivore-induced plant volatile production has been documented only for a few systems, including *P. rapae*. Geervliet et al. (1998) found that Brussels sprouts were most attractive to braconid parasitoids after 3 d of feeding by *P. rapae*. Additionally, a wind tunnel study found that Brussels sprouts fed on by *P. brassicae*, a close relative to *P. rapae*, were most attractive to *C. glomerata* 3 d after feeding and ceased being attractive to the parasitoids 1 d after the herbivores were removed (Mattiacci et al. 2001).

P. rapae larvae go through five instars of approximately 3 d each. Daily mortality rates were taken from Dempster (1967; see Table 2). Because *C. glomerata* survives only on the first two instars, in this model, encountering a plant with a fifth instar larva would have the same effect as encountering an empty plant because no host could be attacked. To simulate this system, the mechanisms for classifying signaling from non-signaling states as described previously were maintained, but the definitions of empty and occupied were reassessed to include the parasitoid's age preference. The term "attack preference" refers to the maximum age of larva a parasitoid is able to attack successfully. We examined the impact that attack preference has on signal relevance by setting the attack preference at 3 d (first instar), 6 d (second instar), 9 d (third instar), 12 d (fourth instar), and 15 d (fifth instar). It is possible that parasitoids encountering a host that is beyond the attack preference may spend more or less time investigating before leaving the plant (and thus "c" would not be the same as it would for a truly empty plant), but we chose not to consider those special conditions in this initial model.

To calculate signal relevance for this system, we can use parameter estimates derived from the literature (see Table 1). The parasitoids are most active in the late morning and early afternoon (Kaiser and Carde 1991), so we assumed a maximum of 4 hr of foraging per day; the total time (T_t) is 14,400 sec. Sato and Ohsaki (2004) observed that, for *C. glomerata* searching for *Pieris* larvae, the time spent searching one leaf (*c*) was 73.5±11.9 sec, and the handling time (*b*) was 13.1±3.9 sec. We can use the mean of these observations as our parameter estimates. The recorded flight speed for *C. rubecula*, a closely related species that also parasitizes *P. rapae*, was 0.33 m/sec (Kaiser et al. 1994), so we can use this to estimate the parameter *a*; area of discovery as 0.33 plants per second.

Table 2 Daily mortality of larvae as a proportion of the individuals at a specific age within a larval instar

Heliothis virescens[a]				*Pieris rapae*[b]	
Day	Instar	Low mortality	High mortality	Instar	Mortality
1	1	0.12	0.33	1	0.187
2	1	0.12	0.33	1	0.187
3	1	0.12	0.33	1	0.187
4	1	0.12	0.33	2	0.087
5	2	0.03	0.26	2	0.087
6	2	0.03	0.26	2	0.087
7	2	0.03	0.26	3	0.084
8	3	0.03	0.21	3	0.084
9	3	0.03	0.21	3	0.084
10	3	0.03	0.21	4	0.137
11	4	0	0.03	4	0.137
12	4	0	0.03	4	0.137
13	4	0	0.03	5	0.233
14	5	0	0	5	0.233
15	5	0	0	5	0.233
16	5	0	0	0	0
Cumulative		0.51	0.96		0.91

[a] Johnson and Gould 1992
[b] Dempster 1967

Results

Fixed Parameters Total foraging time, handling time, area of discovery for random foraging, and giving-up time were held constant for all simulations investigated for a particular herbivore system. Handling time and giving up time were shorter for the *Pieris* system, while area of discovery and total foraging time were shorter for the *Heliothis* system. This leads us to predict that for one foraging day, *Cotesia glomerata* would be capable of attacking more *P. rapae* than *C. nigriceps* is capable of attacking *H. virescens*. In fact, if we set all other parameters (induction delay, relaxation delay, host attack preference, occupation rate, high mortality) equal, then the N_A for *Pieris* is between two and four times as large as N_A for *Heliothis*. However, when all other parameters are equal, there is not a considerable difference in signal relevance between the *Pieris* and *Heliothis* systems, except at the lowest occupation rates when, for example, if plant signal delays are both held at 1 day, a parasitoid of *H. virescens* could see a threefold improvement by following plant cues, compared to only a 2.5-fold improvement for parasitoids of *P. rapae*. This means that even though more *P. rapae* larvae can be attacked per day, parasitoids for both herbivores have the same threshold for when they should not follow cues.

Occupation Rate As occupation rate increases, the proportion of plants occupied increases but approaches an asymptote rather than being linearly correlated with occupation rate. The asymptote is a product of the model design. Plants are infested by multiplying each stage class by the occupation rate, ensuring that a fraction of plants will remain unoccupied for any occupation rates less than 1. At very high occupation rates, the proportion of plants unoccupied can be infinitesimally small so that, in a real field, all plants would in fact be occupied. As described in "Methods and Materials," the fact that the multiple oviposition scenario reaches an asymptote below 1.0 is a result of the model following only the youngest larva on a plant. In the limited oviposition scenario, the asymptote is at an even lower proportion due to the ovipositing host's avoidance of plants induced and empty (SE). Although this may make the model less realistic for natural outbreak conditions, in agricultural settings, growers would be advised to spray long before 90% of the field is occupied; therefore, our model is acceptable over the range to probable herbivore infestation rates.

One consistent trend for most parameter combinations is that, as occupation rate is increased from 0.1 to 0.9, the *Rel* value decreases. As occupation rate increases, the proportion of plants infested increases, so the number attacked (N_A) increases for both wasps following signals and wasps randomly foraging. However, the patterns of increase differ between the foraging strategies (Fig. 2a). The N_A for random foraging increases after a type II functional response, with a large initial increase in attacks slowing down as handling and giving up time become a greater limitation. We see this type II response because occupation rate regulates the transition from NE to NO, and thereby directly changes the density of occupied plants (Eq. 5b). The N_A for parasitoids that respond only to signaling plants, however, does not increase as drastically. The transition from SO to SE is primarily due to the mortality rate, so changing the occupation rate does not change the density of occupied signaling plants compared to all signaling plants

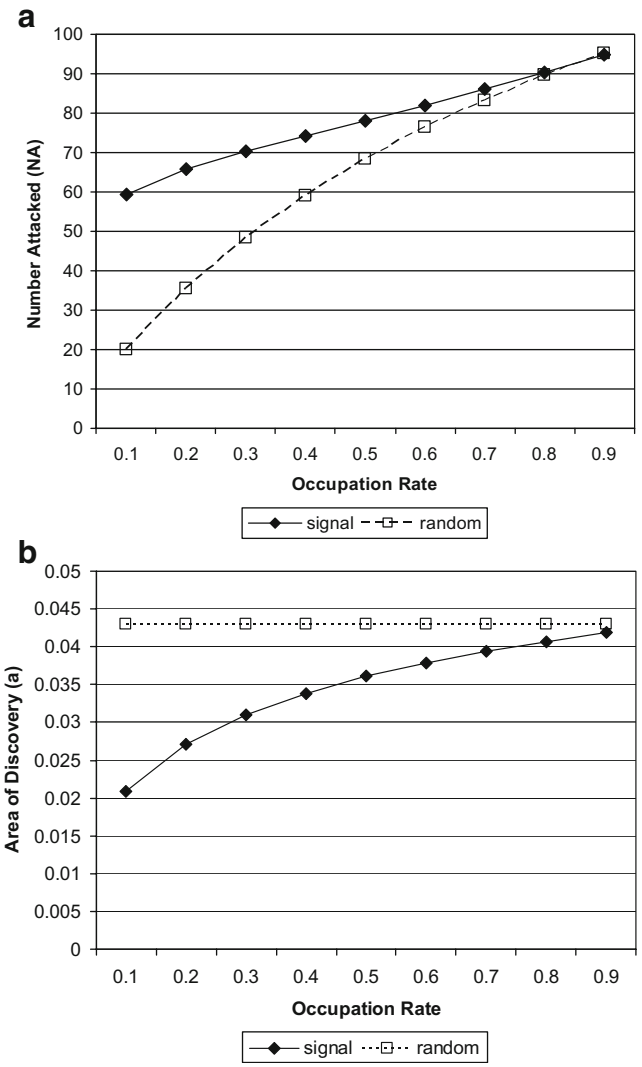

Fig. 2 The impact of occupation rate. These sample data came from the parameters of high mortality and multiple oviposition for *H. virescens*, and plant induction and relaxation delays of one day each. **a** The relationship between occupation rate and number of hosts attacked (N_A) for parasitoids following signals (signal) compared to parasitoids randomly foraging (random). **b** The relationship between occupation rate and area of discovery (a) for parasitoids following signals (signal) compared to parasitoids randomly foraging (random)

(Eq. 5a). However, an increase in occupation rate does change the density of occupied signaling plants compared to all plants (Eq. 9), which results in an increasing area of discovery as occupation rate increases (Fig. 2b). Additionally, in the case of multiple oviposition by *H. virescens*, the chance of a signaling plant being reinfested before relaxing the signal increases with greater occupation rates, which also can lead to a modest increase in density of signaling plants with increasing occupation rates. At high occupation rates, virtually all plants that are occupied are also signaling, resulting in *Rel* approaching one. The only exception to these general patterns was in the case of setting host attack stage to first instars, which will be discussed in the section on host-stage attack.

For the remainder of this paper, we present results for occupation rates less than 0.5, as data for higher occupation rates both represent unrealistic field densities and are less reliable due to the inherent bias in the model toward younger larvae when plants are multiply occupied.

Induction Delay While we are presenting the data only for the *Pieris* system, the impact of induction delay was similar for both *Heliothis* and *Pieris* systems. In most cases, varying induction delay from 1 to 5 d resulted in less than a 0.1 difference in *Rel* values (Fig. 3a). Induction delay determines the transition from NO to SO plants; for randomly foraging parasitoids, this does not change the density of hosts because NO and SO appear in both the numerator and denominator of the density calculations (Eq. 5b). For signal-following parasitoids, once plants are in SO, the transition to SE is not dependent on the induction period, so this does not change the density of available hosts (Eq. 5a). A change in ratio of SO to NO plants can impact the area of discovery, and therefore, we see that a 5-d induction delay leads to a lower *Rel* than a 1-d induction delay, but the change is small, and the parasitoid would benefit from following signals regardless of the induction delay. It should be noted that this result rests on the assumption that the resources in the field are infinitely abundant, so that even though signaling plants are rare in the field, there is still a large enough number of plants in the field to exhaust the parasitoid's total foraging time. The only exception to this general pattern was in the case of setting host-attack stage to first or second instars, which will be discussed in the section on host-stage attack (Fig. 3c).

Relaxation Delay In most cases, as the delay for plant signal relaxation increased from 1 to 5 d, the relevance of the signal decreased (Fig. 3b); the only exception was the case when host-stage attack was limited to first instars (Fig. 3d). Relaxation delay determines the transition of plants from SE to NE. A long delay increases the number of plants remaining in the SE state, which has the effect of lowering the relative density of occupied signaling plants without changing the overall density of occupied plants. In other words, a long relaxation delay means that parasitoids following signals will spend more time foraging on unoccupied plants, thus decreasing the number of hosts attacked.

For the *Heliothis* system, we compared signal relevance when oviposition was limited to empty plants and when there was no limitation. When multiple ovipositions per plant are allowed, the impact of relaxation delay decreases at higher occupation rates because signaling plants are likely to be reinfested. However, when oviposition is limited, the effect of relaxation delay continues even at higher occupation rates.

Mortality Rate Because reported mortality rates vary greatly for *H. virescens*, we looked at the impact of herbivore mortality for that system only. At low occupation rates, signal relevance was higher when the host mortality rate was higher (Fig. 4); however, at higher occupation rates, lower mortality led to higher signal relevance. Mortality can either cause plants to shift from NO to NE states or from SO to SE states, and the impact of mortality on signal relevance depends on the balance of these two transitions. A transition from NO to NE will decrease the density of occupied plants while having no effect on the density of signaling plants that are occupied; thus, x_{Sig} will not be affected by the transition but x_{Ran} will. However, the transition from NO to NE also limits the number of plants in the NO state that can then enter the SO state, thus reducing the n_{SO}, which has the effect of decreasing a_{Sig}. A transition from SO to SE will decrease both the overall density of occupied plants and the density of signaling occupied plants. Because mortality is concentrated at the earliest part of the *H. virescens* life table, the number of transitions from NO to NE is greater than from SO to SE, and therefore, initially increasing mortality causes an increase in signal relevance. In other words, at low occupation rates, the distance between signaling plants is so great that a parasitoid stopping at an empty signaling plant is at a great disadvantage; when mortality is high, herbivores die before plants begin signaling, thus reducing the number of false signals in the field. However, once the herbivore density becomes high enough so that the area of discovery is similar for both foraging strategies, the impact that mortality has on herbivore density becomes much more important. The number of signaling plants is less than the number of plants in the field, and, therefore, the transition of a single plant due to herbivore mortality will have a greater impact on the signaling density than on the overall density. Increasing the mortality decreases the signal relevance as occupation rate increases.

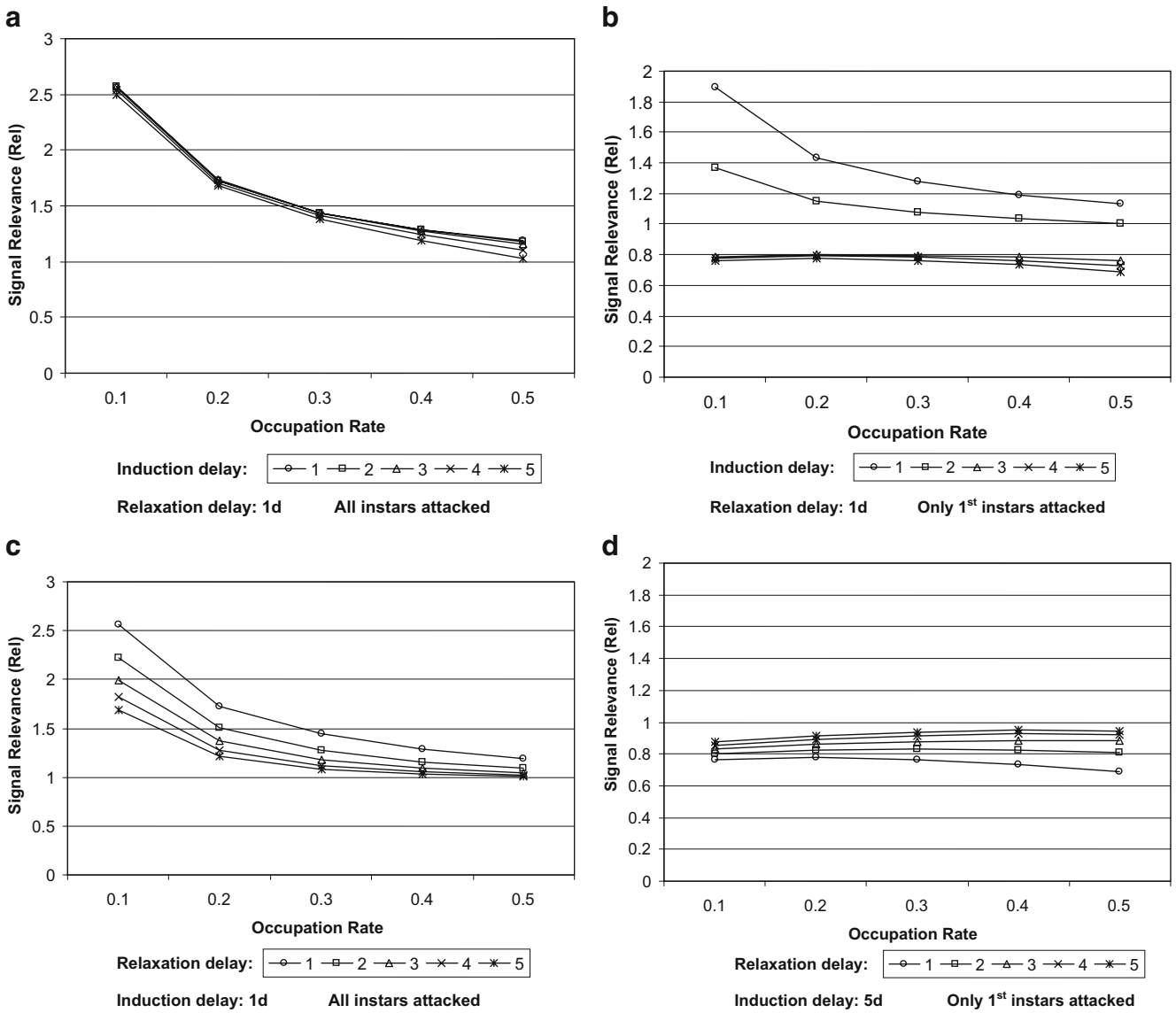

Fig. 3 The relationship between induction delay, relaxation delay, host-stage, attack preference, and signal relevance. For all four cases shown, mortality rates and parasitoid foraging parameters were set to resemble those of *P. rapae* and *Cotesia glomerata*. **a** Numbers in the legend reflect days for induction delay. Relaxation delay was fixed at 1 d. Host-stage attack preference was fixed at fifth instar. **b** *Numbers in the legend* reflect days for relaxation delay. Induction delay was fixed at 1 d, and host-stage attack preference was fixed at all instars attacked. **c** *Numbers in the legend* reflect days for induction delay. Relaxation delay was fixed at 1 d. Host-stage attack preference was fixed at first instar. **d** Numbers in the legend reflect days for relaxation delay. Induction delay was fixed at 5 d, and host-stage attack preference was fixed at first instar

Oviposition Preference We only tested oviposition preference for the *Heliothis* system because *Pieris* females do not avoid ovipositing on occupied or signaling plants. When herbivores limit their oviposition to only NE plants, the signal relevance to the parasitoids decreases (Fig. 4). By avoiding signaling plants, herbivores are increasing the proportion of SE plants relative to SO plants, so parasitoids following signals are more likely to waste time encountering empty plants. When multiple ovipositions per plant are allowed, signaling empty plants can be occupied before the entire relaxation period is complete, and thus, the signal created by the previous herbivore can still be an indicator of the current herbivore. The impact of this behavior depends on the density of signaling plants in the field. At very low densities, limiting oviposition does not greatly decrease signal relevance because the probability of a moth laying multiple eggs on the same plant is low, so there is not much difference in field distribution for the limited or multiple oviposition conditions. At higher densities, *Rel* for multiple oviposition is greater than for the limited oviposition case because more plants can be reinfested before the signal turns off. When herbivores limit their oviposition, it narrows the density range where parasitoids benefit from following signals.

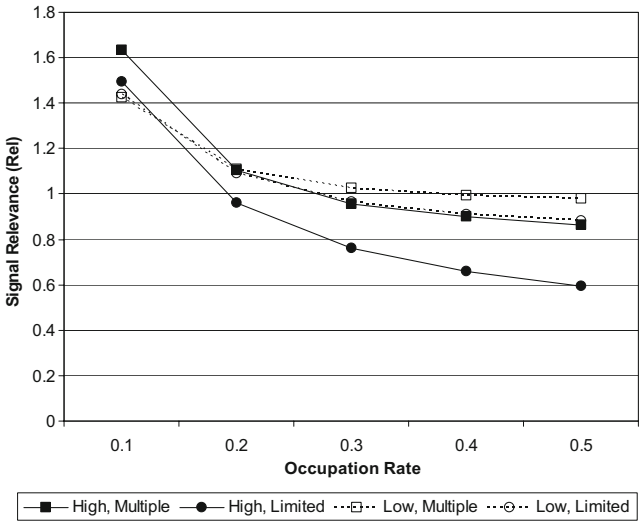

Fig. 4 The relationship between mortality rate, host oviposition, and signal relevance. Parameters were set for *H. virescens*; plant induction and relaxation delays were both set at 5 d. In the legend, "high" and "low" refer to the high and low mortality values on Table 2, "multiple" and "limited" refer to whether a plant could be infested multiple times or if new host infestations were limited to only non-signaling, empty (NE) plants

Host-Stage Attack Preference As alluded to above, host-stage attack preference can interact with other parameters to decrease signal relevance. Specifically, when the parasitoid was limited to a host stage that was shorter than the induction period, the signal relevance decreased. For example, a parasitoid that could attack only first-instar *P. rapae* would have a $Rel>1$ if the host-plant induced after 1 or 2 d but $Rel<1$ if host plant induction took longer than 3 d (Fig. 3c). In the case of higher induction delays and a parasitoid limited to first instars, the SO state occurred only if the plant was reinfested with a new larva, while an older larva induced the signal, which happened at a greater frequency with high occupation rates. In this limited case, *Rel* increases as occupation rate increases, but it never is greater than one.

Discussion

Parasitoid foraging in response to herbivore-induced plant volatiles is a complex behavior, and it will take a combination of multiple models that focus on different aspects of the same system to understand fully this phenomenon. Models that focus on how parasitoids forage in the micro-habitat of the plant canopy (such as Vos and Hemerik 2003) complement this model's approach of looking at parasitoid foraging across an agricultural field. Results from this model identify several biological parameters that should be more thoroughly studied empirically. First, the relationship between herbivore density, plant volatile production, and parasitoid response has traditionally been studied at the single-plant level. However, in this model, field-level herbivore density was shown to affect the relevance of plant volatile signals when the same quality of volatiles was produced by all signaling plants. This indicates that to understand the value of a plant's volatiles to a foraging parasitoid, herbivore population dynamics at the landscape spatial scale must be considered.

The second point is that a delay in the initiation or cessation of volatile signal production may, in certain conditions, decrease signal relevance for a foraging parasitoid. It is not enough to measure simply whether volatiles are produced and whether parasitoids can respond physically to the cue. It is also important to ask if these volatiles are being produced in a time frame relevant to the parasitoid's foraging behavior. This assessment should include relevant physiological constraints on the parasitoid, such as which instars are viable hosts.

The possibility has been raised in several papers of breeding for "calling" plants to enhance biological control (Takabayashi and Dicke 1996; Dicke et al. 2003). In a few studies, it has been found that parasitoids respond more strongly to some cultivars or genotypes within a plant species, indicating that genes for the volatile cues may have inadvertently been bred out of other cultivars (Fritzsche Hoballah et al. 2002; Lou et al. 2006). Part of this discrimination may be due to the specific chemicals that comprise the plants' volatile cues. These preferences may have developed because certain plant–host complexes produce more relevant signals than others.

In addition to examining the presence or absence of volatile signal production in plants, it may be important for plant breeders to look at the relevance of signal production by plants to the parasitoids and predators of interest. A plant volatile that is useful in one geographical region may be useless in another area that has a different insect community. For example, in regions where the main parasitoid is *Cotesia rubecula*, which can successfully attack *P. rapae* in later instars, plants with later volatile releases can still manipulate the third trophic level. However, in geographic areas where the main parasitoid is *C. glomerata*, which can only attack the first two instars of *P. rapae*, the same plants will produce volatiles that are irrelevant to the third trophic level. Additionally, in some geographic regions, the presence of potential parasitoid hosts may overlap with herbivores that are non-hosts but that also trigger plants to produce volatile signals. If these volatiles are not unique for host herbivore, there may be a disproportionate number of signaling but empty (SE) plants induced by the non-host, resulting in irrelevant signals. While past emphasis has been placed on the ability to breed plants that are capable of turning on signals, it may be as

important to focus on breeding plants that can also quickly cease signaling when herbivory ceases. Within the scenarios examined with our model, we found that the greatest increase in parasitoid attack rate due to the presence of signals was fourfold. Plant breeders must therefore determine if less than a fourfold increase in attack rate will lead to economic and environmental gains substantial enough to justify a complex breeding program.

How signal relevance impacts a parasitoid species depends on their plasticity of response. Parasitoids have shown both inherent (Fritzsche Hoballah et al. 2002) and learned ability (Dicke 1999; Fukishima et al. 2002) to follow plant-produced volatile cues. A parasitoid that can learn may adjust its foraging strategy among days or even within one foraging bout based on the relevance of the signal. For a parasitoid with inherent preferences, signal relevance is more likely to act on an evolutionary timescale.

There are many possible ecological pathways through which herbivore-induced plant volatiles could affect parasitoid attack rates. We chose to focus on bypassing non-signaling plants, but there are other processes that may be at work. By altering the assumptions we made in this study, modelers can address some of these other differences. We assumed that the spatial unit of induction was a plant. Some plants begin producing volatiles when nearby plants are induced to create neighborhood effects (Karban 2001). We did not include this type of interaction, but we can speculate that this would have an effect of increasing the signaling empty (SE) plants and may also impact the area of discovery, a, if parasitoids increase their time spent foraging in signal-rich areas. If a parasitoid approaches a field and the overall volatile cloud causes it to fly slower and increase its turning radius, then this impacts the number of plants the parasitoid can land on over time ($a_{\mathrm{Sig}} \leq a_{\mathrm{Ran}}$).

We assume that the field in which the parasitoid forages is sufficiently large so that the parasitoid will run out of time before it runs out of available hosts. The number of signaling plants in a field must be less than or equal to the total number of plants in the field, so a parasitoid's relevant environment is smaller if it is restricted to signaling plants. If a parasitoid is capable of exhausting all the hosts in its environment, we can assume it will leave the field. If then we assume that a parasitoid leaving one field will simply fly to another field with similar characteristics and continue foraging until the total time has expired, then there is no change to the variables. However, once abandoning one field, if the time it takes to reach another field is considerable or a high risk of death during transit occurs, this can effectively reduce the total time the parasitoid has available for foraging. Thus, $T_{\mathrm{tSig}} \leq T_{\mathrm{tRan}}$ in all cases where hosts are a limiting factor and fields are isolated.

We assumed that handling time was constant regardless of host-plant volatiles. If handling time increases on signaling plants where the parasitoid has a positive oviposition experience, as would occur when the parasitoid spends extra time learning the cues of a plant after a successful oviposition, then this could lead to a difference in handling time for signaling and random plants ($b_{\mathrm{Sig}} > b_{\mathrm{Ran}}$).

Because plants were categorized by their youngest infesting larvae, cases where the youngest larva dies first were misclassified. This biases the model toward empty plants in the multiple occupation scenarios because there is higher mortality for first instar larvae, especially in the case of *H. virescens*. This bias is especially troublesome at the higher occupation rates where multiple occupation is more likely to occur. This shortcoming is addressed in the following companion paper (Puente et al. 2008) that reports results with a spatially explicit model that follows the fate of multiple larvae on a single plant. The value of this general model lies in its attempt to capture various interactions brought about by behaviors of all three trophic levels, and its ability to produce system-specific predictions.

The oviposition behavior of *H. virescens* poses a challenge to foraging parasitoids. If *H. virescens* limits oviposition to uninfested plants, the signal relevance that plants provide to parasitoids at high herbivore densities is reduced. However, as long as the density of herbivores remains low, the model predicts that parasitoids will benefit from following plant signals.

Additionally, we looked at two different life tables for *H. virescens* and found that, at low herbivore densities, plant volatiles were more relevant when mortality was high. This indicates that using other pest control methods to suppress the population produces conditions where plant signals are more relevant to parasitoids for biological control. This model supports the notion that complementary methods of pest control are better than relying on a single method such as biological control.

Because *C. glomerata* has a narrow range of host stages, we were curious to see if that would make signals less relevant. As long as *C. glomerata* is able to successfully attack second instars, following plant signals is the preferred strategy for the entire range of parameters we tested for *P. rapae*. However, when parasitoids are limited to just the first instar, they would be better off randomly foraging than following plants that take longer than 2 d to induce signals. We can predict that it would be detrimental for *C. glomerata* to follow plant signals that are not induced until larvae reach the third instar because *C. glomerata* specifically forages on the first two instars. However, in systems where it has been measured, plants responded to herbivory with volatile production well before the third instar would have been reached (Geervliet et al. 1998; Mattiacci et al. 2001; Scascighini et al. 2005).

The general deterministic model presented here quantifies the potential impact of a number of ecological factors

on the importance of induced volatiles to parasitoids. As the first biologically based model of this general system, it brings forth more questions than it answers. More detailed models tailored to specific tritrophic systems and more detailed experimental data on those systems will be needed to answer these questions.

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References

AGRAWAL, A. A., and KARBAN, R. 1999. Why induced defenses may be favored over constitutive strategies in plants, pp. 45–61, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible DefensesPrinceton University Press, Princeton, NJ.

BOTTRELL, D. G., BARBOSA, P., and GOULD, F. 1998. Manipulating natural enemies by plant variety selection and modification: a realistic strategy. *Annu. Rev. Entomol.* 43:347–367.

BRODEUR, J., and VET, L. E. M. 1995. Relationship between parasitoid host-range and host-defense—a comparative study of egg encapsulation in 2 related parasitoid species. *Physiol. Entomol.* 20:7–11.

COURTNEY, S. P. 1986. The ecology of Pierid butterflies: dynamics and interactions. *Adv. Ecol. Res.* 15:51–131.

DEGENHARDT, J., GERSHENZON, J., BALDWIN, I. T., and KESSLER, A. 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14:169–176.

DEMORAES, C. M., and LEWIS, W. J. 1999. Analyses of two parasitoids with convergent foraging strategies. *J. Insect Behav.* 12:571–583.

DEMORAES, C. M., LEWIS, W. J., PARE, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivory-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DEMORAES, C. M., MESCHER, M. C., and TUMLINSON, J. H. 2001. Caterpillar-induced nocturnal plant volatiles repel conspecific females. *Nature* 410:577–580.

DEMPSTER, J. P. 1967. The control of *Pieris rapae* with DDT. I. The natural mortality of the young stages of *Pieris*. *J. Appl. Ecol.* 4: 485–500.

DICKE, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods. *Entomol. Exp. Appl.* 91:131–142.

DICKE, M., VAN POECKE, R. M. P., and DE BOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological function. *Basic Appl. Ecol.* 4:27–42.

FRITZSCHE HOBALLAH, M. E., TAMO, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important. *J. Chem. Ecol.* 28:951–968.

FUKUSHIMA, J., KAINOH, Y., HONDA, H., and TAKABAYASHI, J. 2002. Learning of herbivore-induced and nonspecific plant volatiles by a parasitoid, *Cotesia kariyai*. *J. Chem. Ecol.* 28:579–586.

GEERVLIET, J. B. F., ARIENS, S., DICKE, M., and VET, L. E. M. 1998. Long-distance assessment of patch profitability through volatile infochemicals by the parasitoids *Cotesia glomerata* and *Cotesia rubecula* (Hymenoptera: Braconidae). *Biol. Control* 11:113–121.

HOLLING, C. S. 1959. Some characteristics of simple types of predation and parasitism. *Can. Entomol.* 91:385–398.

HORIKOSHI, M., TAKABAYASHI, J., YANO, S., YAMAOKA, R., OHSAKI, N., and SATO, Y. 1997. *Cotesia glomerata* wasps use fatty acids from plant-herbivore complex in host searching. *J. Chem. Ecol.* 23:1505–1515.

JANSSEN, A., SABELIS, M. W., and BRUIN, J. 2002. Evolution of herbivore-induced plant volatiles. *Oikos* 97:134–138.

JOHNSON, M. T., and GOULD, F. 1992. Interactions of genetically engineered host plant resistance and natural enemies of *Heliothis virescens* (Lepidoptera: Noctuidae) in tobacco. *Environ. Entomol.* 21:586–597.

KAISER, L., and CARDE, R. T. 1991. Plasticity in in-flight orientation to plant and host odours in the specialist parasitoid *Cotesia rubecula*. *Redia* 74:265–271.

KAISER, L., WILLIS, M. A., and CARDE, R. T. 1994. Flight manoevers used by a parasitic wasp to locate host-infested plant. *Entomol. Exp. Appl.* 70:285–294.

KARBAN, R. 2001. Communication between sagebrush and wild tobacco in the field. *Biochem. Sys. Ecol.* 29:995–1005.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. The University of Chicago Press, Chicago.

KAREIVA, P., and ODELL, G. 1987. Swarms of predators exhibit "preytaxis" if individual predators use area-restricted search. *Am. Nat.* 130:233–270.

KESSLER, A., and BALDWIN, I. T. 2002. Plant responses to insect herbivores: the emerging molecular analysis. *Annu. Rev. Plant Biol.* 53:299–328.

LEWIS, W. J., and VINSON, B. 1971. Suitability of certain *Heliothis* (Lepidoptera–Noctuidae) as hosts for parasite *Cardiochiles nigriceps* (Hymenoptera–Braconidae). *Ann. Entomol. Soc. Am.* 64:970–971.

LOU, Y., HUA, X., TURLINGS, T. C. J., CHENG, J., CHEN, X., and YE, G. 2006. Differences in induced volatile emissions among rice varieties result in differential attraction and parasitism of *Nilaparvata lugens* eggs by the parasitoid *Anagrus nilaparvatae* in the field.. *J. Chem. Ecol.* 32:2375–2387.

MATTIACCI, L., and DICKE, M. 1995. Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. *Entomol. Exp. Appl.* 76:37–48.

MATTIACCI, L., ROCCA, B. A., SCASCIGHINI, N., D'ALESSANDRO, M., HERN, A., and DORN, S. 2001. Systemically induced plant volatiles emitted at the time of danger. *J. Chem. Ecol.* 27:2233–2252.

NEALIS, V. G. 1990. Factors affecting the rate of attack by *Cotesia rubecula* (Hymenoptera: Braconidae). *Ecol. Entomol.* 15:163–168.

NEUNZIG, H. H. 1969. The biology of the tobacco budworm and the corn earworm in North Carolina. North Carolina Agricultural Experiment Station, Technical Bulletin no. 196

NORLUND, D. A. 1981. Semiochemicals: a review of the terminology, pp. 13–28, in D. A. Norlund, R. L. Jones, and W. Joe Lewis (eds.). Semiochemicals: Their Role in Pest ControlWiley, New York.

OPPENHEIM, S. J., and GOULD, F. 2002. Is attraction fatal? The effects of herbivore-induced plant volatiles on herbivore parasitism. *Ecology* 83:3416–3425.

PUENTE, M. E. 2007. Synchrony of herbivore presence, induced plant volatiles, and parasitoid response. PhD dissertation. North Carolina State University, Raleigh. http://www.lib.ncsu.edu/theses/available/etd-03202007-165428/unrestricted/etd.pdf.

PUENTE, M. E., MAGORI, K., KENNEDY, G. G., and GOULD, F. 2008. Impact of herbivore-induced plant volatiles on parasitoid foraging success: a spatial simulation of the *Cotesia rubecula*, *Pieris rapae*, and *Brassica oleracea* system. *J. Chem. Ecol*. (in press).

ROSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1998. Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. *J. Chem. Ecol.* 24:303–319.

SATO, Y., and OHSAKI, N. 2004. Response of the wasp (*Cotesia glomerata*) to larvae of the large white butterfly (*Pieris brassicae*). *Ecol. Res.* 19:445–449.

SCASCIGHINI, N., MATTIACCI, L., D'ALESSANDRO, M., HERN, A., ROTT, A. S., and DORN, S. 2005. New insights in analysing parasitoid attracting synomones: early volatile emission and use of stir bar sorptive extraction. *Chemoecology* 15:97–104.

SIEKMANN, G., KELLER, M. A., and TENHUMBERG, B. 2004. The sweet tooth of adult parasitoid Cotesia rubecula: Ignoring hosts for nectar. *J. Insect Behav.* 17:459–476.

TAKABAYASHI, J., and DICKE, M. 1996. Plant-carnivore mutualism through herbivore-induced carnivore attractants. *Trends Plant Sci.* 1:109–113.

TAKAGI, M. 1999. Perspective of practical biological control and population theories. *Res. Popul. Ecol.* 41:121–126.

TILLMAN, P. G., and MULLINIX, B. G. Jr. 2003. Comparison of host-searching and ovipositional behavior of *Cardiochiles nigriceps* Viereck (Hymenoptera: Braconidae), a parasitoid of *Heliothis virescens* (Fabricius) (Lepidoptera: Noctuidae), in tobacco and cotton. *J. Insect Behav.* 10:555–569.

VAN POECKE, R. M. P., ROOSJEN, M., PUMARINO, L., and DICKE, M. 2003. Attraction of the specialist parasitoid *Cotesia rubecula* to *Arabidopsis thaliana* infested by host or non-host herbivore species. *Entomol. Exp. Appl.* 107:229–236.

VET, L. E. M., WACKERS, F. L., and DICKE, M. 1991. The reliability–detectability problem for foraging parasitoids: usability of 1st and 2nd trophic level stimuli. *Redia* 74:115–119.

VOS, M., and HEMERIK, L. 2003. Linking foraging behavior to lifetime reproductive success for an insect parasitoid: adaptation to host distribution. *Behav. Ecol.* 14:236–245.

VOS, M., and VET, E. M. 2004. Geographic variation in host acceptance by an insect parasitoid: genotype versus experience. *Evol. Ecol. Res.* 6:1021–1035.

Some argue that artificially enhancing volatiles in the field will arrest parasitoids in the area, leading to better control, while others argue that artificially enhanced volatiles will produce misleading signals, thus, reducing the receptiveness of parasitoids to volatiles. In a few field studies conducted in small plots, artificially enhanced volatiles have led to higher parasitism, but it is unclear whether this is due to better attraction of parasitoids from outside the plot, better retention of parasitoids within the plot, or increased efficiency of parasitoids within the plot (e.g., James and Price 2004).

Research on the quality of volatile signals has looked at the specificity of volatile production (Dicke and Takabayashi 1991; Blaakmeer et al. 1994) and the parasitoid's ability to discriminate between odors (Agelopoulos and Keller 1994; Fritzsche-Hoballah et al. 2002). However, being able to discriminate between volatiles does not mean that predators and parasitoids will respond to specific plant volatiles in the wild. Many parasitoids have the ability to learn new signals based on past experience (Lewis and Martin 1990). Additionally, there is heritable variation in parasitoids for responding to plant volatiles (Lewis and Martin 1990; Wang et al. 2004). The proximate incentives for a parasitoid to learn a response to a signal or for a population of parasitoids to evolve a response to a signal are the same; the herbivore-induced volatiles must correlate in time and space with herbivore availability. When the volatiles are not consistently correlated with herbivore presence, there is selection for parasitoids to maintain variation in this response, either through maintenance of genetic variation or evolution of phenotypic plasticity (Wang et al. 2004). In studies where the volatile signal has been uncoupled from herbivore presence, such as by saturating fields with volatiles, parasitoid response to the signals has decreased (Lewis and Martin 1990).

Some work has been done to document the lag time between herbivore damage and production of volatiles that elicit parasitoid responses (Mattiacci et al. 2001). Relating this time course to parasitoid efficiency at host finding, however, has not been thoroughly investigated. Mattiacci et al. (2001) found that *Cotesia glomerata* responded to Brussels sprout plants only after herbivores had been feeding for at least 3 days, but they stopped responding to plants if the herbivores had been removed for more than 1 day. Additionally, Agelopoulos and Keller (1994) found that induced cabbage plants where the herbivores had been removed were just as attractive to *C. rubecula* 22 h after herbivore removal as immediately after removal. This seems to indicate that the plant's lag time for commencing volatile production is longer than the lag time needed to cease volatile production, but that both lag times are expected to be of biological importance. This leads to the question addressed in this study: how long can a plant's lag time be without reducing the relevance of the volatiles as signals to the parasitoid?

While it has been shown that herbivore-induced volatile emission can result in attraction of parasitoids to plants within a wind tunnel type of environment (Agelopoulos and Keller 1994) and that induced plants can arrest predators and parasitoids (Agelopoulos and Keller 1994), we have no record of the distances over which these induced cues may act in the field. Designing field experiments to test attraction to induced plants is a difficult task. Identifying the point at which an insect switches from random movement to directed flight as well as identifying which environmental cues out of the complex volatile environment triggered the change is a challenge that has yet to be successfully tackled in the field.

While not a replacement for field studies, computer simulation models can be used to predict how parasitoids should respond to a complex environment (e.g., Dunning et al. 1995). This allows simultaneous testing of more variables than is possible in a laboratory setting while affording more control over parameters than is possible in most field experiments. By artificially establishing diverse patterns of environmental parameters, we can identify which combinations of plant, herbivore, and parasitoid behaviors are predicted to have the strongest impact on parasitoid foraging success. This information can then be used in designing more efficient field studies.

In this paper, we use the tritrophic system of *Cotesia rubecula*, *Pieris rapae*, and *Brassica oleracea* to ask how the temporal and spatial patterns of herbivore-induced plant volatiles impact parasitoid foraging success. The extensive work on volatile signals in this system, as well as the extensive life history data available for these three species, renders this an ideal tritrophic system for detailed simulation analysis. *P. rapae*, the cabbage white butterfly, is a cosmopolitan herbivore that feeds primarily on Brassicaceae (Kaiser and Cardé 1992). While these plants generally share similar chemical defensive profiles (Kaiser and Cardé 1992; Geervliet et al. 1994), there is also variation in form and distribution of the plants, and it has been shown that *P. rapae* prefers certain host types over others, even within the species *B. oleracea* (Jones and Ives 1979). *C. rubecula* is a specialist parasitoid that can attack all stages of *P. rapae* caterpillars, but this parasitoid's larvae suffer greater mortality when attacking older hosts (Nealis 1990; van Driesche et al. 2003). This species has been shown repeatedly to be responsive to herbivore-induced plant volatiles with positive experience reinforcing this response (Kaiser and Cardé 1992; Blaakmeer et al. 1994; Geervliet et al. 1994). The larval parasitoid *C. rubecula* will respond to plant volatiles multiple times after caterpillars have left (Nealis 1990), so they could be negatively affected by asynchronous signals in the field.

Methods and Materials

Plants We modeled a rectangular, contiguous, grid-like field of *Brassica oleracea* plants with the center of each plant located 1 m from its nearest neighbors. For the analyses, the field was fixed at a 20×20 size (a total of 400 plants). Individual *Brassica* plants were assumed to be induced by the feeding of *Pieris* larvae to produce volatiles that attract parasitoids. Plants were assumed to start effective volatile production following the onset of herbivore feeding (*induction delay*, ranging from 1 to 5 days). Plants continued to emit volatiles throughout herbivore feeding, and stopped the emission of volatiles (*relaxation delay*, ranging from 1 to 5 days) after the cessation of herbivore feeding (due to death or pupation of all herbivore larvae on the plant). Therefore, some plants in the field without herbivores did emit volatiles and potentially misguide parasitoids that were attracted to the volatiles.

Population Dynamics of Herbivores Population dynamics of *Pieris rapae* infesting a field were simulated by using an age-structured model based on field-collected life table data. The model tracked up to 20 *Pieris* larvae per plant. The model assumed that all damage-induced plants produced the same concentrations of volatiles, regardless of how many larvae were infesting the plant. However, it has been found that *C. rubecula* is more responsive to plants that host more larvae (Kaiser and Cardé 1992; Kaiser et al. 1994; Geervliet et al. 1998), so being able to follow multiple larvae opened up the possibility in the future of simulating a change in volatile strength due to the herbivore load on a particular plant.

Life table data for *P. rapae* immature stages were gathered from several sources (Harcourt 1966a; Dempster 1967; Parker 1970; Jones et al. 1987). Overall preadult mortality ranged from 69.1 to 95.9%. If the data provided by Harcourt (1966a) which included no egg parasitism were ignored, the average preadult mortality was 91.6%± 3.0% SD (N=7); this is surprisingly consistent considering the variety of host plants and geographic regions these data were collected from. We chose to use the life table from Dempster (1967) because it was consistent with the majority of published life tables and covered the herbivore's lifespan in more detail. The daily herbivore mortality rates used in the reported simulations are given in Table 1. Individual larval death was a stochastic process; for each simulated larva on each day, a random number between 0 and 1 was selected by the computer, and if the number was less than the probability of mortality, the larva was "killed". In a large field, this process should produce roughly the same overall mortality rate as in found in Table 1.

Table 1 Life table for *Pieris rapae*

Stage	Day	Mortality	Mortality (Deterministic)
Egg	1	0.024	0
	2	0.024	0
	3	0.024	0
	4	0.024	0
	5	0.024	0
1st instar	6	0.187	0.187
	7	0.187	0.187
	8	0.187	0.187
2nd instar	9	0.087	0.087
	10	0.087	0.087
	11	0.087	0.087
3rd instar	12	0.084	0.084
	13	0.084	0.084
	14	0.084	0.084
4th instar	15	0.137	0.137
	16	0.137	0.137
	17	0.137	0.137
5th instar	18	0.233	0.233
	19	0.233	0.233
	20	0.233	0.233
Pupae	21	0.007	0
	22	0.007	0
	23	0.007	0
	24	0.007	0
	25	0.007	0
	26	0.007	0
	27	0.007	0
	28	0.007	0

All adult female *P. rapae* were stored in a single cohort regardless of the day they eclosed. Richards (1940) found that adult *P. rapae* populations usually had a 1:1 sex ratio; therefore, half of all final stage pupae were assumed to be males and were excluded from the adult cohort upon eclosion. Adult female *P. rapae* live for about 3 weeks, so for each daily time step, the number of adults was reduced by the number of new adults present 21 days prior to that time step. No other adult mortality is considered.

P. rapae eggs are disproportionately aggregated at the edges of fields (Harcourt 1966b). This is primarily due to the movement patterns of adult butterflies, which have been studied extensively (e.g., Jones 1977; Root and Kareiva 1984). In natural populations, individual butterflies were observed to fly in a roughly straight path (Jones 1977; Root and Kareiva 1984).We based our herbivore distribution on an algorithm developed by Jones (1977) to recreate the movement patterns of ovipositing *P. rapae* females. In the model, each adult was given a starting position and a directional bias at random. The adult either remained at her current location or moved to an adjoining plant; the direction the adult moved was determined by using the probability of a butterfly turning from an initial directional

flight bias (Root and Kareiva 1984) (see Fig. 1). Following movement, the probability that the adult would oviposit was 0.23, the median probability found by Jones (1977). Field observations found that butterflies crossed patch boundaries without stopping (e.g., Root and Kareiva 1984). In order to reflect this and maintain the same field densities, adults that reached the edges of the simulated field are 'mirrored' to the opposite side of the field. Adults continued to follow this algorithm until they had laid a set number of new eggs. To prevent butterflies remaining indefinitely in a field that had reached its capacity of eggs and larvae, when butterflies encounter ten successive plants that are fully occupied, they leave the model field.

We wanted herbivore populations to reflect natural population sizes, so we set the initial adult population size to 1, included adult movement dynamics, and set the number of eggs to 1, 2, 5, 10, 15, or 20 eggs/butterfly/day. After five runs, the daily average of eggs, larvae, and adults for each of the oviposition rates were calculated for 100 days trials. We then compared the average simulated values to reported field densities, correcting for field size. The density of eggs observed in Parker (1970) was higher than any values we obtained, but the densities observed in Jones et al. (1987) were approximated relatively well by either 15 or 20 eggs/butterfly/day, especially in the second and third generations. The first instar densities observed by van Driesche (1988) in kale were closely approximated by 10 eggs/butterfly/day (see Fig. 2), while the first instar densities observed by van Driesche and Bellows (1988) in collards were approximated by the second generation of simulations for 15 or 20 eggs/butterfly/day (an offset of about 30 days) (see Fig. 2). The number of adults in the field observed by van Driesche (1988) was similar to the results of 10 eggs/butterfly/day. Therefore, for the remainder of the experiments, we assumed 10 eggs/butterfly/day as "low herbivore density" and 20 eggs/butterfly/day as "high herbivore density".

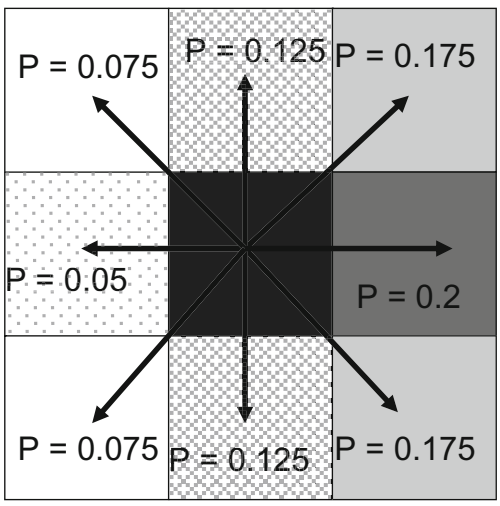

Fig. 1 Spatial bias for *Pieris rapae* butterflies. Assuming a butterfly begins on a plant in the center square and has a bias to the right, the probability that a butterfly will travel to each square is shown by the *P* value in that square

Parasitoid Behavior and Parasitism In essence, the model assesses the fitness of a single parasitoid entering an herbivore-infested field that has had no prior parasitism. Therefore, the parasitism behavior of individual adult *Cotesia* was modeled in detail but its population dynamics were not included. The range of host stages suitable for parasitoid development has been shown to be an important biological parameter for many parasitoids, including the closely related species, *C. glomerata* and *C. rubecula*. The parasitoid *C. glomerata* is unable to attack *P. rapae* larvae older than the third instar because the hosts are able to encapsulate the parasitoid larvae at that stage, so there is higher parasitism on host plants that slow the growth of *P. rapae* larvae (Benrey and Denno 1997). Similarly, *C. rubecula* face higher risks of damage from counterattacks by larvae older than the second instar (Nealis 1990). It has been shown that *C. glomerata* cannot identify the stage of an herbivore based on plant volatile cues (Mattiacci and Dicke 1995) and, therefore, may be attracted to plants with unsuitable larval stages. In the model, parasitoids are only able to successfully attack larvae of the host up to a model-specified instar (*host stage*). Since, in the model, plants that harbor larvae too old to be attacked by parasitoids still emit volatiles, parasitoids that follow signals might be further misled. We varied model parameter values to consider cases where parasitoids could attack first instars only, first and second instars only, or all five instars.

Because we are specifically interested in how herbivore-induced plant volatiles impact parasitoid foraging success, we chose to focus on how underlying host distributions, timing of plant volatiles responses, and parasitoid sensitivity to odor impact parasitoid efficiency. Numerous environmental factors that influence parasitoid foraging efficiency such as wind speed (Keller 1990), light intensity, or previous experience (Keller 1990; Kaiser and Cardé 1992; Geervliet et al. 1998) can also be important, but they are beyond the scope of this paper. We assumed the following constants for both randomly foraging and selectively foraging parasitoids. Sato and Ohsaki (2004) observed that for the closely related *C. glomerata* searching for *Pieris* larvae, the time spent searching one leaf was 73.5±11.9 s, so this was used as the time spent in fruitless search if the parasitoid arrived on a plant with no host. There is some evidence that *C. glomerata* avoids superparasitizing already parasitized larvae (Fatouros et al. 2005), so a parasitized larva was considered "nonhost" but was still capable of inducing a plant to produce volatiles. We assumed a

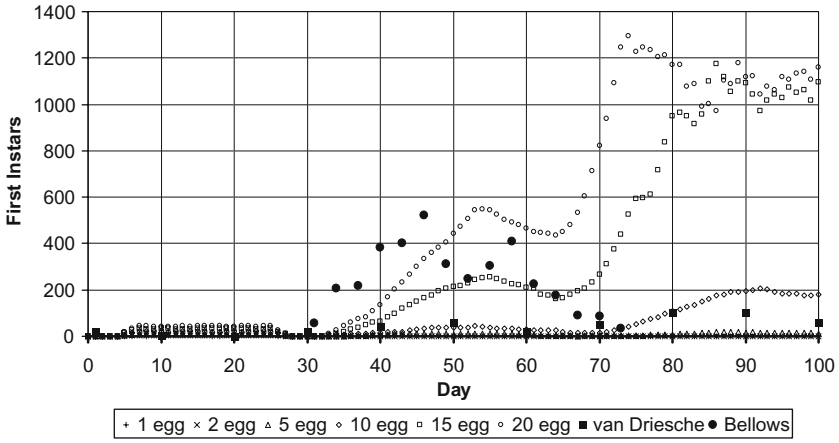

Fig. 2 Comparison of simulated herbivore population dynamics with published field data. The *larger black symbols* are counts of first instars obtained from published studies by van Driesche (1988) and van Driesche and Bellows (1988), adjusted to match the size of our simulated field (400 plants). The *numbers in the legend* refer to the maximum number of eggs each female butterfly could lay per day

parasitoid would be equally likely to find a host early or late in that search time interval; so on average, a parasitoid would spend half as much time searching if it encountered a host on that plant. Although there are circumstances that would prevent parasitoids from discovering available hosts (e.g., plant architecture), we assumed that if a viable host was available, the parasitoid would find it. The time it took for a parasitoid to successfully sting a host was 13.1±3.9 s (Sato and Ohsaki 2004). The recorded flight speed for *C. rubecula* was 0.33 m/s (Kaiser et al. 1994).

Parasitoids were given 3,600 s (i.e., 1 h) of total foraging time per day. The exact amount of time they spend foraging in the field is unknown, but 1 h per day was considered a low but feasible estimate given that most parasitoids only forage during the brightest hours of daylight and must divide time between foraging for food and foraging for hosts (Bartlett 1964). A greater period of time for foraging per day often resulted in all volatile-emitting plants being visited in our small field and produced artifactual results. A parasitoid started its foraging in a field location randomly selected by the simulation model and immediately searched the plant it was on. If no viable host were present, the giving-up time was discounted from the total foraging time and the parasitoid moved to the next plant. If a viable host were present, the host was marked as parasitized, the handling time was discounted from the total foraging time, and half the giving-up time was discounted from the total foraging time to account for search time. After successfully parasitizing a host, parasitoids had a 0.67 probability of leaving a plant. Otherwise they remained to continue searching (Tenhumberg et al. 2001) with a new giving-up time discounted from the foraging time. For many other systems, a more complicated algorithm for calculating giving-up time would be more appropriate; however, because *P. rapae* is not a gregarious host, this method of resetting the giving-up time following each successful oviposition is a close approximation of what occurs in nature (Vos et al. 1998). In wind tunnel experiments, the presence and concentration of host odors did not affect *C. rubecula*'s flight speed or direction of travel, but did impact a parasitoid's "willingness" to take off and whether a parasitoid completed a flight away from the initial location (Keller 1990; Kaiser et al. 1994). Therefore, we used a 0.67 flight probability for both randomly and selectively foraging parasitoids.

The spatial aspects of parasitoid foraging are poorly understood, so we had to make many assumptions in this part of the model. The following assumptions we believe to be reasonable:

- Plant volatiles are diluted over space, so the closer an emitting plant is to a parasitoid the more likely it is to be detected.
- Parasitoids use volatiles to detect a potential host plant, even if they are not *herbivore-induced plant volatiles*, so a randomly foraging parasitoid is also more likely to detect plants that are closest to it (Nordlund et al. 1988).
- Parasitoids decide which plant they will fly to before they leave the plant they are on (Keller 1990).

We used two different shapes of dispersal for the weighting: linear and exponential. According to Elkinton et al. (1987), over short distances, volatiles such as pheromone plumes spread out in a linear fashion, therefore, one dispersal shape was "linear". We assumed that the strength of signal had a value of one at the distance of 1 m and zero at a distance of 6 m and decreased linearly from the highest to lowest value (Table 2, Linear Diffusion). This created a bias where a parasitoid was five times as likely to go to a plant 1 m away compared to a plant 5 m away. The other dispersal shape we assumed was an exponential diffusion. In diffusion models, where plumes are not as well delineated, dilution of volatile concentrations happen at a

Table 2 Volatile distance biases

Distance	Linear		Exponential		
	Expected	Bias	Expected	Relative	Bias
1	1	5	1	25	25
2	0.8	4	0.25	6.25	6
3	0.6	3	0.11	2.78	3
4	0.4	2	0.06	1.56	2
5	0.2	1	0.04	1	1

rate relative to the inverse of the radius squared (Murlis et al. 2000). This created a bias in the model where a parasitoid was 25 times as likely to go to a plant 1 m away compared to a plant 5 m away (Table 2, Exponential Diffusion). Although it is likely that the actual spatial dynamics of detection distances is not either of these two options, we felt this would be adequate for looking at the sensitivity of the model to a parasitoid's odor detection bias.

The plant the parasitoid would fly to was randomly selected from the weighted list of possible destination plants. Parasitoids were prevented from immediately returning to the plant they came from. In the case of parasitoids that responded to herbivore-induced volatiles, only induced plants (emitting volatiles) were added to the weighted list of possible destination plants. If none of the plants on the weighted list of possible destination plants was signaling, then the weighted list was remade with the random movement algorithm, and the parasitoid was moved to a random non-induced plant.

Model Verification Predictions of the present model cannot be immediately tested against lack of empirical data. However, it was possible to assess whether there were errors in the coding of this simulation model by comparing predictions of this stochastic model to predictions of a simpler stage-specific model described in Puente et al. (2008, preceding article) that modeled the same phenomena in a nonspatial, deterministic manner.

The maximum number of hosts a parasitoid could attack in the deterministic model is limited by the time it has to forage rather than the number of hosts in the field, Therefore, by comparing the results of our stochastic model with the deterministic model run with the same parameters, we could identify the minimum field size that led to time-limited, rather than space-limited, foraging. For all parameter combinations explored, randomly foraging parasitoids were able to exhaust their time in fields with greater than 70 plants. Parasitoids foraging with signals needed larger fields to exhaust their time budget. Except for unrealistic parameters, a field size of 400 plants was sufficient for the signal-foraging parasitoids.

Additionally, we were also able to use results from the deterministic comparison to identify how many runs were necessary to account for the stochastic variation caused by herbivore oviposition and mortality. We calculated the mean and standard error of the number of hosts attacked for both signal-following and randomly foraging parasitoids for a number of successive runs with extreme values of parameters (induction and relaxation delays, viable host stage, etc.), and determined how many runs were necessary for the standard error to fall within 5% and 10% of the mean. Under most circumstances, the standard error was within 10% of the mean after fewer than five runs, and 20 runs were sufficient to reach a standard error within 5% of the mean for both number of hosts attacked for signal-following parasitoids and number of hosts attacked for randomly foraging parasitoids. This indicated that for most parameters 20 runs are sufficient. A summary of the variables and constants used in this simulation can be found in Table 3.

Results

We examined time series of the mean number of herbivores attacked by parasitoids randomly foraging and by parasitoids following signals, for both linear and exponential distance biases, for the 54 combinations of parameters we considered. In all parameter combinations, the mean number of hosts attacked by randomly foraging parasitoids with linear biases was within one standard deviation of the mean number of hosts attacked by randomly foraging parasitoids with exponential dispersal of volatile compounds. Averaging over all parameter combinations, parasitoids that randomly foraged visited about eight more plants per day than parasitoids following signals, regardless

Table 3 Parameters used in the model

Parameter	Values
(Oldest viable) Host stage	1, 2, 5
Induction delay (in days)	1, 3, 5
Relaxation delay (in days)	1, 3, 5
Occupation rate (in eggs/plants/field)[a]	0.1–0.9
Herbivore density (in eggs/butterfly/day)	Low (10), high (20)
Total foraging time (T_t) (in seconds)	3,600
Flight speed (a) (in meters/second)	0.33
Handling time (b) (in seconds/host)	13.1
Giving-up time (c) (in seconds/plant)	73.5
Foraging style	Random, signal following
Distance bias[b]	Linear, exponential

[a] Occupation rate was used solely in validating the model; for actual runs, herbivore density values were used
[b] Distance bias is more thoroughly explained in Table 2

of the type of volatile dispersal. By looking at the difference in mean number of larvae attacked for each 5-day-sampling interval for parasitoids following signals versus parasitoids randomly foraging, each of the combinations of induction delays, relaxation delays, herbivore densities, and viable host stages could be classified as one of four patterns:

A. (6 of 54) Following signals was *on average disadvantageous*, but individual runs could be advantageous due to large variances,
B. (12 of 54) Following signals was *not significantly different* from random foraging for any time interval sampled,
C. (5 of 54) Following signals was *advantageous* for at least one host generation, as long as the parasitoid flight bias was *linear*,
D. (31 of 54) Following signals was *advantageous* for at least one host generation, regardless of parasitoid flight bias.

In pattern A, parasitoids following signals generally attacked as many hosts as parasitoids not following signals through the first two generations of herbivores, but fell much lower in the third generation (Fig. 3a). Of the 54 parameter combinations, six combinations fell into this pattern. In the most extreme loss of efficiency, parasitoids that followed signals attacked 40% fewer hosts than randomly foraging parasitoids, under the conditions of a 3-day induction, 1-day relaxation, low herbivore density, only first instars as viable hosts, and linear distance bias. In these simulations, the variance of hosts attacked in the third generation of herbivores was quite large such that the mean for parasitoids randomly searching was well within a single standard deviation of the parasitoids following signals. This first pattern was seen only when the induction delay was greater than one and the oldest viable hosts were either first or second instars.

In pattern B, parasitoids that followed signals generally attacked as many hosts as parasitoids not following signals throughout the year (Fig. 3b). Over a season, this could result in average of up to 40% more hosts attacked or 20% fewer hosts attacked, but for any day sampled, the means for number of hosts attacked if the parasitoids followed signals were well within one standard deviation of the means for number attacked if the parasitoids foraged randomly. Of the 54 combinations we tested, 12 combinations fell into this pattern. In all such cases, the oldest susceptible host stage was either first or second instars, and in almost all of these cases, the induction delay was 5 days; the only exception being two cases where the induction delay was 3 days and only first instars were attacked. In all of these cases, the signals were produced after the inducing host had matured beyond the viable attack stages; therefore, it is not surprising that the resulting host attack rates for parasitoids following signals should not be significantly different from randomly foraging parasitoids.

In pattern C, there was an effect of volatile dispersion on parasitoid success in the first generation of herbivores (Fig. 3c). When volatiles were assumed to have a linear dispersal pattern, parasitoids that followed volatiles attacked between 50% to 200% more hosts than randomly foraging parasitoids. For these same conditions, when volatiles were assumed to have an exponential dispersal pattern, the difference between the signal foraging parasitoids and the randomly foraging parasitoids ranged from attacking 10% fewer hosts to attacking 25% more hosts than randomly foraging parasitoids. Pattern C occurred in five of the 54 combinations, all of which had first and second instars as viable hosts and an induction delay of either 1 or 3 days. Because this advantage was apparent only in the first generation of the herbivores when they are rare in the field, it is likely that a linear dispersal of the volatiles that allowed parasitoids to detect plants from greater distances also allowed them to encounter more patches of viable hosts, and a well synchronized induction then led to their remaining in the patch of viable hosts. Randomly foraging parasitoids with linear volatile dispersion patterns were just as likely to leave a patch of viable hosts as they were to enter a patch, thus giving parasitoids following signals the advantage. Signal foraging parasitoids with exponential volatiles dispersion patterns were less likely to encounter a patch because they did not stray far from a patch they had already exploited.

The remaining 31 parameter combinations (57.5% of all combinations) fell into pattern D, where parasitoids that followed signals attacked more hosts than randomly foraging parasitoids in at least one generation of herbivores, regardless of parasitoid distance bias (Fig. 3d). The advantage occurred primarily in the first herbivore generation but sometimes extended into the second generation. By the third generation, there were sufficiently dense populations of hosts that parasitoids were reaching their saturation point regardless of their foraging strategy. This can be seen by the small standard deviations from day 80 onward. If all five instars were viable hosts, parasitoids benefited from following signals for all herbivore generations regardless of the volatile dispersal patterns. When only first instars were viable hosts, pattern D occurred only when the induction delay was 1 day. When second instars were the oldest viable hosts, if the host density was high and the induction delay was 1 or 3 days, pattern D occurred, but when host densities were low, pattern C occurred.

A summary of all simulations can be found in Supplementary Table 1, where results are reported as "relative advantage" to the parasitoid from following volatiles (i.e., the number of hosts attacked by parasitoids following signals minus the number attacked by parasitoids randomly foraging divided by the number attacked by

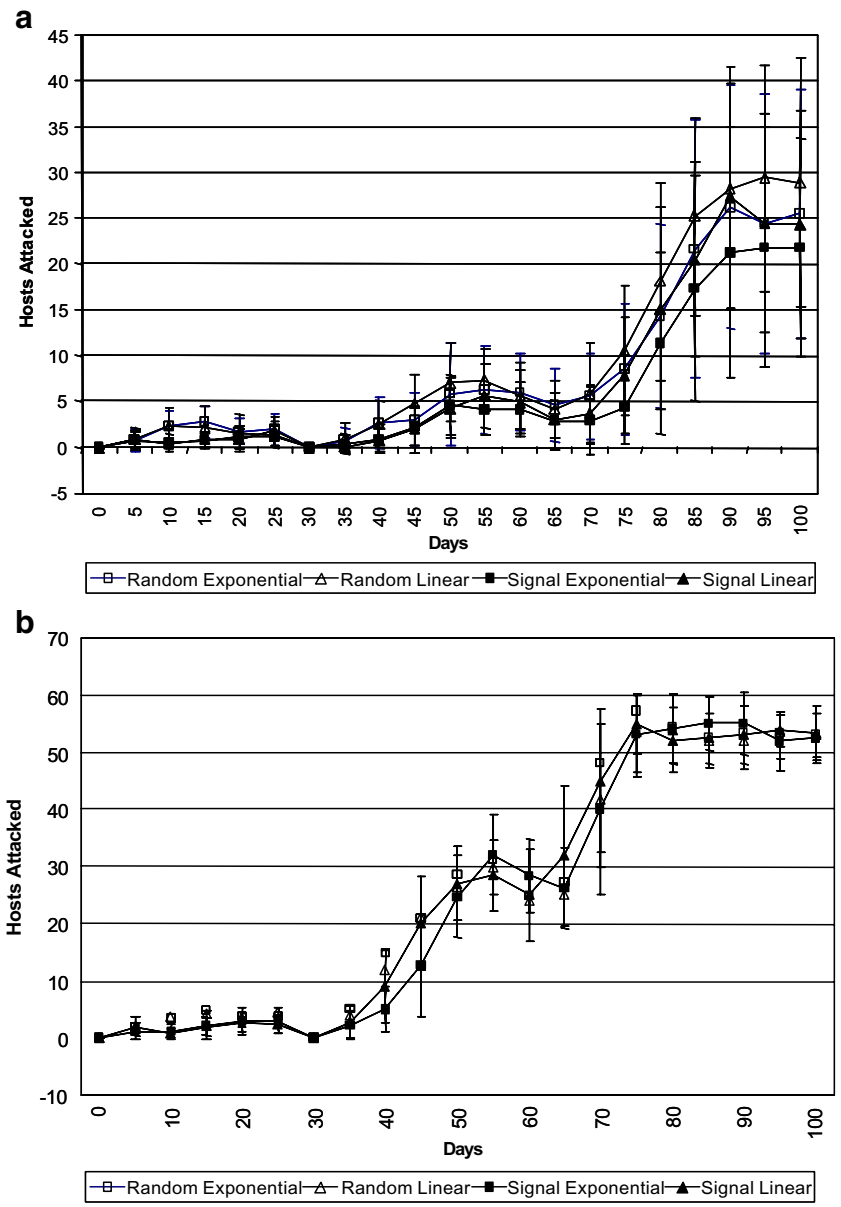

Fig. 3 Examples of how signals can impact the number of hosts attacked. **a** Detrimental signals (*Pattern A*); shown here is the case where induction delay is 3 days, relaxation delay is 3 days, herbivore density is low, and viable host stage is first instars only. **b** Signals no better or worse (*Pattern B*); shown here is the case where induction delay is 5 days, relaxation delay is 5 days, herbivore density is high, and viable host stage is first instars only. **c** Signals beneficial as long as the parasitoid distance bias is linear (*Pattern C*); shown here is the case where induction delay is 1 day, relaxation delay is 1 day, herbivore density is low, and viable host stages are second and first instars. **d** Signals beneficial (*Pattern D*); shown here is the case where induction delay is 1 day, relaxation delay is 1 day, herbivore density is high, and all instars are viable host stages. For each example, four cases were plotted: parasitoids randomly foraging where plants produce volatiles with an exponential diffusion (random exponential), parasitoids randomly foraging where plants produce volatiles with a linear diffusion (random linear), parasitoids following volatile plant cues where plants produce volatiles with an exponential diffusion (signal exponential), and parasitoids following volatile plant cues where plants produce volatiles with a linear diffusion (signal linear). For all four graphs, vertical bars are ±1 SD

randomly foraging parasitoids.). The overall patterns of when parasitoids benefited from following signals was the same if we looked at relative advantage or absolute number of hosts attacked; however, by looking at relative advantage, the gains from the beginning of the season were more pronounced compared to the gains at the end of the season. This is a more biologically relevant measure, as a fitness gain for a parasitoid early in the season would account for a larger overall gain in fitness if we had followed the long-term population dynamics of the parasitoid population.

Although relaxation delay did not impact qualitatively which pattern a simulation fell into, the relaxation delay did impact the overall gain in expected fitness over a season. For example, in the case where all five instars were viable

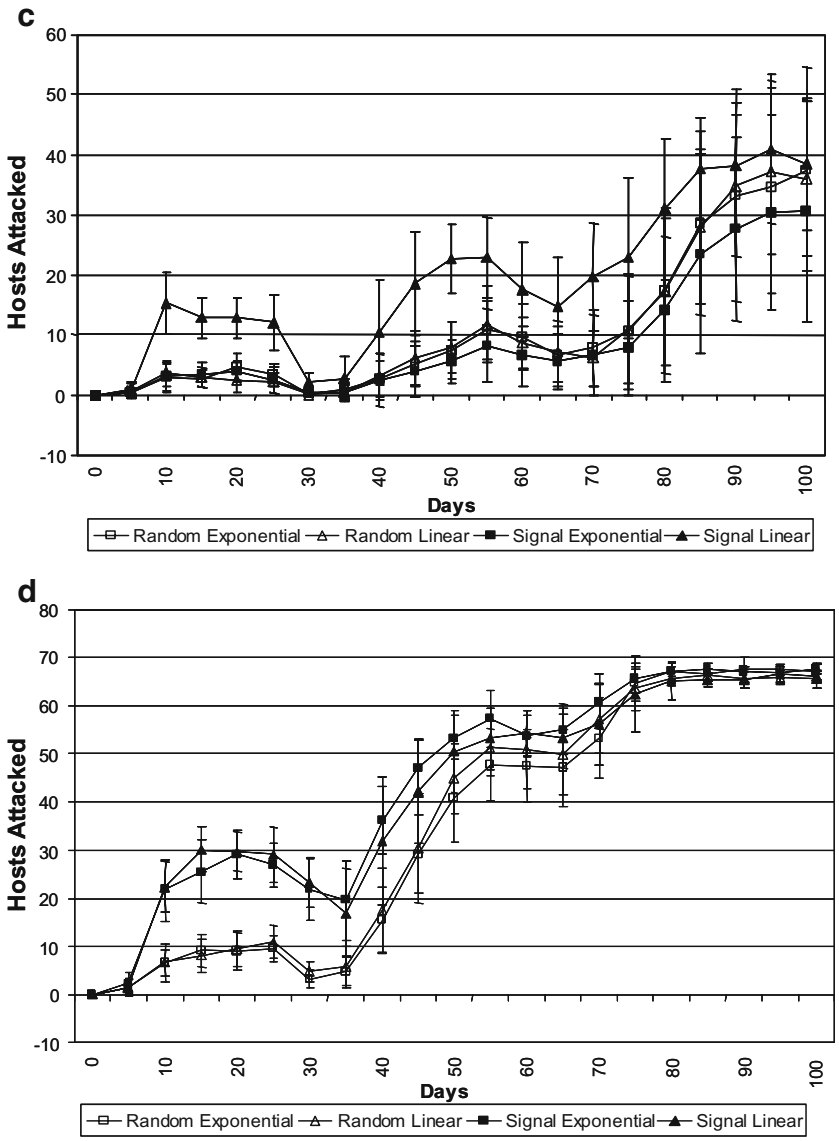

Fig. 3 (continued)

hosts, induction delay was 5 days and host density was high; if the relaxation delay was 1 day, the parasitoids that followed signals could attack on average 130% more hosts compared to randomly foraging parasitoids; if the relaxation delay was 3 days, this gain was reduced to 92% more hosts; and if the relaxation delay was 5 days, this gain was reduced to 78% more hosts.

Discussion

For the majority of parameter combinations (57.5% with exponential shape of dispersal and 66.6% with linear shapes of dispersal), the following of herbivore-induced plant volatiles was a beneficial strategy for parasitoids, but the degree of benefit varied depending on values of a number of parameters. While this result indicates that herbivore-induced plant volatile cues can be a robust indicator for parasitoids in many types of environments, we note that the most likely environment, (where host attacks are limited to first or second instars, and relaxation and induction delay vary between 1 and 3 days), is where there is a transition between the volatile cues being useful all of the time (pattern D) and only in certain volatile dispersal conditions (pattern C). We found that induction delay had an important impact on the utility of volatile signals. Plants with patterns A and B, where plants with induced volatiles were irrelevant or possibly detrimental to the parasitoids, tended to have an induction delay of 3 or 5 days. Relaxation delay was also important for determining the magnitude of effect of following signals could have on parasitoids. These results differ slightly from our deterministic model (Puente et al. 2008, preceding article), where relaxation delay was more important than induction

delay in determining relevance. The difference between results of the two models is due to the fact that the deterministic model assesses attack rate when the plant population has reached an equilibrium frequency of individuals in each of the four states. In the stochastic spatial model, the impact of following signals is assessed throughout the season while frequencies of plants in each state are changing and herbivore density is changing.

Both models show that understanding the molecular mechanism responsible for inducing signals will be important for engineering volatile producing plants that optimize parasitoid foraging efficiency. Future work in the *P. rapae* system can test our prediction by examining how the natural variation among cultivars in volatile induction and cessation impacts parasitoid behavior and attack rates. We predict that *C. rubecula* should have a preference for varieties of *B. oleracea* that can begin volatile production within a day of onset of herbivory and can cease volatile production within a day of the end of herbivore attack.

Our model shows that how parasitoids perceive volatiles in space can be important for determining whether or not it gains an advantage by following signals. Volatiles are considered important cues for "long-distance" foraging (Geervliet et al. 1998), but what constitutes "long-distance" has not been described quantitatively in the literature. If this means that parasitoids can detect signals 1 m away, that would be comparable to our exponential volatile dispersion where parasitoids were 25 times more likely to visit a plant one meter away compared to a plant 5 m away; in this case, parasitoids gained nothing from following signals in several cases. However, if parasitoids can detect and respond to signals from five times that distance, as in our linear bias example, signals more often had at least some positive impact on parasitoid foraging success. While this differentiation (pattern C) only occurred in five cases, these cases all occurred in simulations where second instars were the oldest viable host stage, which is biologically relevant for *C. rubecula*. In all of these cases, herbivore density was low, which would be the desirable state for an agricultural setting. Because of the potential likelihood of these parameter conditions occurring in nature, understanding how volatile plumes disperse in space and how parasitoids perceive these volatiles in space could be important for knowing whether breeding for inducing plants will be a successful endeavor.

Finally, as with our deterministic model, the current model shows the importance of herbivore density to the relevance of volatile cues. In the first herbivore generation, when the number of herbivores was at its lowest (Fig. 2), signals had the greatest impact (Fig. 3c,d). In the third generation, when herbivores were at their highest densities, volatile signals were typically irrelevant or had a negative impact. Most studies of induced volatiles examine just a single volatile source, which misses the potential importance herbivore densities can have on parasitoid foraging success. Predictions from our model argue for looking at larger populations of plants and herbivores before determining whether a volatile signal has a substantially positive effect on parasitoids. We also argue that if breeding plants for volatile production is going to be a successful strategy, the appropriate volatile production by young plants is needed early in the season if it is to improve parasitoid foraging efficiency.

While our simulation model has identified important parameters that should be studied more closely, several modifications to the model could be made in the future that would improve its value. One assumption we made in this model is that parasitoids are either foraging randomly or foraging in response to signals. We did not allow for parasitoids to change patterns within a lifetime. Other models have shown that evolving parasitoid systems can change host-parasitoid dynamics (Abrams and Kawecki 1999). Therefore, incorporating parasitoid learning into this model would be an important next step.

We specifically chose to focus on a naïve parasitoid entering a field of nonparasitized larvae, rather than following an entire parasitoid population's dynamics over a season. Following parasitoid population dynamics would be an interesting model extension but would require considerably more parameters. Parasitoid and host eclosion are not always synchronized in nature (van der Meijden and Klinkhamer 2000); parasitized herbivores can consume different amounts of foods than their nonparasitized congeners (Fatouros et al. 2005) and parasitized larvae can have different mortality from nonparasitized larvae (Jones 1987). These factors may all alter the relevance of volatiles in host plants over several herbivore generations. In many cases, less efficient individual predators or parasitoids will lead to more stable population dynamics over longer periods of time (van der Meijden and Klinkhamer 2000); therefore, following long-term dynamics could lead to different conclusions.

We framed our research question from the naive parasitoid's perspective: when should a parasitoid follow an induced volatile coming from a plant, and how much of a fitness benefit can it obtain from following the volatile plume? These questions could also be framed from the plant's perspective: how accurate does a plant need to be to attract parasitoids? However, this implies that parasitoids can exert a positive evolutionary pressure on plant fitness. There is some evidence that parasitized *Pieris* species consume less, and that the plant can gain fitness by recruiting parasitoids (Fatouros et al. 2005). It is also possible, however, that parasitism has no effect on individual plant fitness within a single generation of parasitoids and hosts (Coleman et al. 1999), thus, making

the question from the plant's point of view inconclusive (Janssen et al. 2002). To have evolutionary relevance this model needs to be modified to include long term plant fitness.

We hope the model will stimulate future research on the timing and spatial dynamics of herbivore-induced plant volatiles. While these are difficult parameters to measure in natural systems, they appear to be ecologically relevant and are important aspects to study, especially, if this phenomenon is to be applied practically in agriculture.

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References

ABRAMS, P. A., and KAWECKI, T. J. 1999. Adaptive host preference and the dynamics of host-parasitoid interactions. *Theor. Popul. Biol*. 56:307–324.

AGELOPOULOS, N. G., and KELLER, M. A. 1994. Plant-natural enemy association in tritrophic system, Cotesia rubecula–Pieris rapae–Brassicaceae (Cruciferae). III: Collection and identification of plant and frass volatiles. *J. Chem. Ecol*. 20:1955–1967.

BARTLETT, B. R. 1964. Patterns in the host feeding habit of adult parasitic Hymenoptera. *Ann. Entomol. Soc. Amer.* 57:344–350.

BENREY, B., and DENNO, R. F. 1997. The Slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. *Ecology* 78:987–999.

BLAAKMEER, A., GEERVLIET, J. B. F., VAN LOON, J. J. A., POSTHUMUS, M. A., VAN BEEK, T. A., and DE GROOT, A. 1994. Comparative headspace analysis of cabbage plants damaged by two species of *Pieris* caterpillars: consequences for in-flight host location by *Cotesia* parasitoids. *Entomol. Exp. Appl.* 73:175–182.

BOTTRELL, D. G., BARBOSA, P., and GOULD, F. 1998. Manipulating natural enemies by plant variety selection and modification: a realistic strategy? *Annu. Rev. Entomol.* 43:347–367.

COLEMAN, R. A., BARKER, A. M., and FENNER, M. 1999. Parasitism of the herbivore *Pieris brassicae L*. (Lep., Pieridae) by *Cotesia glomerata* (Hym., Braconidae) does not benefit the host plant by reduction of herbivory.. *J. Appl. Entomol.* 123:171–177.

DEGENHARDT, J., GERSHENZON, J., BALDWIN, I. T., and KESSLER, A. 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14:169–176.

DEMPSTER, J. P. 1967. The control of *Pieris rapae* with DDT. I. The natural mortality of the young stages of *Pieris*. *J. Appl. Ecol.* 4:485–500.

DICKE, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods. *Entomol. Exp. Appl.* 91:131–142.

DICKE, M., and TAKABAYASHI, J. 1991. Specificity of induced indirect defence of plants against herbivores. *Redia* 74:105–113.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990. Plant strategies of manipulating predator-prey interactions through allelochemicals: prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

DUNNING, J. B. J., STEWART, D. J., DANIELSON, B. J., NOON, B. R., ROOT, T. L., LAMBERSON, R. H., and STEVENS, E. E. 1995. Spatially explicit population models: current forms and future uses. *Ecol. Appl.* 5:3–11.

ELKINTON, J. S., SCHAL, C., ONO, T., and CARDE, R. T. 1987. Pheromone puff trajectory and upwind flight of male gypsy moths in a forest. *Physiol. Entomol*. 12:399–406.

FATOUROS, N. E., VAN LOON, J. J. A., HORDIJK, K. A., SMID, H. M., and DICKE, M. 2005. Herbivore-induced plant volatiles mediate in-flight host discrimination by parasitoids. *J. Chem. Ecol*. 31:2033–2047.

FRITZSCHE-HOBALLAH, M. E.F., TAMO, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important? *J. Chem. Ecol.* 28:951–968.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73:289–297.

GEERVLIET, J. B. F., ARIENS, S., DICKE, M., and VET, L. E. M. 1998. Long-distance assessment of patch profitability through volatile infochemicals by the parasitoids *Cotesia glomerata* and *Cotesia rubecula* (Hymenoptera: Braconidae). *Biol. Control* 11:113–121.

GOUINGUENE, S., ALBORN, H., and TURLINGS, T. C. J. 2003. Induction of volatile emissions in maize by different larval instars of *Spodoptera littoralis*. *J. Chem. Ecol.* 29:145–162.

HARCOURT, D. G. 1966a. Major factors in survival of the immature stages of *Pieris rapae* (L.). *Can. Entomol.* 98:653–662.

HARCOURT, D. G. 1966b. Sequential sampling for the imported cabbageworm, *Pieris rapae* (L.). *Can. Entomol.* 98:741–746.

JAMES, D. G., and PRICE, T. S. 2004. Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. *J. Chem. Ecol.* 30:1613–1628.

JANSSEN, A., SABELIS, M. W., and BRUIN, J. 2002. Evolution of herbivore-induced plant volatiles. *Oikos* 97:134–138.

JONES, R. E. 1977. Movement patterns and egg distribution in cabbage butterflies. *J. Anim. Ecol.* 46:195–212.

JONES, R. E. 1987. Ants, parasitoids, and the cabbage butterfly *Pieris rapae*. *J. Anim. Ecol.* 56:739–749.

JONES, R. E., and IVES, P. M. 1979. The adaptiveness of searching and host selection behaviour in *Pieris rapae*. *J. Anim. Ecol.* 4:75–86.

JONES, R. E., NEALIS, V. G., IVES, P. M., and SCHEERMEYER, E. 1987. Seasonal and spatial variation in the juvenile survival of cabbage butterfly *Pieris rapae*: evidence for patchy density dependence. *J. Anim. Ecol.* 56:723–737.

KAISER, L., and CARDE, R. T. 1992. In-flight orientation to volatiles from the plant-host complex in *Cotesia rubecula* (Hymenoptera: Braconidae): increased sensitivity through olfactory experience. *Physiol. Entomol.* 17:62–67.

KAISER, L., WILLIS, M. A., and CARDE, R. T. 1994. Flight manoevers used by a parasitic wasp to locate host-infested plant. *Entomol. Exp. Appl.* 70:285–294.

KELLER, M. A. 1990. Response of the parasitoid *Cotesia rubecula* to its host *Pieris rapae* in a flight tunnel. *Entomol. Exp. Appl.* 57:243–249.

LEWIS, W. J., and MARTIN, W. R. Jr. 1990. Semiochemicals for use with parasitoids: status and future. *J. Chem. Ecol.* 16:3067–3089.

MATTIACCI, L., and DICKE, M. 1995. Host-age discrimination during host location by *Cotesia glomerata*, a larval parasitoid of *Pieris brassicae*. *Entomol. Exp. Appl.* 76:37–48.

MATTIACCI, L., ROCCA, B. A., SCASCIGHINI, N., D'ALESSANDRO, M., HERN, A., and DORN, S. 2001. Systemically induced plant volatiles emitted at the time of danger. *J. Chem. Ecol.* 27:2233–2252.

MURLIS, J., WILLIS, M. A., and CARDE, R. T. 2000. Spatial and temporal structures of pheromone plumes in fields and forests. *Physiol. Entomol.* 25:211–222.

NEALIS, V. G. 1990. Factors affecting the rate of attack by *Cotesia rubecula* (Hymenoptera: Braconidae). *Ecol. Entomol.* 15:163–168.

NORDLUND, D. A., LEWIS, W. J., and ALTIERI, M. A. 1988. Influences of plant-produced allelochemicals on the host/prey selection behavior of entomophagous insects, pp. 65–96, in P. Barbosa, and D. K. Letourneau (eds.). Novel Aspects of Insect-Plant InteractionsWiley, New York.

PARKER, F. D. 1970. Seasonal mortality and survival of *Pieris rapae* (Lepidoptera: *Pieridae*) in Missouri and the effect of introducing an egg parasite, *Trichogramma evanescens. Ann*. *Entomol. Soc. Amer.* 63:985–994.

PUENTE, M. E. 2007. Synchrony of herbivore presence, induced plant volatiles, and parasitoid response. PhD Dissertation. North Carolina State University, Raleigh. http://www.lib.ncsu.edu/theses/available/etd-03202007–165428/unrestricted/etd.pdf

PUENTE, M. E., KENNEDY, G. C., and GOULD, F. 2008. The impact of herbivore-induced plant volatiles on parasitoid foraging success: a general deterministic model. *J. Chem. Ecol*. DOI 10.1007/s10886-008-9471-x.

RICHARDS, O. W. 1940. The biology of the small white butterfly (*Pieris rapae*) with special reference to the factors controlling its abundance. *J. Anim. Ecol.* 9:234–288.

ROOT, R. B., and KAREIVA, P. M. 1984. The search for resources by cabbage butterflies (*Pieris rapae*): ecological consequences and adaptive significance of Markovian movements in a patchy environment. *Ecology* 65:147–165.

SATO, Y., and OHSAKI, N. 2004. Response of the wasp (*Cotesia glomerata*) to larvae of the large white butterfly (*Pieris brassicae*). *Ecol. Res.* 19:445–449.

TENHUMBERG, B., KELLER, M. A., POSSINGHAM, H. P., and TYRE, A. J. 2001. Optimal patch-leaving behaviour: a case study using the parasitoid *Cotesia rubecula*. *J. Anim. Ecol.* 70:683–691.

VAN DER MEIJDEN, E., and KLINKHAMER, P. G. L. 2000. Conflicting interests of plants and the natural enemies of herbivores. *Oikos* 89:202–208.

VAN DRIESCHE, R. G. 1988. Survivorship patterns of larvae of *Pieris rapae* (L.) (Lepidoptera: Pieridae) in Massachusetts kale, with special reference to mortality due to *Apanteles glomeratus* (L.) (Hymenoptera: Braconidae). *Bull. Entomol. Res.* 78:397–405.

VAN DRIESCHE, R. G., and BELLOWS, T. S. Jr. 1988. Host and parasitoid recruitment for quantifying losses from parasitism, with reference to *Pieris rapae* and *Cotesia glomerata*. *Ecol. Entomol.* 13:215–222.

VAN DRIESCHE, R. G., NUNN, C., KREKE, N., GOLDSTEIN, G., and BENSON, J. 2003. Laboratory and field host preferences of introduced *Cotesia spp*. parasitoids (Hymenoptera: Braconidae) between native and invasive *Pieris* butterflies. *Biol. Control* 28:214–221.

VOS, M., HEMERIK, L., and VET, L. E. M. 1998. Patch exploitation by the parasitoids Cotesia rubecula and Cotesia glomerata in multi-patch environments with different host distributions. *J. Anim. Ecol.* 67:774–783.

WANG, Q., GU, H., and DORN, S. 2004. Genetic relationship between olfactory response and fitness in *Cotesia glomerata* (L.). *Heredity* 92:579–584.

Some compounds emitted as part of the sex pheromone blend of a particular species can cause reduction in or cessation of upwind flight when they are admixed with the pheromone blend of another species. Such compounds, originally termed behavioral "inhibitors", have more recently come to be known as heterospecific behavioral "antagonists" because they interfere with the attraction of heterospecific males. Thus, these *behavioral* antagonists have been considered to act differently from conspecific pheromone components, which are sometimes termed behavioral "agonists" (c.f., Linn et al. 2003; Baker and Heath 2004). However, one critical factor that usually has been overlooked is that pheromone components themselves have the ability to decrease or even eliminate attraction of conspecifics, depending on their amounts in a blend relative to other components. When emitted at the wrong rates relative to other components, the resulting blends no longer comprise the "pheromone" of that species, and it follows that the compounds, in terms of the responses that they elicit, can be both pheromone components and behavioral antagonists, with the behavioral outcome dependent upon the ratio in which they are presented. Here, I propose that the underlying mechanism that results in these various behavioral outcomes is olfactory antagonistic balance, a concept that may facilitate our understanding of the evolution of moth pheromone systems.

Odorants, Odors, and Pheromone Components

It is important to recognize the difference between odorants and odors. Odorants are single volatile compounds; the use of odorants as stimuli allows us to understand neurophysiological and behavioral responses at a simple chemosensory level. Whereas a particular odorant may cause neurophysiological activity in designated olfactory receptor neurons (ORNs), behavioral activity may not result from exposure to this odorant. In contrast, odors are blends of odorants, and odors often produce behavioral responses only when presented in a limited range of blend ratios. Pheromones are odors, inasmuch as they are blends of odorants (i.e., the individual pheromone components), presented in the "correct" blend ratios for a particular species.

Olfactory Antagonistic Balance

I suggest that we should rethink the way we have traditionally distinguished pheromone components as being distinct from heterospecific pheromone antagonists, in terms of olfactory processing. I propose that *all* sex-pheromone-related compounds (including heterospecific antagonists) that mediate behavioral responses should be considered to act as antagonists through olfactory pathways, resulting in a continuum of various levels of attraction. When the antagonistic olfactory inputs are not balanced the compounds causing the imbalance, even including the compounds that at the optimal ratios would have comprised the pheromone, result in reduced attraction. Olfactory balance with regard to sex-pheromone-related compounds is consonant with the concept of "combinatorial coding" (c.f., Hildebrand and Shepard 1997; Vickers et al. 1998; Christensen et al. 2000) that has prevailed since the days of Vincent Dethier (1971) when it was called "across-fiber patterning". For sex-pheromone-related compounds, however, an advantage of the olfactory antagonistic balance concept is that it eliminates heterospecific, behavioral antagonist compounds from being viewed as acting differently on olfaction and subsequent behavioral responses than do conspecific pheromone components.

Consider, for instance, two-component sex pheromones that involve specific ratios of the components in *Yponomeuta* spp. moths [(i.e., (*E*)-11-tetradecenyl acetate and (*Z*)-11-tetradecenyl acetate; (E11-14:OAc and Z11-14:OAc)] (Löfstedt et al. 1990, 1991). For a given *Yponomeuta* species (Fig. 1), when one component is present in too high a ratio, attraction is diminished, or eliminated altogether. In such cases, the blend can no longer be considered to comprise that species' sex pheromone, and the out-of-range "pheromone component" now acts as a behavioral antagonist. For example, when E11-14:OAc is emitted in excessive amounts relative to Z11-14:OAc in any of the species in Fig. 1, creating an off-blend ratio, it acts antagonistically to stop or reduce attraction. Similarly, too much Z11-14:OAc that creates an off-ratio also stops or reduces attraction, and thus Z11-14:OAc can also act antagonistically.

Furthermore, it can be seen that blending the two pheromones of *Yponomeuta cagnagellus* and *Yponomeuta plumbellus*, which share only these same two 'components' but at widely disparate ratios, will result in the blended component ratios from the two species exerting behavioral antagonism on males of the other species, to the extent that little or no attraction will occur. Thus, pheromone components are only components of the pheromone odor when they are emitted at optimal ratios for the pheromone blend of that species. Otherwise, these olfactory antagonists also act as behavioral antagonists.

The only time the two components do not seem to act as behavioral antagonists is when they produce olfactory inputs, relative to the other pheromone-related odorants, that are centered around that species' pheromone blend ratio. I propose that in the behaviorally optimal pheromone blend, pheromone components could appropriately be viewed as balancing each others' olfactory antagonism at a particular position in that species' defined sex pheromone

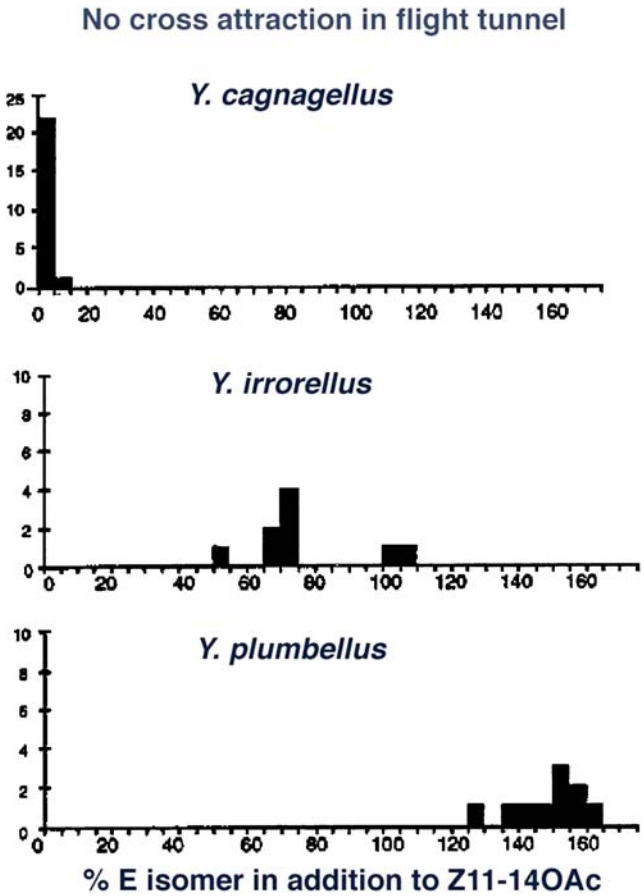

Fig. 1 Attraction of three *Yponomeuta* species that share the same host-plant (European spindle tree) to blends of their pheromone components in the wind tunnel (Löfstedt et al. 1991). There is no overlap in attraction to any of the cross-specific blends. Löfstedt et al. (1991) hypothesized that the species-specific blends arose as a result of blend interference between these sympatric and synchronic species that share the same host plant

"odor space" (Hallem and Carlson 2006). To use an analogy, opposing muscle groups are termed 'antagonists', and it is the balance of the forces exerted by each muscle group that results in various limb positions. Similarly, olfactory neuronal elements may be considered to act antagonistically, producing optimal behavioral outcomes only when their antagonism is balanced.

All pheromone-related inputs to the brain from the ORNs on the antennae, even those tuned to heterospecific behavioral antagonists, are transmitted in the form of excitatory action potentials to glomeruli in the macroglomerular complex (MGC). Among the first synaptic transmissions from the axon terminals of ORNs in the glomeruli are the excitation of gamma-aminobutyric acid (GABA)-ergic local interneurons, as well as direct synapses of ORN terminals with the dendrites of projection interneurons (Anton and Homberg 1999). There is evidence from neurophysiological studies of such synapses (Christensen et al. 1993) that the GABA-transmitted inhibition of one local interneuron can disinhibit a second local interneuron, resulting in excitation of projection interneurons that exit that glomerulus. These projection interneurons then send their excitatory outputs to higher centers such as the mushroom body and lateral protocerebrum (De Belle and Kanzaki 1999). Here, two negatives, through mutual disinhibition, can produce a positive, and this might be one of the neuronal bases for balanced olfactory antagonism. There may be other neurophysiological contributing factors as well. Excitation and further possible balanced antagonism in higher centers, such as the lateral protocerebrum and mushroom body, could also contribute to the ultimate behavioral outcome, that is, olfaction-stimulated, visually mediated flight that results in attraction to the correct blend.

I also propose that with regard to sex pheromone olfaction, the addition of a heterospecific behavioral antagonist to the pheromone blend is no different than the addition of excessive amounts of one of the sex pheromone components to the blend. In both instances, the new blend falls outside an optimal olfactory odor space, and the off-ratios result in an olfactory antagonistic imbalance due to an excessive amount of a given compound. The increased level of inhibition alters the balance of mutual disinhibition-related excitatory outputs, and ultimately results in reduction or even elimination of attraction.

This concept of olfactory antagonistic balance of sex-pheromone-related mediators of behavior does not require changing our definition of sex pheromones or of heterospecific behavioral antagonists. Rather, it provides a different way of thinking about the olfaction-related effects of the individual chemical components, and how they might influence sex pheromone olfaction-related pathways in ways that result in optimal or suboptimal attraction to the odor source.

Another reason that it makes sense not to label one set of pheromone component molecules as agonists (pheromone components, attractants) and another set the other way (antagonists) is because pharmacologically and neurophysiologically, they all act in the same way, as *agonists* to the odorant receptor (OR) that receive them on the ORNs. The ORNs all produce various frequencies of excitatory action potentials both in response to pheromone components and heterospecific antagonists. General odorants can produce both excitatory and inhibitory effects on action potential outputs of the ORNs on which the specific ORs are expressed (c.f., Hallem et al. 2004), but the odorants' initial interactions with their ORs are as pharmacological agonists, and the ORNs' outputs are still various levels of excitatory action potential frequencies.

In pheromone systems comprised of two or more pheromone components, attraction of males can occur in response to a partial blend or even a single component of the blend, but such partial blends are not optimal for

attraction (Linn et al. 1986, 1987). Even when the complete blend *is* emitted, impairment of a component-specific olfactory pathway through habituation or sensory adaptation due to pre-exposure to that single component can temporarily shift the balance of olfactory inputs to create a new, optimal blend ratio and render the true pheromone blend ratio as suboptimal (Linn and Roelofs 1981). In other cases that involve sensory impairment but with no pre-exposure, if the optimal blend ratio is emitted but at an excessively high rate such that upwind flight ceases, especially near the pheromone source, the underlying cause of suboptimal attraction can be found to be due to a suboptimal balance of inputs due to sensory adaptation. That is, the ORNs for one of the component-specific olfactory pathways can become differentially adapted to the excessive flux of that component in the blend, whereas the other ORNs tuned to the remaining two components retain their fidelity in reporting the flux specific to those components, thus creating an imbalance in the antagonistic interactions (Baker et al. 1989; Hansson and Baker 1991).

If we continue to compartmentalize the olfaction of pheromone components and heterospecific antagonists in our minds, instead of considering them as elements of an olfactory continuum, it will hamper progress in understanding the olfactory basis for shifts in male behavioral responses to novel sex pheromone blends, and our understanding of the evolution of divergent blends. With our ever-advancing knowledge of olfactory pathways, it is time to unify our thinking regarding the underlying processes involved in pheromone component and heterospecific antagonist olfaction. A unified model of pheromone and antagonist olfaction also may enhance and unify discourse between those who work on the evolution of sex pheromone olfaction and those who study the molecular and evolutionary aspects of olfaction of general odorants. For example, host-fruit odors attractive to one species of tephritid fruit fly cause a cessation of upwind flight by a second tephritid species when they are added to the latter species' preferred blend (Linn et al. 2005). Labeling the first species' host-fruit volatiles as being behaviorally antagonistic to the second species' is certainly appropriate, and yet I suggest that progress in understanding the evolution of these responses will be slowed if we structure our thinking along the lines of searching for special "antagonist"- or "agonist"-related *olfactory* pathways in such general odorant systems.

Asymmetric Tracking

In the concept of sex pheromone component antagonistic balance, a relaxation of the antagonism that would have otherwise occurred in response to excessive amounts of one compound, which we termed "olfactory antagonistic release" (Domingue et al. 2007), can be envisioned as a way for pheromone blends to shift away from their mean ratio in a first stage of reproductive character displacement, as particular types of "rare" males "track" the widely disparate blends of odd females, while retaining their responsiveness to the majority of females emitting blends centered around the norm of the population (Löfstedt 1990, 1993; Löfstedt et al. 1991; Phelan 1992, 1997). Such a scenario might explain the broadening of behavioral response profiles of male *Trichoplusia ni* (Liu and Haynes 1994; Haynes 1997). That is, after more than 40 generations of laboratory breeding, male *T. ni* subsequently were attracted as readily to the off-ratio blends produced by mutant females as they were to the blend of normal females. At the outset of these breeding experiments, males were attracted to mutant females' blends only at very low response rates, well below their level of attraction to the normal blend (Liu and Haynes 1994; Haynes 1997).

To illustrate this scenario in another way, the assortment of Z11/E11-14:OAc ratios (Fig. 1) seen in the three species of *Yponomeuta spp.* briefly discussed above were suggested to be the result of adaptive responses to "blend interference" from the pheromone blends of sympatric species sharing the same host plant (Löfstedt et al. 1991). We might view the three ratios, each of which is optimal for its respective species, as the result of an optimal antagonistic olfactory balance that is specific to that species. One of many ways we can hypothesize this end result as having occurred is that all three species' blends were originally closer to a 50:50 E/Z ratio (Fig. 2a). A broadening of males' responses then could have occurred in two of the species to include responses to rare (mutant) females that emit off-ratios different from the original blends. This broadening would have been facilitated by a relaxation ("olfactory antagonistic release") (Domingue et al. 2007) of the original degree of antagonism that otherwise would have been imposed in response to excessive amounts of either component. Olfactory antagonistic release could have occurred due to a desensitization or reduction in the number of neuronal elements somewhere along the ORN or central nervous system (CNS) pathway specific for carrying information initiated by the excitation of one of the two ORN types that respond to either Z11- or E11-14:OAc (Fig. 2b, left and right response curves, respectively).

Reproductive Character Displacement and Reinforcement

On the other hand, "olfactory antagonistic imposition" (strengthening of antagonism) (Domingue et al. 2007) would subsequently be involved in moving the shift in mean male responsiveness further away from the former mean, with the strength of antagonism on the other isomer's olfactory pathway being increased through increased ORN

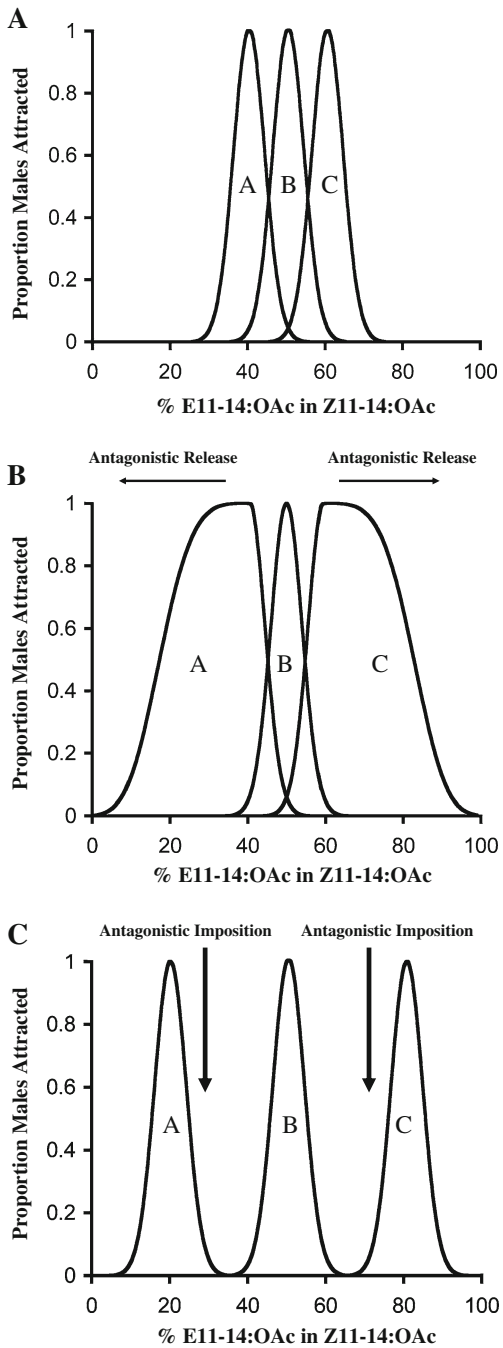

Fig. 2 Illustration of antagonistic release and imposition of changes in blend ratios that elicit male attraction in three hypothetical species that utilize Z11- and E11-14:OAc. **a** Original populations, in which there is a narrow range of Z11- and E11-14:OAc ratios to which males will be attracted. **b** After antagonistic release has occurred in males, the blend ratios to which males can respond are expanded in either direction to include off-ratios that may be emitted by rare mutant females in the population. The males' ranges of responsiveness include these variant blends, but males retain responsiveness to ratios in the previous norm so that such males can respond to both ancestral and derived pheromone blends. **c** After antagonistic imposition has occurred, males respond to new, tighter blend ratios in the derived populations, and thus assortative mating occurs in the new populations

or CNS sensitivity to that second isomer (Fig. 2c). If such a shift occurred as a response to blend interference (Löfstedt et al. 1991) between already existing species, then reproductive character displacement would result (Butlin and Ritchie 1989; Butlin and Trickett 1997). Alternatively, if this two-part process occurred during a speciation event, the imposition of increased antagonism would be implicated in reinforcement and subsequent reproductive isolation that is favored by selection (as the fitness of hybrids between the derived and ancestral populations decreases) so that males and females from the two populations can avoid fitness-related mating mistakes (Phelan 1992, 1997).

The addition of a third component to the sex pheromone blend can also impart species specificity to the new blend through this same two-part process. *Yponomeuta padellus*, a derived species of *Yponomeuta*, may have diverged in this way (Löfstedt et al. 1991), and its three-component blend sets it apart from two other *Yponomeuta* species that have similar two-component E11- and Z11-14:OAc blend ratios (Fig. 3). The process by which the third component might have been added, thus creating a three-component blend that males tracked, is hypothetically depicted in Fig. 3.

We hypothesize that there was first a broadening of responsiveness in rare males to include behaviorally beneficial olfactory antagonistic input from Z11-16:OAc, so they could respond to and track rare females emitting small amounts of this compound in addition to the original Z11- and E11-14:OAc blend (antagonistic release). This would have been followed by olfactory antagonistic imposition to keep *Y. padellus* males in the derived population from being attracted to the ancestral blends of *Yponomeuta viginctipunctatus* or *Yponomeuta evonomellus* females, which were emitting *only* ratios of the original two components, without Z11-16:OAc. These latter females, therefore, will have had antagonistically excessive amounts of this two-component blend in their emissions (Fig. 3).

Similar evolutionary scenarios for shifts in balanced antagonism can likely be constructed for the well-investigated pheromone systems in the Tortricidae and in heliothine moths. Two- and three-component pheromones in sympatric tortricid species, as in yponomeutids, often rely on tightly regulated, differing ratios of E11- and Z11-14:OAc, as well as on various additional components (Cardé et al. 1977; Roelofs and Brown 1982). In the sex pheromone systems of heliothine moths, although male behavioral response profiles to varying 2-component blend ratios are not as tightly regulated as in tortricids and yponomeutids, there is at least some degree of ratio specificity (Vickers et al. 1991; Vickers 2002). Also, upwind flight is inhibited in response to single "components" (Vickers et al. 1991), and by addition of heterospecific compounds to otherwise optimal pheromone component blends (Vickers and Baker 1997; Baker et al. 1998; Quero and Baker 1999). Notably,

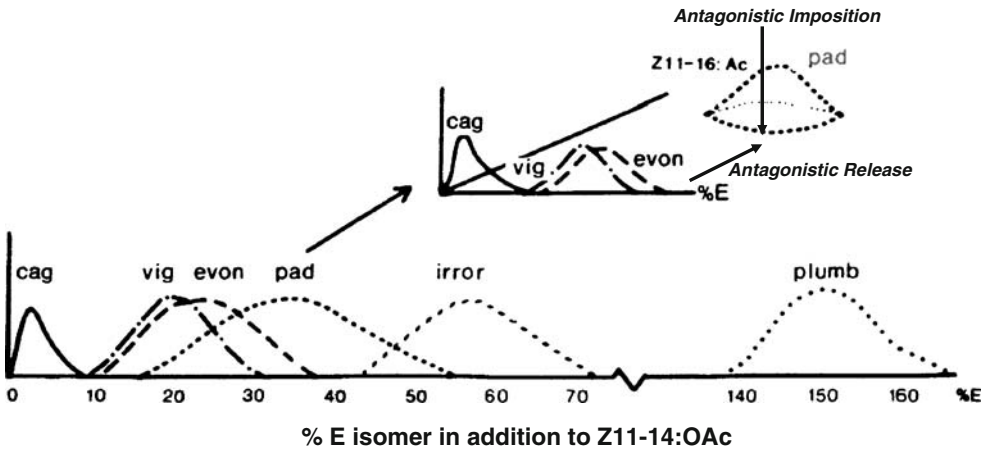

Fig. 3 Illustration of the pattern of antagonistic release, followed by antagonistic imposition, in the *Yponomeuta* species depicted by Löfstedt et al. (1991). *Y. padellus*, a derived species, has added a third pheromone component, Z11-16:OAc, to its blend, along with Z11- and E11-14:OAc. First, we hypothesize that antagonistic release has occurred to allow expansion of male responsiveness to this new, three-component blend. Then, we hypothesize that antagonistic imposition evolved to reinforce the assortative mating that will occur because of decreased fitness of hybrids from matings between the derived and ancestral populations, through increased sensitivity to blends emitting now-excessive amounts of the Z11- plus E11-14:OAc's (lacking Z11-16:OAc)

as in all other moth species in which antennal lobe neuroanatomy has been studied (Hansson and Christensen 1999), the glomeruli in heliothine males that receive inputs from ORNs tuned to heterspecific antagonists occur together in the MGC in close association with glomeruli that receive inputs from ORNs tuned to conspecific pheromone components (Christensen et al. 1995; Vickers et al. 1998; Berg et al. 1998, 2005; Vickers and Christensen 2003; Lee et al. 2006a, b). Alterations in balanced antagonism due to the mutual-inhibition (disinhibition) activities of GABA-ergic local interneurons would be most pronounced in this typical lepidopteran arrangement of closely spaced glomeruli for conspecific and heterospecific pheromone components in the MGC.

Single-component Pheromones and Balanced Antagonism

A saltational pheromone shift (Baker 2002; Roelofs et al. 2002) seems to have occurred in another derived species of yponomeutid, *Yponomeuta rorellus* (Löfstedt et al. 1986, 1990, 1991). This species' pheromone odor appears to be solely comprised of the unusual pheromone component, tetradecyl acetate (14:OAc). One of the types of ORN known to be involved in attraction in *Y. rorellus* responds with high activity to 14:OAc, but it also responds to E11- and Z11-14:OAc that are emitted by other sympatric *Yponomeuta* species in the environment. Attraction to other *Yponomeuta spp.* females that emit the ancestral blends containing Z11- or E11-14:OAc is prevented because of the activity of an ORN involved in behavioral antagonism that is stimulated by both E11- and Z11-14:OAc (Löfstedt et al. 1990, 1991). We view the broadened activity of this ORN that accepts either E11- and Z11-14:OAc as ligands as an example of olfactory antagonistic imposition (Domingue et al. 2007). Importantly, this behaviorally antagonistic ORN pathway is not merely responsive to any generic type of pheromone-related compound. Its lack of response to 14:OAc, as well as to the odd compounds (*E*)-6-tetradecenyl acetate and (*E*)-12-tetradecenyl acetate (E12-14:OAc) is apparently responsible for allowing equally high levels of attraction of *Y. rorellus* males to all three of these single compounds, all of which cause high firing rates in the attraction-related ORN (Löfstedt et al. 1990), despite the fact that only 14:OAc is the *Y. rorellus* pheromone component.

Single-component pheromone blends are rare in the Lepidoptera, but I maintain that they still fit the concept of attraction being due to balanced olfactory antagonism. First of all, even single component pheromones must be considered to be *blends* at the neurophysiological level because of the combinatorial coding process that compares the ratio of inputs into a single-component glomerulus (and out through projection interneurons) relative to the lack of activity occurring in other glomeruli in the absence of other pheromone-like odorants. Thus, even for single-component pheromones, inputs to the MGC are analyzed by the CNS as patterns of stimuli, i.e., "blends", with parts of the pattern consisting of the absence as well as the presence of activity in various glomeruli and along the rest of the pathways in the system. To make an analogy, in the auditory realm, the combinatorial code that produces recognition of single musical notes, as it does for chords, depends as much on the absence of many notes as it does on the presence of others.

Olfactory Pathways

Studies of *Drosophila* ORNs that respond to general odorants have shown that ORN activity is determined almost exclusively by whatever OR is expressed on that ORN (Hallem et al. 2004). In pheromone olfaction, modulating perireceptor factors such as binding proteins seem to affect the presentation of the pheromone component ligand to the OR more than in general odorant systems (Du and Prestwich 1995; Leal et al. 2005). The effects of degradative enzymes specific for pheromone components also seem to affect the time-course of ORN excitation more than in general odorant systems (Syed et al. 2006). Nevertheless, olfactory antagonistic release and imposition in moth sex pheromone systems will depend to a large degree on the up- or downregulation of OR gene expression on particular pheromone-component sensitive ORNs. Coexpression of two ORs on single ORNs is also possible in insects (Dobritsa et al. 2003; Goldman et al. 2005) and in moths might potentially contribute to shifts that broaden behavioral responsiveness to a wider array of pheromone blends (Baker et al. 2006), that affect both reinforcement and assortative mating.

Alternatively, a single OR itself might possibly have biochemical cross-affinities to two or more structurally related pheromone odorants, just as many ORs for general odorants do (c.f., Hallem and Carlson 2006). There are many examples in moths of ORNs that are tuned to a particular sex pheromone component, but they are also highly responsive to other molecules, even including totally synthetic analogs of the pheromone component that could not possibly comprise part of a sex pheromone blend of any species (c.f., Grant et al. 1989; Löfstedt et al. 1990; Berg et al. 1995).

It is difficult to imagine that there are OR genes in the genome specific for producing ORs having specific affinities for every one of such odd, non-naturally occurring molecules, and that they have suddenly become coexpressed on the dendrites of pheromone-component-responsive ORNs to cause this cross-responsiveness. Rather, cross-reactivity to structurally similar compounds by a single, broadly accommodating pheromone-component-tuned OR might provide a more logical explanation for such phenomena. Such ORs might be viewed as being preadapted to respond to odd conspecific females emitting such compounds, should such rare occurrences ever happen. Thus, such pheromone-odorant-related ORs that (perhaps serendipitously) accommodate structurally similar compounds might provide the basis for broader sex-pheromone-related ORN responsiveness (Linn et al. 2007a), which can result in either broader or narrower behavioral responsiveness, depending upon on which ORNs the ORs reside. Learning more about ORN coexpression of two ORs versus single expression of broadly tuned ORs should help guide our interpretations of the evolution of sex pheromone blends, particularly as they relate to shifts in sex-pheromone-related behavior.

Because the glomerular projection addresses of ORNs in insects do not change with whatever ORs are expressed on the ORNs (Dobritsa et al. 2003; Hallem et al. 2004; Goldman et al. 2005), shifts in the olfactory balance that change behavioral response specificity to certain sex pheromone blend ratios, therefore, might begin with ORN response profile shifts. Ultimately, the response profiles of ORNs, both chemically and temporally, rely on the expression of ORs that dictate stereotypical temporal spike-train characteristics and odorant tuning profiles of the ORNs (Hallem et al. 2004). Of course, changes in synaptic connectivity in the antennal lobe, such as the number, types, and targets of synapses in particular glomeruli, could affect the amount of inhibitory local interneuron activity as well as projection interneuron output to the mushroom body and lateral protocerebrum (Anton and Homberg 1999; De Belle and Kanzaki 1999; Hansson and Christensen 1999). Thus, shifts in olfactory antagonistic balance are not necessarily entirely dependent upon pheromone odorant OR gene expression and levels of ORN excitation.

Nevertheless, the predominance of OR gene expression in insects in determining both the overall activity levels and temporal characteristics of ORN action potential output (Hallem et al. 2004) indicates that the study of the response profiles of ORNs (DeBruyne et al. 1999, 2001) may to a large extent explain what occurs during shifts in pheromone-related olfactory pathways and in optimal attraction to various blends. This perspective has been supported by the neuroethological studies that concern geographic variation in moth sex pheromone communication systems, as exemplified in *Agrotis segetum* (c.f. Löfstedt 1990, 1993; Hansson et al. 1990). Further neuroethological studies on species that utilize highly "unusual" (from an anthropomorphic perspective) sex pheromone blend components should be particularly instructive for understanding how shifts in olfactory antagonistic balance might explain the evolution of sex pheromone blend shifts.

Possible Examples With *Ostrinia spp.*

Recent studies with *Ostrinia furnacalis*, the Asian corn borer (ACB), and *Ostrinia nubilalis*, the European corn borer (ECB), have implicated a mechanism involving ORNs on male antennae that explains how a few "rare" males (Roelofs et al. 2002; Linn et al. 2003, 2007b) in the population are attracted to both their own ECB or ACB sex pheromone blend, as well as to the entirely different blend of the other species (Domingue et al. 2007; Linn et al. 2007b). The ACB is considered to be a derived species

(Ishikawa et al. 1999), and is the only *Ostrinia* species that uses ~ 1:2 to 1:1 E:Z blends of E12-14:OAc and (*Z*)-12-tetradecenyl acetate (Z12-14:OAc) as its sex pheromone. The vast majority of ACB males are attracted only to their own ACB blend, and Takanashi et al. (2006) and Domingue et al. (2007) found that these "normal" ACB males, as with the rare males, have attraction-related ORNs that respond to both the Z11-/E11-14:OAc components of ECB and also to the Z12-/E12-14:OAc ACB components. However, normal ACB males have an ORN tuned to (*Z*)-9-tetradecenyl acetate (Z9-14:OAc) that is involved in heterospecific behavioral antagonism and which responds also to Z11-14:OAc (Takanashi et al. 2006; Domingue et al. 2007), preventing attraction to the ECB blend (Domingue et al. 2007). This heterospecific behavioral antagonism-related ORN in *rare* ACB males does not respond to Z11-/E11-14:OAc, and thus it does not impede attraction to the ECB blend, nor does it impede attraction to the ACB blend due to its lack of activity to Z12-/E12-14:OAc (Domingue et al. 2007).

Domingue et al. (2007) considered the rare ACB males as being similar to the type of male that existed when ACB diverged from Z11/E11 species, during the first stage of asymmetric tracking (Phelan 1992, 1997). In this context, broadly tuned males would be able to be attracted to both the unusual ACB females emitting Z12- and E12-14:Oac, while retaining their responsiveness to the ancestral pheromone blend comprised of Z11- and E11-14:OAc. This stage of divergence of pheromone blends in the Lepidoptera had been previously noted as producing 'asymmetrical reproductive isolation" (Löfstedt et al. 1991) (as represented by the rare ACB males in our study) because males from the derived population (Fig. 2b) could respond to both the derived and the ancestral females, whereas ancestral population males could only respond to ancestral females.

The second stage of asymmetric tracking (Phelan 1992, 1997) involves the occurrence of assortative mating between females that emit the new blend and the derived males that respond to it. In a speciation event, the impetus for assortative mating would be a fitness disadvantage that arises in hybrids resulting from matings between the ancestral population females and males from the derived population (Phelan 1992, 1997). Ancestral females then should be selected to reject derived males for mating, and such males should subsequently be selected to not be attracted to these females because of the (ultimately) fruitless mating encounters with these females. The lack of responsiveness in derived males to the ancestral blend (here represented by the normal ACB males) could be accomplished by the emergence of behavioral antagonism to the old blend. Domingue et al. (2007) suggested that the responsiveness of the Z9-14:OAc behaviorally antagonistic pathway-related ORNs to Z11-/E11-14:OAc in these normal ACB males is evidence for this second step. After olfactory antagonistic imposition has occurred (Fig. 2c), full premating reproductive isolation would result (Löfstedt et al. 1991; Phelan 1992, 1997).

There are species of *Ostrinia* in Asia that have three-component pheromone blends comprised of Z11- and E11-14:OAc plus Z9-14:OAc (Ishikawa et al. 1999). These appear to have been derived from ancestral populations that use only Z11- and E11-14:OAc (Ishikawa et al. 1999). The scenario for the addition of this third component may have been the same as outlined above for the addition of Z11-16:OAc into the *Y. padellus* blend (Fig. 3).

Other studies are emerging that support the concept of olfactory antagonistic balance, with OR gene expression being a predominant factor (Hansson et al. 2007). The ECB "E"- and "Z"- pheromone strains' ORN glomerular projection destinations in MGC glomeruli, the MGC morphologies, and the projection interneuron sex pheromone component tuning profiles were investigated. The only difference between the blend-specific behavioral responsiveness of males of the two ECB strains was shown to be due to a swapping of the expression of ORs specific for Z11-14:OAc and E11-14:OAc in one strain onto the opposite colocalized ORNs (in the same sensillum) in the other strain (Hansson et al. 2007).

The specificities of the two ORNs thus seem to have been switched to respond now to the opposite isomer, without a concomitant switch in the glomeruli of the MGC to which the ORNs project. The new olfactory antagonistic balance that allows for the potential responses to the two widely disparate blends of the ECB Z-strain and E-strain females apparently has occurred only by a swapping of expression of the ORs, leaving the rest of the pathways unchanged to impart the same balanced olfactory inputs in the antennal lobe and farther up in the CNS, but now through reversed ORN specificities of response.

A similar interpretation that uses antagonistic olfactory balance also can be made regarding the study of Cossé et al. (1995) on F2 hybrids between the E- and Z-strains of ECB. This study showed that as predicted (W.L. Roelofs, unpublished), a significant portion of the F2s in one cross that were behaviorally E-strain males would readily fly upwind to the E-strain pheromone blend, even though neurophysiological studies on these males showed that they had Z-strain ORN architecture (spike-size relationships). The large-spiking ORNs in the antennal sensilla of this portion of the F2 males responded to Z11-14:Oac, and the co-compartmentalized small-spiking ORN responded to E11-14:OAc. Normally, E-strain behavioral responders have the reverse situation, with the large-spiking ORN responding to E11-14:OAc and the small-spiking ORN responding to Z11-14:OAc.

As in the study by Hansson et al. (2007), the results of Cossé et al. (1995) lend themselves to the interpretation that the ORs specifically responsive to each of the two pheromone components in these males have been swapped onto the neighboring, colocalized ORN, perhaps due to regulatory genes (Clyne et al. 1999; Endo et al. 2007; Ray et al. 2007). Because the same olfactory antagonistic balance has been maintained, even though the balance is now the result of a different ratio of components, strong upwind flight behavior can occur. The two MGC glomeruli receiving input from the same two ORNs cannot discern that the inputs are now coming from these ORNs responding to the geometric isomers opposite to those than they normally do. No changes in CNS wiring or CNS integration of these inputs need to have occurred in order for optimal attraction to continue to take place. Rather, the fact that strong behavioral responses are elicited provides strong evidence that the remainder of the pathway has remained unchanged, and that only a switch of ORs has occurred.

Summary

I propose this new model of olfactory antagonistic balance of sex-pheromone-related compounds as a possible way of unifying our thinking and discussions about the sex-pheromone-mediated behaviors that we observe, the olfactory pathways involved, and the evolution of sex pheromones. I suggest that the concept of olfactory antagonistic balance can revitalize our thinking and suggest new possibilities for research on the evolution of divergent sex pheromone blends, and on the combinatorial coding that is involved in the positive or negative behavioral outcomes displayed by individual male moths based on their olfactory discrimination abilities.

The upwind flight behaviors of male moths that are elicited or not elicited by correct or incorrect blends of moth sex pheromone "components" are directly related to reproductive success. Thus, a detailed knowledge of such behaviors provides a foundation for understanding speciation, reproductive character displacement, and hence the evolution of insect communication systems and olfaction in general. Studies of shifts in sex-pheromone related ORN response profiles and their regulation by expression, coexpression, or shifts in expression of putative OR genes, may uncover general principles that govern how such factors might cause evolutionarily important olfactory and behavioral shifts in response to general odorants, such as the host cues that mediate feeding and oviposition in insect general odorant olfactory systems (c.f. Linn et al. 2005; Olsson et al. 2006a, b). Finally, I suggest that studies of possible shifts in sex-pheromone-related ORN response profiles that are related to shifts in behavior and to pheromone OR gene expression will complement and augment continuing advances with identified OR/ORN systems such as those in *Drosophila, Anopheles, Aedes*, and *Culex spp*.

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References

ANTON, S., and HOMBERG, U. 1999. Antennal lobe structure, pp. 97–124, in B. S. Hansson (ed.). Insect OlfactionSpringer, Berlin.

BAKER, T. C. 2002. Mechanism for saltational shifts in pheromone communication systems. *Proc. Nat. Acad. Sci. USA* 99:13368–13370.

BAKER, T. C., and HEATH, J. J. 2004. Pheromones—function and use in insect control, pp. 407–460, in L. I. Gilbert, K. Iatro, and S. S. Gill (eds.). Molecular Insect Science, vol. 6. Elsevier, The Netherlands.

BAKER, T. C., HANSSON, B. S., LÖFSTEDT, C., and LÖFQVIST, J. 1989. Adaptation of male moth antennal neurons in a pheromone plume is associated with cessation of pheromone-mediated flight. *Chem. Senses* 14:439–448.

BAKER, T. C., FADAMIRO, H. Y., and COSSÉ, A. A. 1998. Moth uses fine tuning for odour resolution. *Nature (London)* 393:530.

BAKER, T. C., QUERO, C., OCHIENG, S. A., and VICKERS, N. J. 2006. Inheritance of olfactory preferences. II. Olfactory receptor neuron responses from *Heliothis subflexa* x *Heliothis virescens* hybrid moths. *Brain Behav. Evol.* 68:75–89.

BERG, B. G., TUMLINSON, J. H., and MUSTAPARTA, H. 1995. Chemical communication in heliothine moths IV. Receptor neuron responses to pheromone compounds and formate analogs in the male tobacco budworm moth *Heliothis virescens*. *J. Comp. Physiol. A* 177:527–534.

BERG, B. G., ALMAAS, T. J., BJAALIE, J. G., and MUSTAPARTA, H. 1998. The macroglomerular complex of the antennal lobe in the tobacco budworm *Heliothis virescens*: specified subdivision in four compartments according to information about biologically significant compounds. *J. Comp. Physiol. A* 183:669–682.

BERG, B. G., ALMAAS, T. J., BJAALIE, J. G., and MUSTAPARTA, H. 2005. Projections of male-specific receptor neurons in the antennal lobe of the oriental tobacco budworm moth, *Helicoverpa assulta*: a unique glomerular organization among related species. *J. Comp. Neurol.* 486:209–220.

BUTLIN, R. K., and RITCHIE, M. G. 1989. Genetic coupling in mate recognition systems: what is the evidence? *Biol. J. Linn. Soc.* 37:237–246.

BUTLIN, R. K., and TRICKETT, A. J. 1997. Can population genetics simulations help to interpret pheromone evolution?, pp. 548–562,

in R. T. Cardé, and A. K. Minks (eds.). Insect Pheromone Research: New DirectionsChapman & Hall, New York.

CARDÉ, R. T., CARDÉ, A. M., HILL, A. S., and ROELOFS, W. L. 1977. Sex pheromone specificity as a reproductive isolating mechanism among the sibling species *Archips argyrospilus* and *A. mortuanus* and other sympatric tortricine moths (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 3:71–84.

CHRISTENSEN, T. A., WALDROP, B. R., HARROW, L. D., and HILDEBRAND, J. G. 1993. Local interneurons and information processing in the olfactory glomeruli of the moth *Manduca sexta*. *J. Comp. Physiol. A* 173:385–399.

CHRISTENSEN, T. A., MUSTAPARTA, H., and HILDEBRAND, J. G. 1995. Chemical communication in heliothine moths. VI. Parallel pathways for information processing in the macroglomerular complex of the tobacco budworm moth *Heliothis virescens*. *J. Comp. Physiol. A* 177:545–557.

CHRISTENSEN, T. A., PAWLOWSKI, V. M., LEI, H., and HILDBRAND, J. G. 2000. Multi-unit recordings reveal context-dependent modulation of synchrony in odor-specific neural ensembles. *Nat. Neurosci.* 3:927–931.

CLYNE, P., CERTEL, S., DE BRUYNE, M., ZASLAVSKY, L., JOHNSON, W., and CARLSON, J. 1999. The odor specificities of a subset of olfactory receptor neurons are governed by Acj6, a POU-domain transcription factory. *Neuron* 22:339–347.

COSSÉ, A. A., CAMPBELL, M. G., GLOVER, T. J., LINN, C. E. Jr., TODD, J. L., BAKER, T. C., and ROELOFS, W. L. 1995. Pheromone behavioral responses in unusual male European corn borer hybrid progeny not correlated to electrophysiological phenotypes of their pheromone-specific antennal neurons. *Experientia* 51:809–816.

DE BELLE, S., and KANZAKI, R. 1999. Protocerebral olfactory processing, pp. 97–124, in B. S. Hansson (ed.). Insect Olfaction-Springer, Berlin.

DE BRUYNE, M., CLYNE, P. J., and CARLSON, J. R. 1999. Odor coding in a model olfactory organ: the *Drosophila* maxillary palp. *J. Neurosci.* 19:4520–4532.

DE BRUYNE, M., FOSTER, K., and CARLSON, J. R. 2001. Odor coding in the *Drosophila* antenna. *Neuron* 30:537–552.

DETHIER, V. G. 1971. A surfeit of stimuli, a paucity of receptors. *Am. Sci.* 59:706–715.

DOBRITSA, A. A., VAN DER GOES VAN NATERS, W., WARR, C. G., STEINBRECHT, R. A., and CARLSON, J. R. 2003. Integrating the molecular and cellular basis of odor coding in the *Drosophila* antenna. *Neuron* 37:827–841.

DOMINGUE, M. J., MUSTO, C. J., LINN, C. E. Jr., ROELOFS, W. L., and BAKER, T. C. 2007. Evidence of olfactory antagonistic imposition as a facilitator of evolutionary shifts in pheromone blend usage in *Ostrinia spp.* (Lepidoptera: Crambidae). *J. Insect Physiol.* 53:488–496.

DU, G., and PRESTWICH, G. D. 1995. Protein structure encodes the ligand binding specificity in pheromone binding proteins. *Biochemistry* 34:8726–8732.

ENDO, K., AOKI, T., YODA, Y., KIMURA, K.-I., and HAMA, C. 2007. Notch signal organizes the *Drosophila* olfactory circuitry by diversifying the sensory neuronal lineages. *Nat. Neurosci.* 10:153–160.

GOLDMAN, A., VAN DER GOES VAN NATERS, W., LESSING, D., WARR, C., and CARLSON, J. R. 2005. Coexpression of two functional odor receptors in one neuron. *Neuron* 45:661–666.

GRANT, A. J., MAYER, M. S., and MANKIN, R. W. 1989. Responses from sensilla on the antennae of male *Heliothis zea* to its major pheromone component and two analogs. *J. Chem. Ecol.* 15:2625–2634.

HALLEM, E. A., and CARLSON, J. R. 2006. Coding of odors by a receptor repertoire. *Cell* 125:143–160.

HALLEM, E. A., HO, M., and CARLSON, J. R. 2004. The molecular basis of odor coding in the *Drosophila* antenna. *Cell* 117:965–979.

HANSSON, B. S., and BAKER, T. C. 1991. Differential adaptation rates in a male moth's sex pheromone receptor neurons. *Naturwissenschaften* 78:517–520.

HANSSON, B. S., and CHRISTENSEN, T. A. 1999. Functional characteristics of the antennal lobe, pp. 125–161, in B. S. Hansson (ed.). Insect OlfactionSpringer, Berlin.

HANSSON, B. S., TÓTH, M., LÖFSTEDT, C., SZÖCS, G., SUBCHEV, M., and LÖFQVIST, J. 1990. Pheromone variation among eastern European and a western Asian population of the turnip moth *Agrotis segetum*. *J. Chem. Ecol.* 16:1611–1622.

HANSSON, B. S., DEKKER, T., and KÁRPÁTI, Z. 2007. Strain-specific pheromone processing in the European corn borer antennal lobe. Abstract, 23rd ISCE Annual Meeting. Jena, Germany, July 2007. pp. 43.

HAYNES, K. F. 1997. Genetics of pheromone communication in the cabbage looper, pp. 525–534, in R. T. Cardé, and A. K. Minks (eds.). Pheromone Research: New DirectionsChapman & Hall, New York.

HILDEBRAND, J. G., and SHEPARD, G. 1997. Mechanisms of olfactory discrimination: Converging evidence for common principles across phyla. *Annu. Rev. Neurosci.* 20:595–631.

ISHIKAWA, Y., TAKANASHI, T., KIM, C.-G., HOSHIZAKI, S., TATSUKI, S., and HUANG, Y. 1999. *Ostrinia* spp. in Japan: their host plants and sex pheromones. *Entomol. Exp. Appl.* 91:237–244.

LEAL, W. S., CHEN, A. M., ISHIDA, Y., CHIANG, V. P., ERICKSON, M. L., MORGAN, T. L., and TSURUDA, J. M. 2005. Kinetics and molecular properties of pheromone binding and release. *Proc. Natl. Acad. Sci. USA* 102:5386–5391.

LEE, S.-G., CARLSSON, M. A., HANSSON, B. S., TODD, J. L., and BAKER, T. C. 2006a. Antennal lobe projection destinations of *Helicoverpa zea*. Male olfactory receptor neurons responsive to heliothine sex pheromone components. *J. Comp. Physiol. A.* 192:351–363.

LEE, S.-G., VICKERS, N. J., and BAKER, T. C. 2006b. Glomerular targets of *Helicoverpa subflexa* male olfactory receptor neurons housed within long trichoid sensilla. *Chem. Senses* 9:821–834.

LINN, C. E. Jr., and ROELOFS, W. L. 1981. Modification of sex pheromone blend discrimination in male Oriental fruit moths by pre-exposure to (*E*)-8-dodecenyl acetate. *Physiol. Entomol.* 6:421–429.

LINN, C. E. Jr., CAMPBELL, M. G., and ROELOFS, W. L. 1986. Male moth sensitivity to multicomponent pheromones: the critical role of the female released blend in determining the functional role of components and the active space of the pheromone. *J. Chem. Ecol.* 12:659–668.

LINN, C. E. Jr., CAMPBELL, M. G., and ROELOFS, W. l. 1987. Pheromone components and active spaces: What do male moths smell and where do they smell it? *Science* 237:650–652.

LINN, C. Jr., O'CONNOR, M., and ROELOFS, W. L. 2003. Silent genes and rare males: a fresh look at pheromone response specificity in the European corn borer moth, *Ostrinia nubilalis*. *J. Insect Sci.* 3:151–6.

LINN, C. Jr., NOJIMA, S., and ROELOFS, W. L. 2005. Antagonist effects of non-host fruit volatiles on discrimination of host fruit by *Rhagoletis pomonella* flies infesting apple (*Malus pumila*), hawthorn (*Crataegus spp.*), and flowering dogwood (*Cornus florida*). *Entomol. Exp. Appl.* 114:97–105.

LINN, C. E. Jr., DOMINGUE, M. J., MUSTO, C., BAKER, T. C., and ROELOFS, W. L. 2007a. Support for (*Z*)-11-hexadecanal as a pheromone antagonist in *Ostrinia nubilalis*: flight tunnel and single sensillum studies with a New York population. *J. Chem. Ecol.* 33:909–921.

LINN, C. E. Jr., MUSTO, C. J., and ROELOFS, W. L. 2007b. More rare males in *Ostrinia*: response of Asian corn borer moths to the sex pheromone of the European corn borer. *J. Chem. Ecol.* 33:199–212.

LIU, Y.-B., and HAYNES, K. F. 1994. Evolution of behavioral responses to sex pheromone in mutant laboratory colonies of *Trichoplusia ni*. *J. Chem. Ecol.* 20:231–238.

LÖFSTEDT, C. 1990. Population variation and genetic control of pheromone communication systems in moths. *Entomol. Exp. Appl.* 54:199–218.

LÖFSTEDT, C. 1993. Moth pheromone genetics and evolution. *Phil. Trans. Roy. Soc. B* 340:167–177.

LÖFSTEDT, C., HERREBOUT, W. M., and DU, J.-W. 1986. Evolution of the ermine moth pheromone tetradecyl acetate. *Nature* 323:621–623.

LÖFSTEDT, C., HANSSON, B. S., DIJKERMAN, H. J., and HERREBOUT, W. M. 1990. Behavioral and electrophysiological activity of unsaturated analogues of the pheromone tetradecenyl acetate in the small ermine moth *Yponomeuta rorellus*. *Physiol. Entomol.* 15:47–54.

LÖFSTEDT, C., HERREBOUT, W. M., and MENKEN, J. 1991. Sex pheromones and their potential role in the evolution of reproductive isolation in small ermine moths (Yponomeutidae). *Chemoecology* 2:20–28.

OLSSON, S. B., LINN, C. E. Jr., and ROELOFS, W. L. 2006a. The chemosensory basis for behavioral divergence involved in sympatric host shifts. I. Characterizing olfactory receptor neuron classes responding to key host volatiles. *J. Comp. Physiol. A. Neuroethol. Sens. Neural Behav. Physiol.* 192:279–288.

OLSSON, S. B., LINN, C. E. Jr., and ROELOFS, W. L. 2006b. The chemosensory basis for behavioral divergence involved in sympatric host shifts II: olfactory receptor neuron sensitivity and temporal firing pattern to individual key host volatiles. *J. Comp. Physiol. A. Neuroethol. Sens. Neural Behav. Physiol.* 192:289–300.

PHELAN, P. L. 1992. Evolution of sex pheromones and the role of asymmetric tracking, pp. 265–314, in B. D. Roitberg, and M. B. Isman (eds.). Insect Chemical EcologyChapman & Hall, New York.

PHELAN, P. L. 1997. Genetics and phylogenetics in the evolution of sex pheromones, pp. 563–579, in R. T. Cardé, and A. K. Minks (eds.). Insect Pheromone Research, New DirectionsChapman & Hall, New York.

QUERO, C., and BAKER, T. C. 1999. Antagonistic effect of (Z)-11-hexadecen-1-ol on the pheromone-mediated flight of *Helicoverpa zea* (Boddie) (Lepidoptera: Noctuidae). *J. Insect Behav.* 12:701–709.

RAY, A., VAN DER GOES VAN NATERS, W., SHIRAIWA, T., and CARLSON, J. R. 2007. Mechanisms of odor receptor gene choice in *Drosophila*. *Neuron* 53:353–369.

ROELOFS, W. L., and BROWN, R. L. 1982. Pheromones and evolutionary relationships of Tortricidae. *Ann. Rev. Ecolog. Syst.* 13:395–422.

ROELOFS, W. L., LIU, W., HAO, G., JIAO, H., ROONEY, A. P., and LINN, C. E. Jr. 2002. Evolution of moth sex pheromones via ancestral genes. *Proc. Natl. Acad. Sci. USA* 99:13621–13626.

SYED, Z., ISHIDA, Y., TAYLOR, K., KIMBRELL, D. A., and LEAL, W. S. 2006. Pheromone reception in fruit flies expressing a moth's odorant receptor. *Proc. Natl. Acad. Sci. USA* 103:16538–16543.

TAKANASHI, T., ISHIKAWA, Y., ANDERSON, P., HUANG, Y., LÖFSTEDT, C., TATSUKI, S., and HANSSON, B. S. 2006. Unusual response characteristics of pheromone-specific olfactory receptor neurons in the Asian corn borer moth *Ostrinia furnacalis*. *J. Exp. Biol.* 209:4946–4956.

VICKERS, N. J. 2002. Defining a synthetic pheromone blend attractive to male *Heliothis subflexa* under wind tunnel conditions. *J. Chem. Ecol.* 28:1255–1267.

VICKERS, N. J., and BAKER, T. C. 1997. Chemical communication in heliothine moths. VII. Correlation between diminished responses to point-source plumes and single filaments similarly tainted with a behavioral antagonist. *J. Comp. Physiol.* 180:523–536.

VICKERS, N. J., and CHRISTENSEN, T. A. 2003. Functional divergence of spatially conserved olfactory glomeruli in two related moth species. *Chem. Senses* 28:325–338.

VICKERS, N. J., CHRISTENSEN, T. A., MUSTAPARTA, H., and BAKER, T. C. 1991. Chemical communication in heliothine moths III. Flight behavior of male *Helicoverpa zea* and *Heliothis virescens* in response to varying ratios of intra- and interspecific sex pheromone components. *J. Comp. Physiol. A* 169:275–280.

VICKERS, N. J., CHRISTENSEN, T. A., and HILDEBRAND, J. G. 1998. Combinatorial odor discrimination in the brain: attractive and antagonist odor blends are represented in distinct combinations of uniquely identifiable glomeruli. *J. Comp. Neurol.* 400:35–56.

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General Guidelines for Authors for Submission of Manuscripts that Contain Identifications and Syntheses of Compounds

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These guidelines have been developed to provide assistance to authors, with the goal of ensuring that compounds reported in the Journal, including both compounds that were previously known and compounds new to science, have been rigorously and unequivocally identified. The editors recognize that in some circumstances, because of the small amounts of material available from natural sources, it will not be possible to provide all the information suggested in the guidelines below. It is also impossible for the editors to anticipate all possible scenarios, given the variety of different types of structures, and the relative ease or difficulty of unambiguous identification of different types of compounds. In such cases, the editors will use their best judgment in determining whether:

1. authors are justified in claiming full identification of a compound,
2. the identification should be reported as tentative only, or
3. there is insufficient evidence to support the identification of a compound at all. In such cases, the editor may request that a compound be designated as an unknown.

The editors stress that these guidelines are not intended as barriers to publication. Rather, the intent is to help authors determine the information that is required to identify compounds properly, and to the extent possible, to prevent publication of information or structures that are incorrect. Publication of incorrect information is detrimental to the reputations of both the authors and the Journal. If authors are in doubt with regard to the data required to claim identification of new compounds, please contact the editors for guidance.

The editors also encourage authors to submit reasonable tentative identifications (clearly designated as such) for compounds for which they have partial but not necessarily conclusive identification information. As an example, such compounds might include analogs or homologs of a compound that is completely identified in a manuscript, where the spectral, chromatographic, or other data strongly suggest structural similarities. In short, the editors encourage the careful and responsible publication of results in a way that maximizes new information available to the research community while minimizing errors.

Please note the following:

1. It *will not be acceptable under any circumstances* to claim conclusive identification of a compound based only on a "match" with a computerized mass spectral data base spectrum. Any such claims will be rejected by the editors.
2. Normally, it will not be acceptable to claim *conclusive* identification of a compound when it is possible or even likely that the compound could be, for example, a different isomer or even a different compound than claimed. As an example, it may be difficult or impossible to claim conclusive identification of a monounsaturated alkenyl acetate by close matches of mass spectra and retention times alone because of uncertainty about the position and stereochemistry of the double bond. In cases such as this, wherever possible, the retention index and mass spectral data should be supported by, for example, microscale derivatizations that unambiguously demonstrate the double-bond position and geometry. This is used as an example: Different types of compounds will require different methods and pieces of information for proper identification.

I. Identification of Compounds

A. Conclusive Identification of Compounds New to Science

For the types of data listed below, authors are asked to use their *best judgment* as to the information required to identify a compound unequivocally. This will vary with the types and complexity of the molecules involved. Authors should use as many of the methods listed below (and additional methods as appropriate) as are required to completely and unequivocally identify a compound.

In cases where authors have data that strongly suggest, but do not conclusively prove, a particular structure, they are requested to list identifications as tentative. As mentioned above, a match with a database mass spectrum, *in the absence of other supporting data*, will not be sufficient to claim even a tentative identification.

Acceptable Methods

1. Mass spectral data or other confirmation of molecular weight/molecular formula. Determination of the molecular weight by chemical ionization mass spectrometry (CI-MS) or exact mass determination is encouraged. Elemental composition by combustion or elemental analysis is an acceptable alternative.
2. NMR data:
   (a) Basic proton spectral data, fully analyzed.
   (b) Basic carbon spectral data.
   (c) Other NMR data required to prove unequivocally the structure (e.g., results of NOE, COSY, HMBC, HSQC, other NMR experiments).
3. Matches with a standard synthesized to prove the structure.
4. Optical rotation for chiral compounds is desirable but not mandatory. Chromatographic analysis on a chiral stationary phase, or other more reliable and accurate methods of demonstrating enantiomeric purity, are preferred.
5. Optional: other supporting information such as results of microchemical tests, UV and IR data, chromatographic characteristics/retention indices, boiling or melting points, etc.
6. If applicable, unequivocal X-ray crystal structure data would be an asset.

For Very Small Amounts

1. Mass spectral data.
2. Results of microchemical tests to confirm the presence and positions of functional groups, or other methods that can provide structural information on very small quantities, are recommended.
3. Confirmation of structure with a standard synthesized to prove the structure, including exact matches of chromatographic and mass spectral data, taken under equivalent concentration and other operating conditions.
4. Any other information that helps to prove the structure unequivocally.

B. Previously Known Compounds for Which Data are Available in the Literature

It is not necessary to provide full and detailed structural information on compounds that are well known in the literature. Nevertheless, authors are strongly encouraged to confirm identification with an authentic standard whenever possible to minimize the possibility of errors.

In general terms, authors are requested to provide as many of the following types of information as necessary to support the identification of known compounds:

1. One or more literature references in which the compound has been properly identified from a particular source.
2. Matching mass spectrum with a known standard.
3. Matching chromatographic properties with a known standard. Corroborating analyses by using two or more chromatography columns with different stationary phases.
4. If necessary, matching NMR or other spectral data with a known standard.
5. Any other data required to prove the structure unequivocally. For example, microchemical tests to prove the presence or positions of functional groups, retention index matches, IR or UV spectral matches, etc.
6. If a compound is chiral, determination of its absolute configuration by comparison with known standards by using enantioselective gas chromatography. Chromatographic methods that provide unequivocal information on both absolute configuration and enantiomeric composition are preferred over measurements of optical rotation and comparison with rotations of known standards or literature values, which are affected by impurities, traces of solvent, etc.

II. Synthetic Routes and Synthesized Compounds

A. Synthesis of Compounds New to Science

1. Sufficient information should be provided to enable complete replication of the work by someone with a reasonable knowledge of synthetic chemistry.
2. Any novel or special sets of conditions, *particularly with regard to safety*, should be described in detail.

3. Sufficient spectral data, particularly NMR and MS data, should be provided for purified key intermediates that, in combination with the synthetic route and reagents used, will allow unequivocal identification of those intermediates.
4. For intermediates that were previously known, and which were made according to previously published syntheses, the conditions used should be summarized briefly, with a reference to the previous synthesis. Full spectral data for such intermediates are not required so long as the spectral data matched those previously reported; authors should provide an explicit statement to that effect, along with the relevant citation(s). Any changes of conditions from the published route should be described.
5. For intermediates that were not previously known, and for the final product, full details of the reaction conditions should be provided. In cases of common and well-known reactions (e.g., acetylation, tosylation, $LiAlH_4$ reduction), abbreviated descriptions of conditions are sufficient (reagents, temperature, solvent, time, other relevant conditions).
6. For intermediates that were not previously known, and for the final product, sufficient spectral data should be provided to prove the structures unequivocally. This typically should include proton and carbon NMR data, mass spectral data, and optical rotation data if applicable. Other requisite data might include IR and UV, ORD, etc. For chiral compounds, determinations of enantiomeric excess are recommended. However, if the enantiomeric excess has been determined for an intermediate that is then carried through several subsequent steps, in which there is no chance of epimerization of the chiral center(s), it is not necessary to determine the enantiomeric excess for each intermediate.
7. It is not necessary to provide full spectral data on intermediates that were carried through a series of *straightforward* steps without isolation and purification. However, it is expected that authors will provide corroborative spectral data on, at a minimum, every third or fourth intermediate.
8. Yields, chemical purities, and isomeric purities should be provided for all steps in which compounds are isolated and purified, and particularly for the final product.

B. Synthesis of Previously Known Compounds

a. By a Previously Published Route

1. Authors need only provide a reference, a statement that the compound was made as previously described, and sufficient spectral data to prove that they do indeed have the correct compound. Alternatively, an explicit statement that the spectral and physical properties of their product matched literature values, with the citation (s) given, will be sufficient.
2. Any deviations from the published route should be described.
3. Yield, chemical purity, and isomeric purity (including enantiomeric excess if applicable) should be provided for the final product.

b. By a New Route

1. Requirements are the same as for synthesis of previously unknown compounds, see above.

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New Hemiketal Steroid from the Introduced Soft Coral *Chromonephthea braziliensis* is a Chemical Defense against Predatory Fishes

Beatriz G. Fleury · Bruno G. Lages · Jussara P. Barbosa · Carlos R. Kaiser · Angelo C. Pinto

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Abstract Recent studies show that chemical defenses in the exotic soft coral *Chromonephthea braziliensis* Ofwegen (Nephtheidae, Alcyonacea) can be one of the reasons for the success of this introduced species. We report for the first time the detailed composition of the monohydroxylated sterol fraction and a new hemiketal steroid, 23-keto-cladiellin-A, isolated from the unpalatable hexane extract from *C. braziliensis*. Bioassay-guided fractionation of this extract revealed that this hemiketal steroid exhibits potent feeding deterrent properties against a natural assemblage of fishes at the natural concentration. The major sterol fraction, containing the monohydroxylated sterols, was inactive in the bioassay. The results suggest that this active molecule may be driving the observed success of the invasion of this soft coral along the Brazilian Atlantic coast.

Keywords *Chromonephthea braziliensis* · Nephtheidae · Alcyonacea · Soft coral · Exotic species · Steroid · Antifeeding · Chemical defense · Marine chemical ecology

B. G. Fleury (✉) · B. G. Lages
Departamento de Ecologia, IBRAG,
Universidade do Estado do Rio de Janeiro,
Rua São Francisco Xavier 524, PHLC Sala 220,
20.559-900, Rio de Janeiro, Rio de Janeiro, Brazil
e-mail: bgfleury@uol.com.br

J. P. Barbosa · C. R. Kaiser · A. C. Pinto
Departamento de Química Orgânica,
Universidade Federal do Rio de Janeiro,
Bloco A, CT, Ilha do Fundão,
Rio de Janeiro, Rio de Janeiro, 21.945-970, Brazil

Introduction

Biological invasions in marine environments are one of the lesser understood aspects of global change (Vitousek et al. 1996). They represent a serious ecological and economic threat leading to decreased biodiversity, unbalanced ecosystems, and fishery and tourism impairment (Reise et al. 2006; Occhipinti-Ambrogi 2007; Olenin et al. 2007).

Biological invasions have been considered to be rapid evolutionary changes (Lee 2002), but little is known about their patterns and processes in marine ecosystems (Sax et al. 2007). Recent studies, however, have demonstrated that chemical defensive strategies may facilitate the invasion of the Indo-Pacific exotic soft coral *Chromonephthea braziliensis* Ofwegen (2005) in the Brazilian Atlantic coast (Lages et al. 2006).

Soft corals are among the major benthic invertebrates that compete for space on tropical reefs, particularly in the Indo-Pacific Ocean (Coll 1992). Their evolutionary success in areas of high levels of predation has been attributed to their production of significant amounts of complementary (secondary) metabolites (Sammarco and Coll 1997; Blunt et al. 2007) in their tissues, which may function as predator-deterrents and serve other ecological functions (Coll 1992; Sammarco and Coll 1992; Mizobuchi et al. 1996; Slattery et al. 1999; McClintock and Baker 2001; Fleury et al. 2004, 2006; Paul et al. 2006).

The ecological and evolutionary consequences of complementary metabolites have been considered only recently, and their effects on marine biodiversity are now recognized (Hay and Fenical 1996). There is currently little evidence, however, of the adaptive response of marine invasive species under selective pressure from a new environment.

As part of our continuing interest in the complementary metabolites responsible for the invasive success of *C.*

braziliensis, we conducted a bioassay-guided fractionation of the components of the organic extract of this exotic coral. The results indicate an active molecule that may be driving the observed success. We report the isolation and identification of a new hemiketal steroid, 23-keto-cladiellin-A, that acts as a chemical defense against a natural assemblage of fishes in the natural environment.

Methods and Materials

Coral Sampling Colonies of the exotic soft coral *C. braziliensis* Ofwegen 2005 (Nephtheidae, Alcyonacea) (identified before as *Stereonephthya* aff. *curvata*, Ferreira 2003) were collected in May 2004 in a sheltered rocky shore, by SCUBA diving at about 8-m depth at Saco dos Cardeiros, Arraial do Cabo region (23° 44′ S–42° 02′ W), state of Rio de Janeiro, southeastern coast of Brazil. This region is a marine harvest reserve and sustains unique reef systems characterized by different hydrodynamic regimes. It exhibits a rich benthic community and associated ichthyofauna (over 150 fish species) (Ferreira et al. 2001). Colonies of *C. braziliensis* were kept frozen (−25°) until analysis in the laboratory.

Field Assay Field assays were performed at Pedra Vermelha, Arraial do Cabo, Brazil. Crude extract, fractions, and pure compound were incorporated at their natural volumetrically concentrations reconstituted into a matrix of carrageenan-based artificial diet at the same concentration as occurred in the fresh soft coral tissue (Lages et al. 2006). Carrageenan (Sigma C-1013 type 1) food strips were prepared by an established methodology (Fenical and Pawlik 1991; Pawlik and Fenical 1992), involving combination of 3.75 g carrageenan, 60 ml distilled water, and 30 ml commercial tunafish purée packed in water (Pawlik and Fenical 1992; Epifanio et al. 1999, 2007). The extract, fractions, or pure compound dissolved in methanol were added to this mixture. For each experiment, 20 treated and 20 control food strips (1.0×0.6×5.0 cm each) were prepared and arranged in pairs and attached to 20 ropes that were randomly deployed at Pedra Vermelha coast. During the experiments, several common tropical fishes were observed in the studied area (Chaetodontidae, Haemulidae, Labridae, and Pomacentridae families) (Ferreira et al. 2001). After 4 to 6 h, the ropes were retrieved, and the amount of each strip eaten was measured. The *Wilcoxon paired-sample test* (one-tail) was used to analyze the results (Zar 1996).

Bioassay-Guided Fractionation Freeze-dried colonies of *C. braziliensis* (286 g dry wt) were cut into small pieces and exhaustively extracted with *n*-hexane under ultrasonication at room temperature. After removal of the solvent under reduced pressure, 9.2 g of a brownish gum was obtained and used for assays of antifeeding activity. Part of this organic extract (4.26 g) was fractioned by silica gel chromatography, employing *n*-hexane with increasing concentrations of ethyl acetate. The fractions with similar chemical compositions were combined after thin-layer chromatography (TLC) analysis to yield five fractions (F1 to F5). Fraction 2 (F2; 1.81 g) was further purified by repeated flash chromatography, yielding in order of elution, fractions A and B, which were both crystallized from methanol to give two different white solids, identified by spectroscopic methods [gas chromatography (GC)/mass spectrometry (MS), ultraviolet (UV), infrared (IR), MS, ^{1}H and ^{13}C and 2D nuclear magnetic resonance (NMR)], and comparison with literature data (Fleury et al. 1994; Zhang et al. 2005). Procedures for structure elucidation of fraction 1 and compound 1 are described below.

Analytical Procedures The Fourier transform IR spectrum was recorded on a Nicolet-Magna 760 spectrophotometer in KBr pellets. The UV spectrum was determined with a UV-visible Cary 3E Varian spectrophotometer. Electron spectroscopic imaging (ESI)–MS was obtained in positive ion mode on a Q-TOF mass spectrometer (Micromass, Manchester, UK). GC–MS analysis was carried out on an HP Model 5973 with electron impact ionization at 70 eV. The monohydroxylated sterol fraction was submitted to GC/MS analysis on capillary DB-5 column (i.d.: 0.25 mm; length: 30 m; thickness: 0.33 μm), in program mode from 200 to 290°C, 10°min^{-1}, and 10 min at the upper limit (split injection 1:20). Sterol mixture compositions were calculated (%) from the GC peak areas and identified by comparison with literature data (Fleury et al. 1994) and Wiley 275 Mass Library. The NMR spectrum was performed on a Bruker AVANCE-300 spectrometer operating at 300 and 75 MHz, for ^{1}H and ^{13}C, respectively (c=10 mg/0.6 mL in $CDCl_3$+ 0.05% TMS using a 5-mm tube, at 298 K). Melting point was measured by using a Melt Temp® apparatus and is uncorrected. Isolation procedures were monitored by employing TLC on pre-coated silica gel plates (Merck, Whitehouse Station, NJ, USA, Kieselgel 60 F-254) and UV inspection, and with ceric sulfate/heat pretreatment.

Results

Field Assays Initial field assays performed with the *n*-hexane extract revealed that it deterred a natural assemblage of fish consumers (P<0.05), as previously found (Lages et al. 2006). This assay was a guide to subsequent purification

of the active compound. Five fractions from this crude extract also were incorporated into artificial diets at their natural volumetric concentrations, and tested in situ, against fish consumers. The results showed significant inhibitory activity for F2 ($P<0.05$, Fig. 1).

Similar bioassay-guided separation of the active component F2, via repeated silica gel chromatography purification afforded two fractions A and B. These were further purified by crystallization using methanol, yielding, respectively, the monohydroxylated sterol mixture as fraction 1 (535 mg, 0.20% dry wt soft coral) (see Table 1) and the pure compound, a new hemiketal steroid, 23-keto-cladiellin-A (**1**) (357 mg, 0.12% of dry wt soft coral) (Fig. 2). The major fraction had no significant inhibition on feeding relative to controls ($P>0.05$; Fig. 3). In fact, we observed fish consumers feeding on several test strips. On the other hand, feeding deterrent properties were restricted to the more polar compound (1), which significantly reduced consumption of food strips by fishes relative to controls ($P<0.01$; Fig. 3).

Monohydroxylated Sterols Fraction The mixture of nine monohydroxylated sterols showed four dominant compounds: cholesterol, campesterol, epibrassicasterol, and 22-dehydrocholesterol. Table 1 reports the molecular ion and identification of the sterol fraction by GC/MS in comparison with literature data (Fleury et al. 1994) and Wiley 275 Mass Library. Diagnostic fragment ions at *m/z* 255, 213, 145, and 105 indicated the presence of a δ^{5-}3β-ol structure (Budzikiewicz et al. 1964) substituted with different side chains.

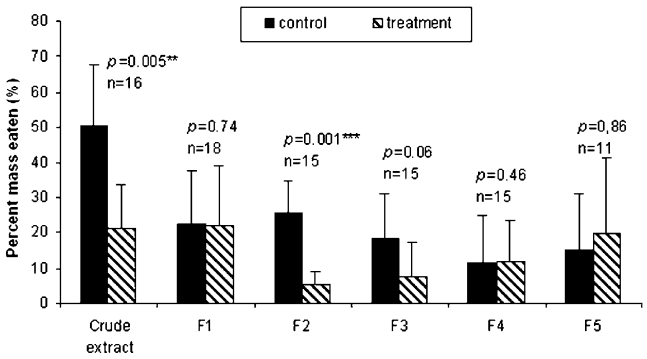

Fig. 1 Consumption of fishes on paired bait-strips with (treated) and without (control) hexane extract (crude extract), and five different fractions (F1 to F5) from this bioactive extract of *C. braziliensis*. *Vertical bars*, ±95% confidence limits. *n*, Number of pairs retrieved of 20 deployed. *P*, probability calculated by the Wilcoxon paired-sample test, one-tailed. *Asterisks*, statistically significant ($P<0.05$) reductions in feeding relative to palatable control

Structural Elucidation of the 23-keto-cladiellin-A Compound 1 (Fig. 2) had a molecular formula $C_{27}H_{38}O_4$, as determined by HRMS-ESI (nine degrees of unsaturation). Carbon-13 and DEPT NMR analysis showed the presence of four methyl groups, eight methylenes (all sp^3), nine methines (three sp^2 and six sp^3), and six quaternary carbon atoms (three sp^2 and three sp^3). The UV maximum at 242 nm was typical of a cross-conjugated cyclohexadienone functionality, which was substantiated by an IR absorption at 1,660 cm^{-1} and $^{13}C/^{1}H$ NMR signals at δ 186.4 (C-3), 123.9 (C-4), and 6.09 (H-4, d, 1.7 Hz), 127.6 (C-2) and 6.26 (H-2, dd, 10.2 and 1.7 Hz), 155.7 (C-1) and 7.09 (H-1, d, 10.2 Hz). The IR spectrum also showed an absorption at 1,716 cm^{-1}, characteristic of a ketonic carbonyl group, supported by ^{13}C NMR chemical shift at δ 208.3 (C-23). The remaining degrees of unsaturation of compound 1 could be accommodated by a steroid nucleus (3) and an additional ring (1). From the HMBC spectrum (Table 2), correlations between H-19/C-1, C-5, C-9, C-10 and H-4/C-2, C-6, C-10 were observed, elucidating rings A and B, as well as correlations between H-8/C-11, H-15β/C-8, C-13 constituting the C and D rings typical of the cholesta-1,4-dien-3-one steroidal skeleton. In this spectrum, two methyl group signals at δ 0.95 (H-26, d, 6.7 Hz) and 0.94 (H-27, d, 6.7 Hz) were correlated with C-25 (δ 23.9) and C-24 (δ 43.6), and H-24α,β was correlated with C-23 (δ 208.3). This framework was confirmed in the mass spectrum by the presence of the base peak at *m/z* 341 (^{+}O = C-CH_2-isopropyl, or M^{+}-85). The unusual quaternary carbon signal at C-22 (δ 98.8) indicated the presence of two oxygen atoms linked to it. Correlations between H-18α/C-12 and C-22, H-18β/C-12, C-13, C17, and the remaining degree of unsaturation demanded by the molecular formula, suggested the presence of a ring, part of an ether bridge. A hemiketal seemed most likely. Correlations in the NOESY spectrum observed between H-19 with H-6β and H-11β and H-18β with H-8β were in agreement with the relative stereochemistry of a normal 5α-cholestane skeleton (8β, 9α, 10β, 13β, 14α, 17β) (Mellado et al. 2005). The β-orientation of H-20 was confirmed by the correlation with H-17. Thus, the NOE correlation between H-21 and the hydrogen from the hydroxyl group on C-22 justify the β position of both. This was also supported by the correlation between H-18α,β and the hydrogen from the hydroxyl group. These assignments enabled us to conclude that the proposed structure represents a new marine steroid (Fig. 2). It was named as 23-keto-cladiellin-A because it is closely related to cladiellin A isolated from the soft coral *Cladiella* sp. (Zhang et al. 2005). However, cladiellin A is unstable in both pyridine-d_6 and $CDCl_3$ solutions yielding dehydration products, but this is not the case of the present 23-keto-cladiellin-A, which is stable even in DMSO-d_6.

Table 1 Monohydroxylated sterols composition of exotic soft coral *C. braziliensis* determined by GC/MS analysis

M	Molecular formula	Area (%)	Compounds[a]
384	$C_{27}H_{44}O$	14.0	22-Dehydrocholesterol = 22(*E*)-cholesta-5,22-dien-3 β-ol
386	$C_{27}H_{46}O$	23.4	Cholesterol = cholesta-5-en-3 β-ol
398	$C_{28}H_{46}O$	16.0	Epibrassicasterol = 22(*E*), 24 (*S*)-Ergosta-5,22–3β-ol
398	$C_{28}H_{46}O$	9.1	Brassicasterol = 22(*E*)-24(*S*)-24-Methylcholesta-5,22-dien-3β-ol
400	$C_{28}H_{48}O$	18.4	Campesterol = 24(*R*)-24-Methylcholesta-5,24-dien-3β-ol
412	$C_{29}H_{48}O$	3.5	28-Isofucosterol = 24(*Z*)-24-Ethylcholesta-5–24(28)-dien-3β-ol
412	$C_{29}H_{48}O$	8.8	28(*E*)-Ethylcholesta-5,22-dien-3β-ol
414	$C_{29}H_{50}O$	5.0	Sitosterol = 24Ethylcholesterol(24ξ)-Ethylcholesta-5–3β-ol
426	$C_{30}H_{50}O$	1.8	24(*E*)-propylcholesta-5,24-dien-3β-ol or 24(*E*)-isopropylcholesta-5,24-dien-3β-ol

DB5-capillary column
M, molecular weight
[a] Wiley 275 Mass Library and Fleury et al. 1994

Discussion

Octocorals provide an interesting model system to test for the presence of defensive interactions because they express physical and chemical defenses (Van Alstyne et al. 1994; Kelman et al. 1999; Koh et al. 2000). Generally, the physical protection afforded by a more highly calcified polypary in Nephtheidae family (Alcyonacea) appears to be associated with the lack of chemical defense (Sammarco et al. 1987). In fact, some Nephtheidae species commonly use spicules as physical defense (Coll 1992), although there are more known examples of chemical defenses in this family (Coll 1992; Lages et al. 2006).

The secondary metabolites from marine invertebrates that play a defensive role against predation usually are structurally complex and frequently present in high concentrations in animals that lack obvious physical defenses (Paul 1992; Pawlik 1993). We found the major sterol fraction, isolated from the exotic soft coral *C. braziliensis*, that contained the monohydroxylated sterols to be inactive in the field assay. A minor component, however, the steroid 23-keto-cladiellin-A, appears to have an antipredatory function. This new compound joins with a small number of metabolites from octocorals with proven antifeedant properties (e.g., Harvell et al. 1988; Wylie and Paul 1989; Fenical and Pawlik 1991; Pawlik and Fenical 1992; Gerhart and Coll 1993; Cronin et al. 1995; Epifanio et al. 1999, 2007; Maia et al. 1999). There are no clear correlations between the active and inactive metabolites. It is still early to make any generalizations about structure–function relationships and chemical defenses. A minor change in the stereochemistry, structure, or functionality of a deterrent compound may render it inactive (Pawlik 1993).

The field assay reported in this work was done under ecologically realistic conditions, in Brazilian waters, far from the natural habitat of *C. braziliensis*. Although the results reported herein were seen to be relevant, further studies, both in the laboratory and in the field, under more

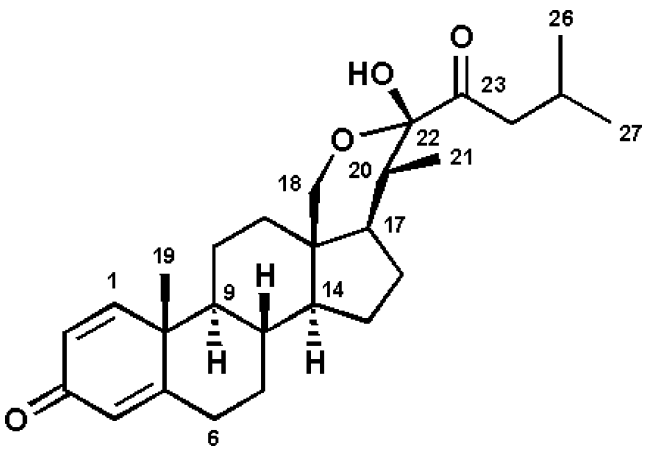

Fig. 2 Compound 1 (23-keto-cladiellin-A)

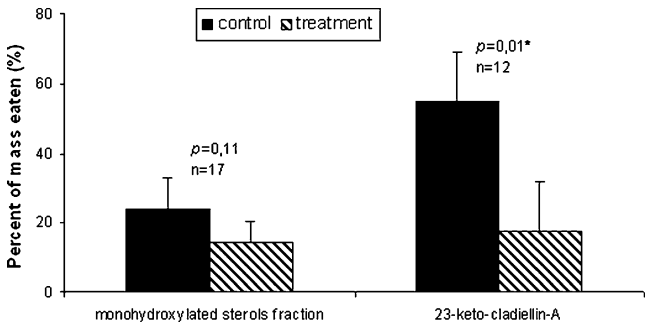

Fig. 3 Consumption of fishes on paired bait-strips with (treated) and without (control) different fractions from the bioactive F2 of *C. braziliensis*: The major fraction, monohydroxylated sterols fraction and compound 1 (23-keto-cladiellin-A). *Vertical bars*, ±95% confidence limits. *n*, Number of pairs retrieved of 20 deployed. *P*, probability calculated by the Wilcoxon paired-sample test, one-tailed. *Asterisk* statistically significant ($P<0.05$) reductions in feeding relative to palatable control

Table 2 NMR data for compound 1 (in $CDCl_3$)

Carbon	δ_C[a]	δ_H(*J*/Hz)	HMBC correlations	NOESY correlations
1	155.7 (d)	7.09 d (10.2)	C-3, C-5, C-9, C-10, C-21	H-2, H-21
2	127.6 (d)	6.26 dd (10.2, 1.7)	C-4, C-10	H-1
3	186.4 (s)	–		
4	123.9 (d)	6.09 d (1.7)	C-2, C-6, C-10	H-6β
5	169.0 (s)	–		
6α	32.7 (t)	2.46 dt (13.8, 13.8, 4.8)	C-7	H-21
6β		2.36 m		H-4
7α	33.8 (t)	1.97 m	C-9	
7β		1.03 m		
8	35.4 (d)	1.42 m	C-11	H-20α, H-20β
9	52.5 (d)	1.15 m	C-8	H-11β
10	43.7 (s)	–		H-1, 2, 4, 19
11α	22.5 (t)	1.86 m	C-9, C-12, C-13	H-8, H-21
11β		1.67 m	C-8	H-9
12α	32.1 (t)	2.37 m		H-17
12β		1.00 m		
13	42.2 (s)	–		
14	54.3 (d)	1.12 m		H-17
15α	24.3 (t)	1.71 m		H-22
15β		1.41 m		
16α	22.6 (t)	2.32 m		H-20α, H-22
16β		1.87 m	C-8, C-13	
17	48.3 (d)	1.63 m		H-12α, H-14, H-18
18α	58.6 (t)	3.80 d (11.4)	C-12, C-19	H-8, H-15α, 19-OH
18β		3.61 d (11.4)	C-12, C-13, C-17, C-19	H-8, H-11α
19	18.7 (q)	1.22 s	C-1, C-5, C-9, C-10	H-1, H-6α, H-11α
20	31.6 (d)	2.20 m	C-17	H-17
21	13.4 (q)	0.73 d (7.1)	C-17, C-18, C-19	H-15α, H-16α, 19-OH
22	98.8 (s)	–		
23	208.3 (s)	–		2H-24
24α	43.6 (t)	2.68 dd (17.8, 7.0)	C-23, C-25, C-26, C-27	H-26, 27
24β		2.38 m	C-23, C-25, C-26, C-27	H-26, 27
25	23.9 (d)	2.21 m	C-26, C-27	
26[b]	22.7 (q)	0.95 d (6.7)	C-24, C-25	H-24α, H-24β
27[b]	22.4 (q)	0.94 d (6.7)	C-24, C-25	H-24α, H-24β
19-OH	–	4.34 s	C-18, C-19, C-23	H-22, H-20α

[a] Multiplicities were determined by Dept-135 and Dept-90 experiments
[b] Signals can be interchanged

controlled conditions, with selected predator fish species are planned.

Recent studies of invasive marine species have highlighted the utility of using exotic species as model organisms (Sax et al. 2007), and, therefore, they outline a conceptual framework uniting the various mechanisms by which exotic species promote evolutionary diversification (Vellend et al. 2007). The absence of coevolved specialist enemies and the preferential consumption of native species by native generalists putatively generally give exotic species a competitive advantage over their native counterparts (Keany and Crawley 2002; Shea and Chesson 2002; Siemann and Rogers 2003). Further research on differential grazing between predators and co-occurring prey species is necessary to yield information that might support the enemy release hypothesis (Keany and Crawley 2002; Colautti et al. 2004). This theory may explain the effectiveness of the invasive species expansion in the new habitat.

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References

BLUNT, J. W., COPP, B. R., HU, W. P., MUNRO, M. H. G., NORTHCOTE, P. T., and PRINSEP, M. R. 2007. Marine natural products. *Nat. Prod. Rep.* 24:31–86.

BUDZIKIEWICZ, H., DJERASSI, C., and WILLIAMS, D. H. 1964. Structure Eclucidation of Natural Products by Mass Spectrometry: Steroids, Terpenoids, Sugars and Miscellaneous Classes, Vol. II. Holden-Day, San Francisco.

COLAUTTI, R. I., RICCIARDI, A., GRITGOROVICH, I. A., and MACISAAC, H. J. 2004. Is invasion success explained by the enemy release hypothesis. *Ecol. Lett.* 7:721–733.

COLL, J. 1992. The chemistry and chemical ecology of octocorals (Coelenterata, Anthozoa, Octocorallia). *Chem. Rev.* 92:613–631.

CRONIN, G., HAY, M. E., FENICAL, W., and LINDQUIST, N. 1995. Distribution, density, and sequestration of host chemical defenses by the specialist nudibranch *Tritonia hamnerorum* found at high densities on the sea fan *Gorgonia ventalina*. *Mar. Ecol. Prog. Ser.* 119:177–189.

EPIFANIO, R. D. A., MARTINS, D. L., VILLAÇA, R., and GABRIEL, R. 1999. Chemical defenses against fish predation in three brazilian octocorals: 11b, 12 b-epoxypukalide as a feeding deterrent in *Phyllogorgia dilatata*. *J. Chem. Ecol.* 25:2255–2265.

EPIFANIO, R. D. A., MAIA, L. F., PAWLIK, J. R., and FENICAL, W. 2007. Antipredatory secosterols from the octocoral *Pseudopterogorgia americana*. *Mar. Ecol. Prog. Ser.* 329:307–310.

FENICAL, W., and PAWLIK, J. R. 1991. Defensive properties of secondary metabolites from the Caribbean gorgonian coral *Erythropodium caribaeorum*. *Mar. Ecol. Prog. Ser.* 75:1–8.

FERREIRA, C. E. L. 2003. Non-indigenous corals at marginal sites. *Coral Reefs* 22:498.

FERREIRA, C. E. L., GONÇALVES, J. E. A., and COUTINHO, R. 2001. Community structure of fishes and habitat complexity on a tropical rocky shore. *Environ. Biol. Fishes* 61:353–369.

FLEURY, B. G., PEREIRA, M. V. G., SILVA, J. R. P., KAISIN, M., TEIXEIRA, V. L., and KELECOM, A. 1994. Sterols from Brazilian marine brown algae. *Phytochemistry* 37:1447–1449.

FLEURY, B. G., COLL, J. C., SAMMARCO, P. W., TENTORI, E., and DUQUESNE, S. 2004. Complementary (secondary) metabolites in an Octocoral competing with a scleractinian coral: effects of varying nutrient regimes. *J. Exp. Mar. Biol. Ecol.* 303:105–131.

FLEURY, B. G., COLL, J. C., and SAMMARCO, P. W. 2006. Complementary (secondary) metabolites in a soft coral: sex-specific variability, inter-clonal variability, and competition. *Mar. Ecol.* 27:204–218.

GERHART, D. J., and COLL, J. C. 1993. Pukalide, a widely distributed octocoral diterpenoid, induces vomiting in fish. *J. Chem. Ecol.* 19:2697–2704.

HARVELL, C. D., FENICAL, W., and GREENE, C. H. 1988. Chemical and structural defenses of Caribbean gorgonians (*Pseudopterogorgia* spp.). I. Development of an in situ feeding assay. *Mar. Ecol. Prog. Ser.* 49:287–294.

HAY, M. E., and FENICAL, W. 1996. Chemical ecology and marine biodiversity: insights and products from the sea. *Oceanography* 9:10–19.

KEANY, R. M., and CRAWLEY, M. J. 2002. Exotic plants invasion and enemy release hypothesis. *Trends Ecol. Evol.* 17:164–170.

KELMAN, D., BENAYAHU, Y., and KASHMAN, Y. 1999. Chemical defense of the soft coral *Parerythropodium fulvum fulvum* (Forskal) in the Red Sea against generalist fish. *J. Exp. Mar. Biol. Ecol.* 238:127–137.

KOH, L. L., GOH, N. K. C., CHOU, L. M., and TAN, Y. W. 2000. Chemical and physical defenses of Singapore gorgonians (Octocorallia: Gorgonacea). *J. Exp. Mar. Biol. Ecol.* 251:103–115.

LAGES, B. G., FLEURY, B. G., FERREIRA, C. E. L., and PEREIRA, R. C. 2006. Chemical defense of an exotic coral as invasion strategy. *J. Exp. Mar. Biol. Ecol.* 328:127–135.

LEE, C. E. 2002. Evolutionary genetics of invasive species. *TREE* 17:386–391.

MAIA, L. F., EPIFANIO, R. D. A., and FENICAL, W. 1999. New fish feeding deterrents, including a novel sesquiterpenoid heterogorgiolide, from the brazilian gorgonian *Heterogorgia uatumani* (Octocorallia, Gorgonacea). *J. Nat. Prod.* 62:1322–1324.

MCCLINTOCK, J. B., and BAKER, B. J. (eds.) 2001p. 610. CRC, New York.

MELLADO, G. G., ZUBÍA, E., ORTEGA, M. J., and LÓPEZ-GONZÁLEZ, P. J. 2005. Steroids from the Antartic Octocoral *Anthomastus bathyproctus*. *J. Nat. Prod.* 68:1111–1115.

MIZOBUCHI, S., ADACHI, K., and MIKI, W. 1996. Antifouling polyhydroxysterols isolated from a palauan octocoral of *Sinularia* sp. *Fish. Sci.* 62:98–100.

OCCHIPINTI-AMBROGI, A. 2007. Global change and marine communities: Alien species and climate change. *Mar. Pol. Bul.* 55:342–352.

OFWEGEN, L. 2005. A new genus of nephtheid soft corals from the Indo-Pacific. *Zool. Meded.* 79:1–236.

OLENIN, S., MINCHIN, D., and DAUNYS, D. 2007. Assessment of biopollution in aquatic ecosystems. *Mar. Pol. Bul.* 55:379–394.

PAUL, V. J. 1992. Ecological Roles of Marine Natural Products. pp. 164–188. Comstock, Ithaca.

PAUL, V. J., PUGLISI, M. P., and RITSON-WILLIAMS, R. 2006. Marine chemical ecology. *Nat. Prod. Rep.* 23:153–180.

PAWLIK, J. R. 1993. Marine invertebrate chemical defenses. *Chem. Rev.* 93:1911–1922.

PAWLIK, J. R., and FENICAL, W. 1992. Chemical defense of *Pterogorgia anceps*, a Caribbean gorgonian corals: a preliminary survey. *J. Exp. Mar. Biol. Ecol.* 108:55–66.

REISE, K., OLENIN, S., and THIELTGES, D. W. 2006. Are aliens threatening European aquatic coastal ecosystems. *Helg. Mar. Res.* 60:106–112.

SAMMARCO, P. W., and COLL, J. C. 1992. Chemical adaptations in the Octocorallia: evolutionary considerations. *Mar. Ecol. Prog. Ser.* 88:93–104.

SAMMARCO, P. W., and COLL, J. C. 1997. Secondary metabolites- or primary? Re-examination of a concept through a marine example. *Proc. 8th Int. Coral Reef Symp.* 2:1245–1250.

SAMMARCO, P. W., LA BARRE, S., and COLL, J. C. 1987. Defensive strategies of soft corals (Coelenterata: Octocorallia) of the Great Barrier Reef. III. The relationship between ichthyotoxicity and morphology. *Oecologia* 74:93–101.

SAX, D. F., STACHOWICZ, J. J., BROWN, J. H., BRUNO, J. F., DAWSON, M. N., GAINES, S. D., GROSBERG, R. K., HASTINGS, A., HOLT, R. D., MAYFIELD, M. M., O`CONNOR, M. I., and RICE, W. R. 2007. Ecological and evolutionary insights from species invasions. *Trends Ecol. Evol.* 22:465–471.

SHEA, K., and CHESSON, P. 2002. Community ecology theory as a framework for biological invasions. *Trends Ecol. Evol.* 17:170–176.

SIEMANN, E., and ROGERS, W. E. 2003. Herbivory, disease, recruitment limitation, and success of alien and native tree species. *Ecology* 84:1489–1505.

SLATERRY, M., HINES, G. A., STARMER, J., and PAUL, V. J. 1999. Chemical signals in gametogenesis, spawning and larval settlement and defense of the soft coral *Sinularia polydactyla*. *Coral Reefs* 18:75–84.

VAN ALSTYNE, K. L., WYLIE, C. R., and PAUL, V. J. 1994. Antipredator defenses in tropical pacific corals (Coelenterata: Alcyonacea): II.

The relative importance of chemical and structural defenses in three species of *Sinularia*. *J. Exp. Mar. Biol. Ecol.* 178:17–34.

VELLEND, M., HARMON, L. J., LOCKWOOD, J. L., MAYFIELD, M. M., HUGHES, A. R., WARES, J. P., and SAX, D. F. 2007. Effects of exotic species on evolutionary diversification. *Trends Ecol. Evol.* 22:481–488.

VITOUSEK, P. M., D´ANTONIO, C. M., LOOPE, L. L., and WESTBROOKS, R. 1996. Biological invasions as global environmental change. *Am. Sci.* 84:218–228.

WYLIE, C. R., and PAUL, V. J. 1989. Chemical defenses in three species of Sinularia (Coelenterata, Alcyonacea): effects against generalist predators and the butterflyfish *Chaetodon unimaculatus.* Bloch. *J. Exp. Mar. Biol. Ecol.* 129:141–160.

ZAR, J. H. 1996. Biostatistical Analysis. 3rd edn.Prentice hall, Englewood Cliffs.

ZHANG, G. W., MA, X. Q., KURIHARA, H., ZHANG, C. X., YAO, X. S., SU, J. Y., and ZENG, L. M. 2005. New hemiketal steroid from the soft coral *Cladiella* sp. *Org. Lett.* 7:991–994.

Essential oils traditionally have been used to repel or kill insects and protect stored grain (Isman 2000). Molluscicidal actions have been less well studied, but certain essential oil constituents are toxic to aquatic snails, including species that act as vectors for disease-causing parasites in vertebrates (De Souza et al. 1991; Singh et al. 1997; Lahlou and Berrada 2001).

Despite the wealth of literature on ecological and pesticidal effects, few studies of essential oils have considered the mechanisms of their toxic action. In insects, several workers have reported that some essential oils such as eugenol show high-affinity binding to octopamine receptors, and they suggest that effects on octopamine receptors are responsible for the insecticidal properties of at least some oils (Enan 2001, 2005; Kostyukovsky et al. 2002). This seems plausible in view of the very low doses needed for octopamine receptor binding and activation compared to most other effects of essential oils. Unfortunately, hardly any electrophysiological studies have been made on effects of the oils in insects (see Price and Berry 2006).

The situation in molluscs is even less clear. Essential oils reduce neuronal excitability, alter spike configuration, and block responses to some neurotransmitters, although octopamine or other monoamines have not been tested (Szabadics and Erdélyi 2000). No attempt has been made to relate the effects to toxic, repellent, or antifeedant actions. In the present study, we tested the effects of three essential oil constituents (citral, geraniol, and eugenol) on the nervous system of the freshwater pulmonate snail *Planorbis corneus*. This snail does not encounter the oils naturally, but we used it as a model because the buccal ganglia, which control feeding, are well characterized in some freshwater pulmonates and contain octopamine receptors (Hiripi et al. 1998). Behavioral experiments showed that the oils stopped the animals from feeding when added to their aquarium water and eventually killed them. Accordingly, we focused on electrophysiological effects in the buccal ganglia, which play a major role in coordination of feeding in pulmonate molluscs (Benjamin 1983; Vehovszky and Elliott 2002). Furthermore, most neuronal octopamine is located in the buccal ganglia (Hiripi et al. 1998), where it acts as a neurotransmitter and neuromodulator (Elliott and Vehovszky 2001), thus giving the opportunity to study effects of the oils on octopamine receptors and hence make comparisons with insects.

Methods and Materials

Animals Specimens of *P. corneus L.* were purchased from Blades Biological Supplies Ltd. (UK). They were maintained in freshwater aquaria (containing dechlorinated tap water) at room temperature (18–22°C) and fed on lettuce leaves.

Chemicals All chemicals and components of the physiological saline were purchased from Sigma-Aldrich (UK). Essential oils were citral [3,7-dimethyl-2,6-octadienal] (95%), geraniol [3,7-dimethyl-2,6-octadien-1-ol] (98%), and eugenol [2-methoxy-4-(2-propenyl)phenol] (99%). Citral is a mixture of the stereoisomers geranial and neral (Nakamura et al. 2003) and is structurally similar to geraniol; eugenol has an unrelated structure. (±)-Octopamine hydrochloride and the octopamine antagonists phentolamine hydrochloride, metoclopramide hydrochloride, and yohimbine hydrochloride were used to distinguish different subtypes of octopamine receptor and to test for activation or block of octopamine receptors by the oils. Dopamine hydrochloride and acetylcholine chloride were used to examine the specificity of effects of the oils on octopamine receptors.

In behavioral experiments, chemicals were dissolved in dechlorinated tap water. For electrophysiology experiments, they were dissolved in physiological saline containing: NaCl, 80 mmol l^{-1}; KCl, 4 mmol l^{-1}; $CaCl_2 \cdot 2H_2O$, 7 mmol l^{-1}; $MgCl_2 \cdot 6H_2O$, 5 mmol l^{-1}; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 20 mmol l^{-1} (pH 7.2). Care was taken to ensure that the essential oils, which are relatively insoluble, were actually in solution (see Price and Berry 2006, for details).

Behavior

Feeding Experiments Single snails, which had been deprived of food for 24 h, were placed separately into 100 ml of test solution (concentration 10^{-5}–10^{-3} mol l^{-1}). The same volume of water alone was used for controls. Solutions were changed every 24 h to prevent buildup of waste products and to avoid any possible loss of chemical potency that could occur through separation, degradation, or evaporation. Thirty animals per treatment were used and 30 nontreated controls.

At the start of experiments, a 4-cm^2 piece of organic lettuce was washed in dechlorinated tap water and placed in the center of each dish, and one snail was placed directly onto the lettuce. After 24 h, the area of lettuce that had been consumed was estimated by drawing its outline onto a graph paper. A new piece of lettuce was then added, and the procedure was repeated for 7 days.

Assessment of Animals The condition of the animals was assessed every 15 min for the first 2 h of exposure, then every 24 h by touching the tentacles with a pair of fine forceps. A healthy individual responded to the stimuli by rapidly retracting the tentacles or its whole body. Dead snails were unresponsive to stimuli, withdrawn into their shell, and often had a shriveled appearance.

Statistical Analysis LC_{50} values were calculated by using probit analysis (Finney 1952). Mean (±SE) amounts of food consumed were calculated and evaluated by two-tailed Student's *t* tests to determine significance between means.

Electrophysiology For intracellular recordings, isolated buccal ganglia were pinned with their dorsal surface uppermost to the Sylgard base of a Perspex experimental bath (volume 1 ml) that was perfused with physiological saline at 3 ml min^{-1} by using a peristaltic pump (Gilson Minipuls3). Individual neurons were impaled with a glass microelectrode filled with 3 mol l^{-1} KCl (15–25 MΩ). Extracellular recordings were made from buccal nerves in isolated preparations of the buccal mass (with attached buccal ganglia, salivary glands, and esophagus) by using a glass-tipped suction electrode. Substances were applied by perfusion at known concentration. Octopamine, dopamine, and acetylcholine also were applied locally by iontophoretic injection from a microelectrode filled with the substance at a concentration of 0.1 mol l^{-1} in distilled water (filtered through a 0.1-μm Millipore filter). Iontophoretic current was monitored to ensure constancy, and the saline flow was stopped during applications. Retaining currents were found to be unnecessary. Sufficient time was allowed between applications (at least 5 min) to avoid receptor desensitization.

Conventional electrophysiological apparatus was used (see Price and Berry 2006, for details). Recordings were further displayed and stored on a computer by using a micro1401 ADC interface (Cambridge Electronic Design, UK; sampling rate 60 KHz) and subsequently analyzed with Spike2 software (Cambridge Electronic Design, UK).

Statistics For effects on spike frequency, the frequency is expressed as mean (±SE), and significance of effects was evaluated by two-tailed Student's *t* tests.

Measurement of Esophageal Contractions The esophagus was isolated, without its connection to the buccal ganglia, and separate pieces of thread were tied to each end. It was then suspended vertically in a glass flask (10 ml) by anchoring it posteriorly to a weight at the base of the flask and tying it anteriorly to a transducer (SRI, UK) to record isotonic contractions that were monitored and displayed in the same way as the electrophysiological data (above). The preparation was maintained in saline, and the oils were added (dissolved in saline at their final concentration) from the bottom of the flask and overflowed at the top. Use of dyes in separate experiments showed that almost full substitution occurred within 20 s. This procedure in itself did not affect spontaneous contractions; initial attempts to remove some or all of the saline and then replace it with saline that contained an essential oil showed that esophageal contractions could change irrespective of the presence of the oil and, therefore, this method was not used. The tension was adjusted to about 2×10^{-4} N.

Results

Behavior

Symptoms of Toxicity Eugenol, citral, and geraniol were all toxic to *P. corneus* (threshold approximately 2.5×10^{-4} mol l^{-1}). During the first 2 h of exposure to the two highest doses (5×10^{-4} and 10^{-3} mol l^{-1}), all snails were inactive compared to controls. The tentacles were limp; the response to tactile stimulation was reduced, and the snails appeared swollen, causing the radula to protrude; these symptoms were labeled as "knockdown." Separate experiments showed that if snails displaying these symptoms were placed into fresh water, they always recovered; however, when kept in the experimental solutions, they were always dead by the end point of the experiment (168 h), thus making these symptoms good indicators of eventual mortality (all dead individuals displayed these symptoms prior to death). Dead snails tended to have a shriveled rather than swollen appearance. About 20% of knocked down individuals had everted genitalia after exposure to the oils for 24 h.

Analysis of Mortality At the end point of the experiment, the order of potency was eugenol > citral > geraniol (LC_{50} values 2.26, 2.93, and 3.28×10^{-4} mol l^{-1}, respectively; see Fig. 1a). LC_{50} values of the oils decreased with time, and the steepest decline occurred within the first 96 h (Fig. 1a).

Feeding The amount of lettuce eaten was recorded for all individuals that were considered healthy at the time of assessment. Figure 1b shows the results after 24 h; later time points are not shown because the number of animals (*N*) became too variable (due to mortality–knockdown). Eugenol caused a significant reduction in food consumption at 10^{-4} mol l^{-1} ($P<0.01$). Citral and geraniol decreased food consumption at 2.5×10^{-4} mol l^{-1} ($P<0.001$ and $P<0.05$, respectively). The two highest doses (5×10^{-4} and 10^{-3} mol l^{-1}) were not included in the analysis because of the high levels of knockdown that occurred after 24 h.

Electrophysiology

Biphasic Dose-Dependent Effects of Essential Oils on Buccal Ganglion Neurons Attention was focused mainly on the largest neuron in each ganglion, referred to here as the giant buccal cell (GBC). Citral and geraniol (5×10^{-5}–10^{-3} mol l^{-1}) produced an increase in the rate of

A.

LC_{50}

(95% confidence limits)

Oil	24 hr	48 hr	72 hr	96 hr	120 hr	144 hr	168 hr
Citral	[b]	5.64 (4.94-6.64)	4.17 (3.69-4.77)	3.24 (2.85-3.70)	3.11 (2.74-3.57)	2.99 (2.62-3.45)	2.93 (2.57-3.38)
Eugenol	17.07 (12.31-39.30)	13.75[a]	7.23 (4.45-19.15)	2.61 (1.67-5.28)	2.55 (1.61-5.17)	2.41 (1.51-4.95)	2.26 (1.41-4.78)
Geraniol	[b]	7.37 (4.64-16.79)	4.03 (3.59-4.51)	3.96 (3.50-4.50)	3.28 (2.57-4.61)	3.28 (2.57-4.61)	3.28 (2.57-4.61)

[a] *Estimates of 95% confidence limits could not be calculated reliably due to limitations of the dose range.*

[b] *No mortality.*

B.

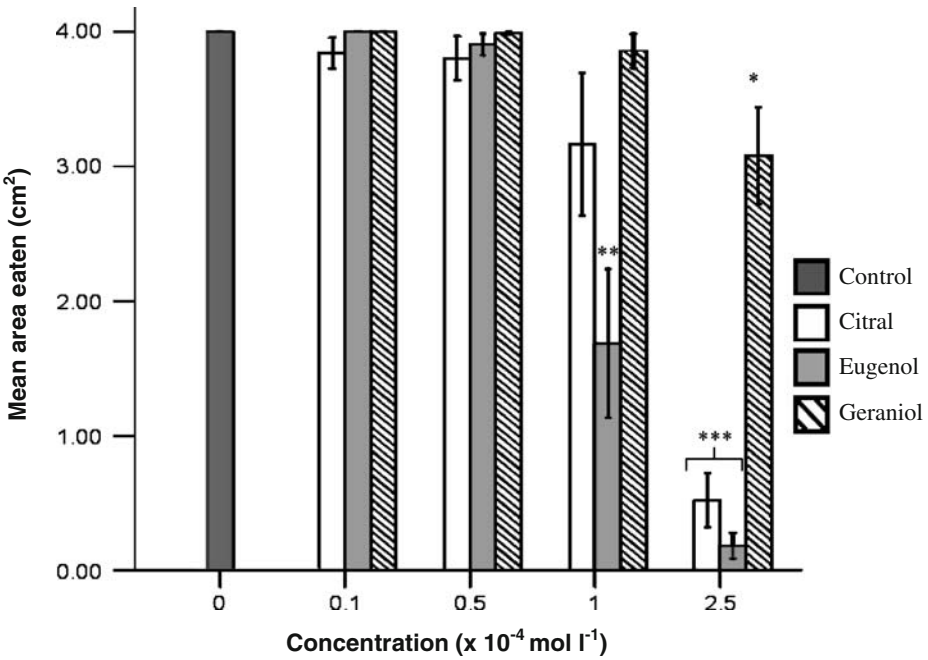

Fig. 1 **a** Table of values of LC_{50} ($\times 10^{-4}$ mol l^{-1}) for *P. corneus* at different times after exposure to citral, eugenol, and geraniol. **b** Bar chart showing effect of increasing concentrations of the oils on the mean area of lettuce eaten after 24-h exposure. *Significantly different from control, $P<0.05$; **$P<0.01$; ***$P<0.001$. $N=30$ for each oil (reduced at 2.5×10^{-4} mol l^{-1} to 29 for geraniol and 27 for citral and eugenol because of death–knockdown of animals). Concentrations of 5×10^{-4} and 10^{-3} mol l^{-1} were omitted from the analysis because of high levels of knockdown

spontaneous firing in the GBCs and all other cells tested in the buccal ganglia, while higher doses produced a reduction in firing rate (Fig. 2a). Eugenol was variable in its effects; at 10^{-3} mol l^{-1}, it decreased or blocked spontaneous firing within 5 min of application ($N=16$; Fig. 2b; threshold approximately 10^{-4} mol l^{-1}). In four of the above cells, however, there was an initial excitation lasting for 1–5 min (not shown; it was similar to the response to citral and geraniol). At doses that reduced spontaneous firing, all three oils reversibly blocked impulses elicited by applied depolarizing current (Fig. 2c).

Essential Oils Mimic Octopamine in Initiating Burst Firing in Buccal Ganglion Neurons The increased firing rate produced by the oils was generally a result of initiation of burst firing. In extracellular recordings from the buccal nerves, burst activity was induced by perfusion of the oils (Fig. 3a) or octopamine (Fig. 3b). Citral and geraniol elicited bursting in the GBCs or any cells recorded intracellularly from groups 3, 4, 5, or 7 (Fig. 3c; see Arshavsky et al. 1988 for classification of groups). Bursting did not appear to be endogenous because the frequency was unchanged when cells were hyperpolarized by applied current, and phase relationships with other neurons in the buccal ganglia were maintained. Spike bursts became shorter at higher doses of geraniol and citral (10^{-3} mol l^{-1}), and firing was eliminated at approximately 2×10^{-3} mol l^{-1}.

Perfusion of octopamine (threshold approximately 10^{-6} mol l^{-1}) also produced burst firing in quiescent and spontaneously active neurons in the buccal ganglia ($N=22$; Fig. 3d, upper). In the GBCs, however, octopamine (threshold approximately 10^{-6} mol l^{-1}) produced a hyperpolarization of 10–15 mV and usually elicited discrete inhibitory postsynaptic potentials that progressively de-

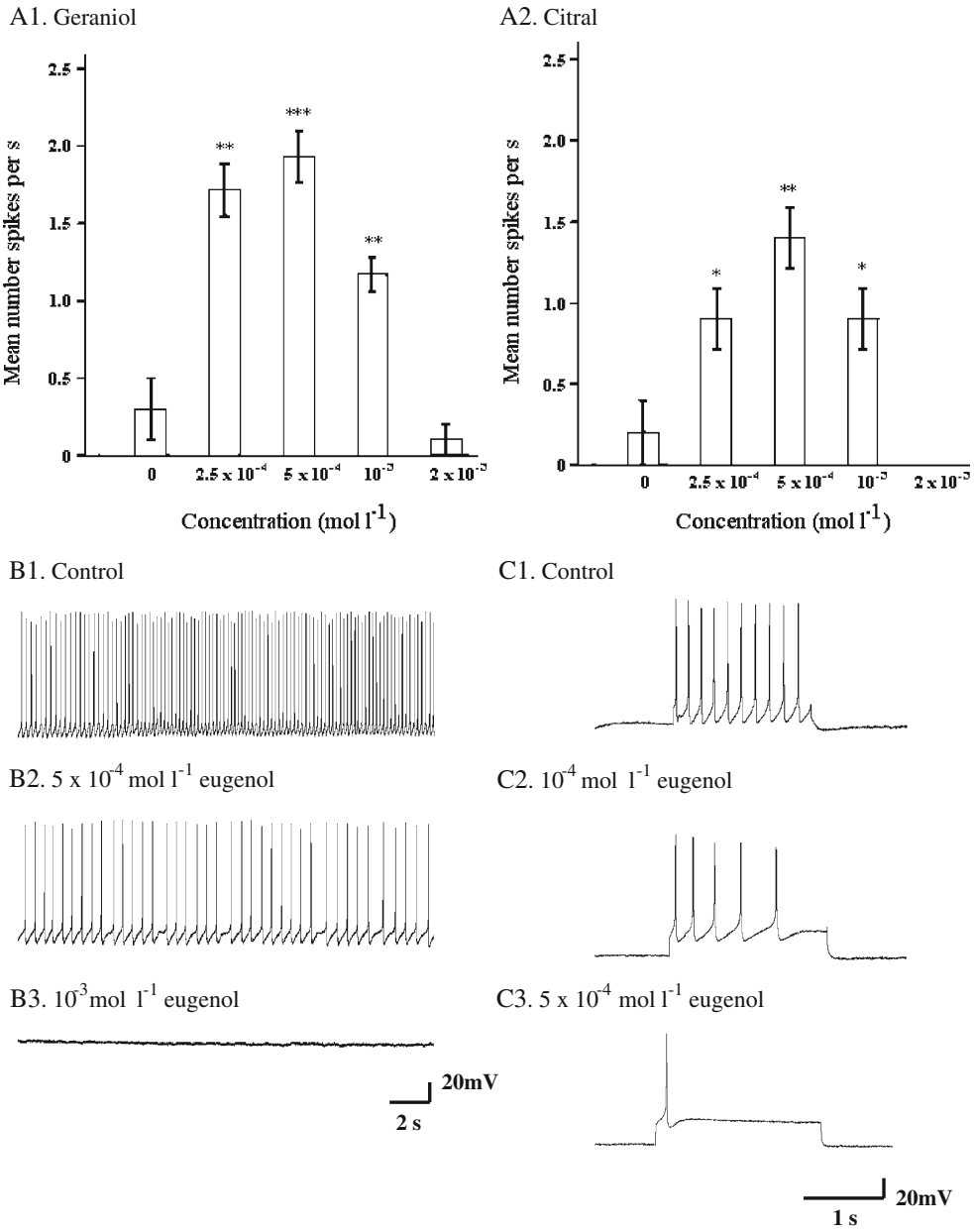

Fig. 2 Geraniol and citral have biphasic dose effects on the spontaneous firing rate of buccal ganglion neurons from groups 3, 4, 5, or 7, while eugenol is mainly depressant. **a** Bar charts showing biphasic effects of geraniol and citral on mean (±SE) spontaneous impulse frequency (N=8). Measurements were made for 5 min before introduction of the compound (control) and for 5 min after introduction (following a delay of 1 min). *Significantly different from control, $P<0.05$; **$P<0.01$ ***$P<0.001$. **b** Eugenol reversibly abolishes spontaneous activity (recovery not shown). (*B1*) Control. (*B2*) 5×10^{-4} mol l^{-1} eugenol reduces spike frequency. (*B3*) 10^{-3} mol l^{-1} eugenol abolishes spikes and synaptic input. **c** Eugenol reversibly abolishes spikes elicited by applied depolarizing pulses (different cell; amplitude of the current was the same in each recording; recovery not shown). (*C1*) Control. (*C2–C3*) Progressive reduction in response with increasing concentration. Similar effects were produced by citral and geraniol at approximately 2×10^{-3} mol l^{-1}; lower doses had no apparent effect on firing threshold

creased in amplitude with the hyperpolarization and were in phase with inputs in other buccal ganglion neurons that were induced to burst fire (N=27; Fig. 3d). Octopamine decreased excitability of GBCs and usually rendered them unexcitable at 10^{-4} mol l^{-1}.

The Oils Do not Appear to be Octopamine Receptor Agonists Since the oils mimicked octopamine in initiating and modulating burst firing, we investigated pharmacological evidence for their possible actions as octopaminergic agonists (as found in insects), and for this purpose we focused on the GBCs. The oils did not appear to activate octopamine receptors that mediate inhibition since they did not mimic octopamine in hyperpolarizing the GBCs; citral, geraniol, and (in some cases) eugenol produced a depolarization and excitation. They did, however, mimic effects of octopamine on GBCs following exposure to phentolamine.

Phentolamine (threshold approximately 10^{-7} mol l^{-1}) blocked the hyperpolarizing response of GBCs to octopamine, converting it to an excitatory response (N=15). This occurred whether octopamine was applied by perfusion (Fig. 4a) or iontophoresis (Fig. 4b) and suggested the unmasking of a depolarizing octopamine receptor on the GBCs (in two GBCs, there was a biphasic response to iontophoresis of octopamine, with a small depolarization preceding the hyperpolarization; not shown). In the presence of phentolamine, perfused octopamine produced a depolarization and (in four out of eight preparations) burst

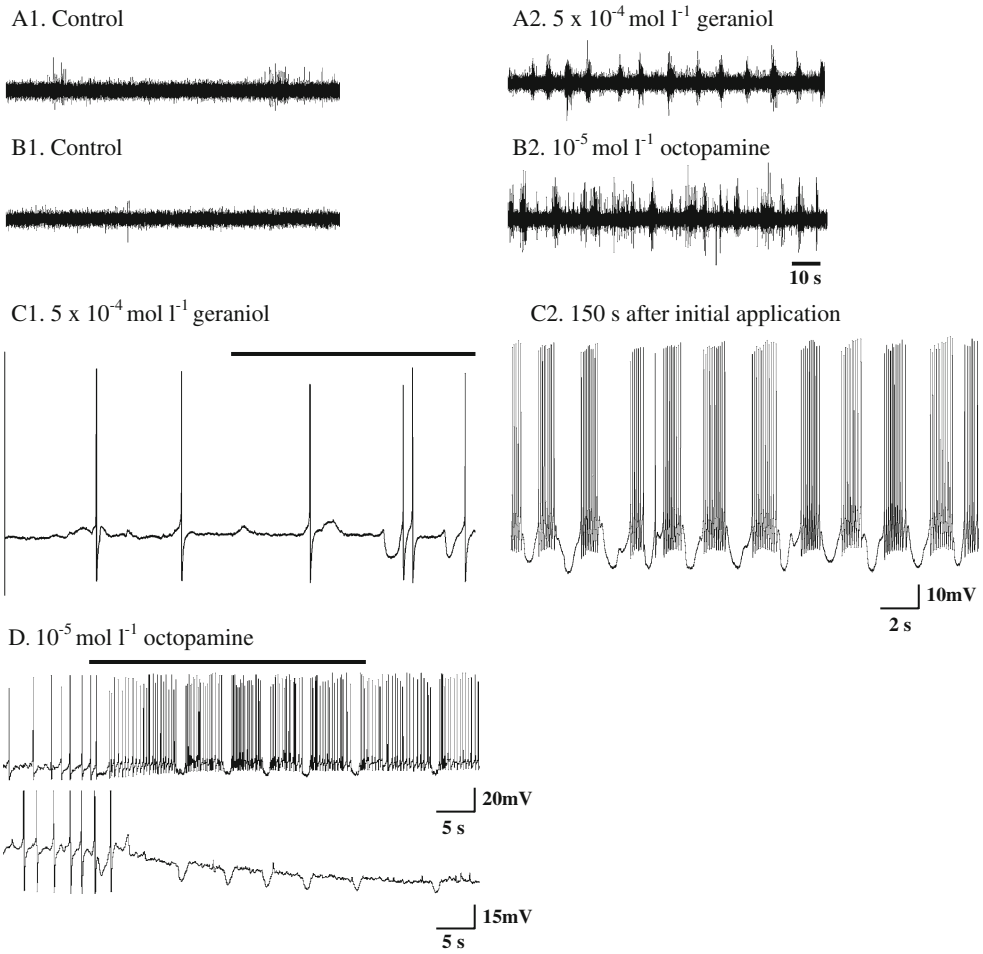

Fig. 3 Recordings to illustrate that citral and geraniol generally mimic octopamine in inducing burst firing in buccal ganglion neurons. Geraniol (**a**) and octopamine (**b**) induce burst firing in the right dorso-buccal nerve of an isolated preparation of the buccal mass. The extracellular recordings are from different preparations. Each pair of recordings is separated by approximately 2 min and the chemical was added within 10 s of the end of the control recording. (**c**) Geraniol elicits burst firing in a giant buccal cell. (**d**) Octopamine elicits burst firing in a group 5 neuron (*upper*) but the effect on a GBC (recorded simultaneously, *lower*) is hyperpolarizing, though it initiates inhibitory postsynaptic potentials that are in phase with those that cause bursting in the other neuron. The black bar represents addition of chemicals

firing in the GBCs similar to that produced by geraniol (Fig. 4, A2, compare Fig. 3c). Recovery from phentolamine was very slow, beginning at least 2 h after washout. Phentolamine (up to 5×10^{-4} mol l^{-1}) did not affect the excitatory (burst firing) response to octopamine in other buccal ganglion neurons (not shown). Yohimbine had similar effects to phentolamine on the response of GBCs to octopamine (conversion of hyperpolarization to depolarization), but higher doses were required (approximately 5×10^{-4} mol l^{-1}; N=4, results not shown).

Metoclopramide (10^{-4} mol l^{-1}) had no effect on hyperpolarizing octopamine responses in GBCs (N=5) but it blocked the excitatory responses to octopamine that occurred after administration of phentolamine (N=5; Fig. 5a). It also blocked the initiation of burst firing normally caused by octopamine in other cells tested (N=5; Fig. 5b). Thus, it provided a useful test for activation of excitatory octopamine receptors by the oils. None of the antagonists (up to 10^{-3} mol l^{-1}), however, had any effect on responses to eugenol, citral, or geraniol (N=5 for each oil; results not shown). There was, therefore, no pharmacological evidence to support activation of octopamine receptors (excitatory or inhibitory) by the oils.

The Oils Are not Specific Octopamine Receptor Antagonists The oils were tested for octopamine receptor antagonism by observing their effects on the response of the GBCs to octopamine. Submillimolar doses of eugenol had no effect on the hyperpolarizing response of GBCs to octopamine applied via perfusion or iontophoresis (N=5). Higher doses (approximately 10^{-3} mol l^{-1}) reduced the response, although these doses also greatly reduced or blocked spike activity. Total block occurred at 2×10^{-3} mol l^{-1} (not shown). Similarly, for citral and geraniol, doses below 10^{-3} mol l^{-1} had no effect on the hyperpolarizing response of GBCs to octopamine (N=5), even when the cell was burst firing as a result of oil application. Higher doses (approximately 2×10^{-3} mol l^{-1}) of geraniol and citral blocked the response (not shown). In other buccal ganglion cells, and in GBCs exposed to phentolamine, excitatory responses to octopamine were reduced by eugenol at 10^{-3} mol l^{-1} and blocked at 2×10^{-3} mol l^{-1} (N=4). Citral and geraniol also blocked excitatory responses at 2×10^{-3} mol l^{-1} (N=4); this dose affected spiking, but lower concentrations could not be tested because they were themselves excitatory. All blocking effects were reversible.

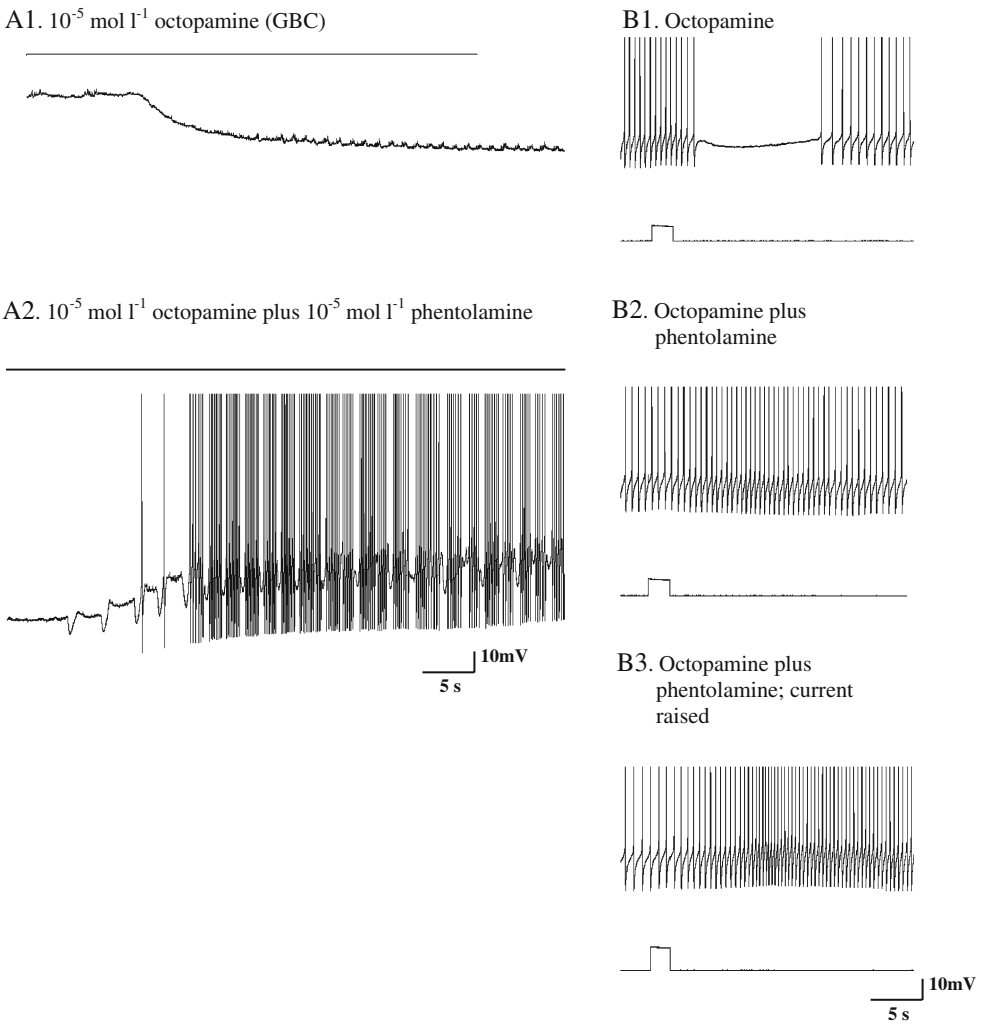

Fig. 4 Phentolamine reverses the inhibitory response of a giant buccal cell (GBC) to octopamine applied by perfusion (**a**) or iontophoresis (**b**). (*A1*) Perfusion of 10^{-5} mol l^{-1} octopamine hyperpolarizes a GBC. (*A2*) After 20-min exposure to 10^{-5} mol l^{-1} phentolamine, octopamine excites the same GBC and elicits burst firing similar to that induced by geraniol (see Fig. 3c). (*B1*) Control. Octopamine produces inhibition (iontophoretic current is shown below the recording; 250 nA). (*B2*) 20 min after introduction of 10^{-5} mol l^{-1} phentolamine, the response is a weak excitation. (*B3*) A larger current (350 nA) increases the excitation. The black bar represents addition of chemicals

The effects of these relatively high concentrations, however, were not specific to octopamine. Depolarizing and hyperpolarizing responses to dopamine also were reduced by the oils; these were tested by iontophoresis and by recording dopamine-mediated postsynaptic potentials in visceral ganglion neurons following stimulation of the giant dopamine neuron in the left pedal ganglion (Berry and Cottrell 1975). Excitatory responses were reduced by the oils at approximately 10^{-3} mol l^{-1}, while hyperpolarizing responses needed 2×10^{-3} mol l^{-1} (not shown; N=3 in each case). Depolarizing responses to iontophoretically applied ACh were reversibly reduced or abolished by all three oils at 5×10^{-4} mol l^{-1} (N=4; not shown).

Effects of Essential Oils on Gut Movements Effects of the oils on movements of the innervated buccal mass were in accord with their effects on the buccal ganglia. The buccal mass was reversibly excited into rhythmic activity by citral and geraniol (5×10^{-5}–10^{-3} mol l^{-1}) for up to 2 h (N=5 for each oil). Eugenol (threshold 10^{-4} mol l^{-1}) reversibly reduced spontaneous movements of the buccal mass (N=5).

Eugenol, citral, and geraniol (10^{-5}–2×10^{-3} mol l^{-1}) all had the same effect on the isolated esophagus of reversibly increasing the frequency of contractions and progressively reducing their amplitude (Fig. 6a; N=9 for each oil). There was often an initial increase in amplitude (Fig. 6b), and at 5×10^{-4} mol l^{-1} and above there was always a transient sustained contraction (Fig. 6c). In preparations that showed complex patterns of activity, this became simplified by abolition of the smaller contractions. At low concentrations, the effects were short lasting (approximately 5 min at 10^{-5}–5×10^{-5} mol l^{-1}), and at higher concentrations there was at least partial recovery of amplitude and frequency in the continued presence of the oil.

The esophagus also responded to octopamine (N=10; Fig. 6d). At 5×10^{-4}–10^{-3} mol l^{-1}, there was always a transient sustained contraction, but at lower concentrations there was considerable variation in sensitivity among preparations. A reduction in amplitude and increase in frequency of contractions occurred at 5×10^{-6} mol l^{-1} in four preparations and at 5×10^{-4} mol l^{-1} in the others. There was at least partial recovery in the continued presence of octopamine.

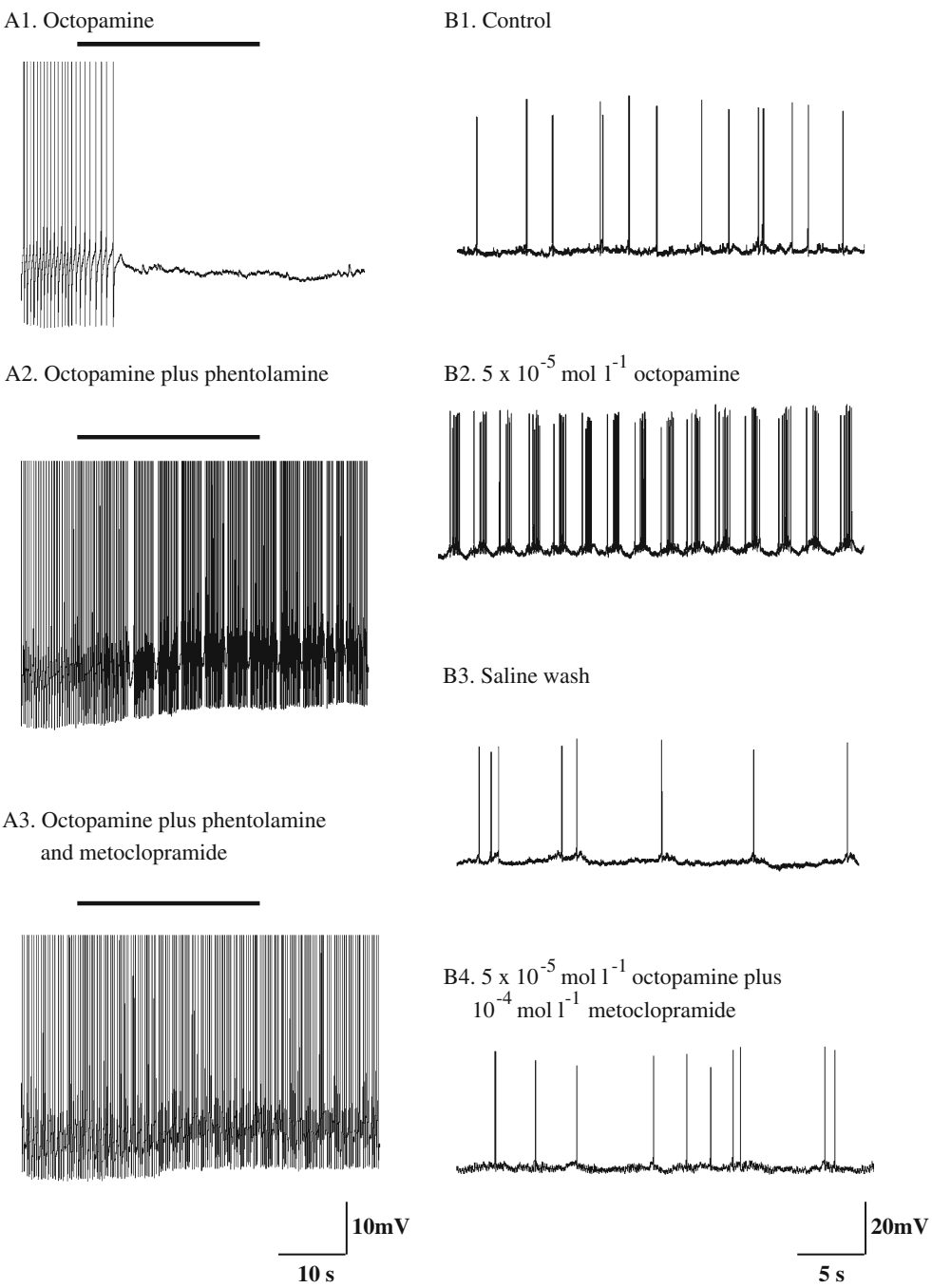

Fig. 5 a Metoclopramide blocks excitatory (burst initiating) responses to octopamine in buccal ganglion neurons. (*A1*) Normal inhibitory response of a giant buccal cell to octopamine (10^{-5} mol l^{-1}, indicated by *horizontal bar*). (*A2*) In the presence of phentolamine (10^{-5} mol l^{-1}), the response to octopamine reverses and becomes excitatory, producing burst firing. (*A3*) Burst firing is eliminated by metoclopramide (10^{-4} mol l^{-1}). **b** Metoclopramide blocks the initiation of burst firing by octopamine in a group 5 neuron. (*B1*) Control. (*B2*) Burst firing 60 s after exposure to octopamine (5×10^{-5} mol l^{-1}). (*B3*) Recovery after washout of octopamine. (*B4*) The same dose of octopamine does not initiate burst firing in the presence of metoclopramide (10^{-4} mol l^{-1})

None of the effects of the oils was blocked by phentolamine (10^{-5} mol l^{-1}) or metoclopramide (10^{-4} mol l^{-1}), applied for at least 1 h. Also, exposure to octopamine (10^{-4} mol l^{-1}) for 30 min to try to desensitize any octopamine receptors did not affect responses to the oils. Thus, in spite of the similarity of effects, the oils did not appear to work via activation of octopamine receptors.

Discussion

All three essential oil constituents were toxic to *P. corneus* in a dose- and time-dependent manner. Acute symptoms of exposure to lethal doses (approximately 2.5×10^{-4} mol l^{-1}) included a lack of response to tactile stimulation of the tentacles, swelling of the body, and sometimes protrusion of

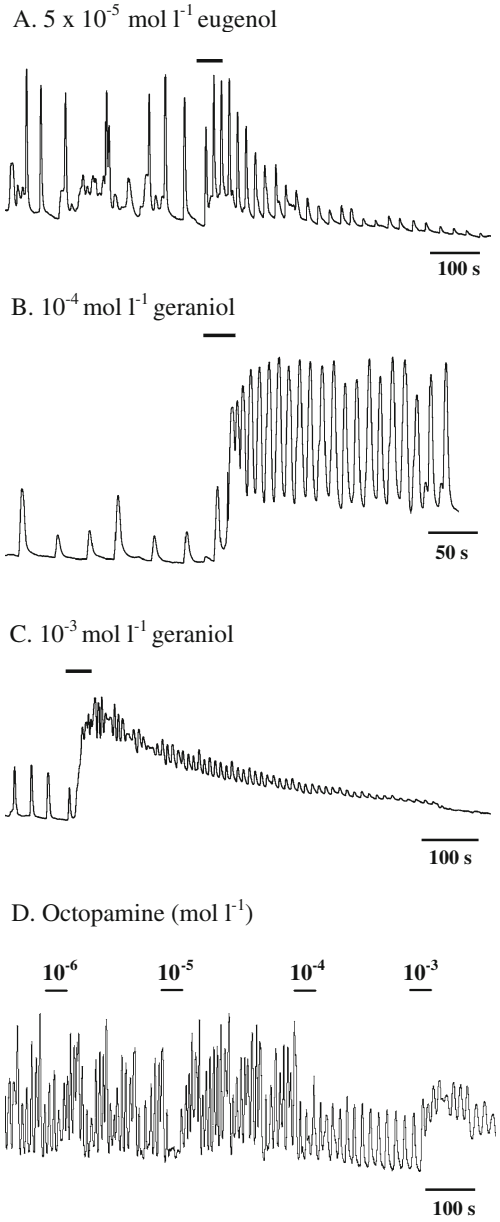

Fig. 6 Effects of essential oils and octopamine on spontaneous contractions of the isolated esophagus. **a** Eugenol (5×10^{-5} mol l^{-1}) produces an increase in frequency and progressive reduction in amplitude. **b** Geraniol (10^{-4} mol l^{-1}) increases frequency and produces an initial increase in amplitude. **c** Geraniol (10^{-3} mol l^{-1}) produces a transient sustained contraction in addition to increasing the frequency and reducing the amplitude of spontaneous contractions. **d** Octopamine has little effect at 10^{-6} or 10^{-5} mol l^{-1} but reduces amplitude of contractions at 10^{-4} mol l^{-1} and produces a transient sustained contraction at 10^{-3} mol l^{-1}

the mouthparts and genitalia, which were good indicators of eventual death (after 7 days) but were reversible up to about 5-h exposure. De Souza et al. (1991) found that *O*-methyleugenol had a similar effect in *Biomphalaria glabrata* and caused the copulatory and urethral organs to project from the body. Lahlou and Berrada (2001) showed that some essential oil constituents caused *Bulinus truncatus* to "inflate and extend out of the shell." In addition to this, in our study, all three essential oil constituents (approximately 2.5×10^{-4} mol l^{-1}) significantly reduced the amount of food consumed within 24 h.

Essential oils have a wide variety of biological effects, possibly related at least in part to their lipophilic nature, so it is difficult to specify precise mechanisms of action. In insects, oils tend to cause symptoms that suggest neurotoxicity (Coats et al. 1991; Kostyukovsky et al. 2002), typified by tremors, hyperactivity, rapid knockdown, and eventual death (Coats et al. 1991; Enan 2001). A possible target in insects is the octopamine receptor in the brain (Enan 2001, 2005). Whereas most effects of the oils generally have been found to occur at millimolar concentrations (in any animal), certain insect octopamine receptors are activated by micromolar doses of some essential oils and show high-affinity binding (Enan 2001, 2005; Kostyukovsky et al. 2002). Price and Berry (2006), by using electrophysiological techniques, were unable to demonstrate specific effects on octopamine receptors in the nerve cord of cockroaches, but these might have been different subtypes from those studied by other workers (see Evans and Robb 1993, for review of octopamine receptor classification).

In the few electrophysiological studies on the nervous system, effects of essential oils generally have been reported as depressant, such as blockage of responses to neurotransmitters (Szabadics and Erdélyi 2000) and reduced neuronal excitability (local and general anesthetic effects have been found in mammals; Brodin and Røed 1984; Ghelardini et al. 1999). The present results also demonstrated a blocking of action potentials and responses to octopamine, dopamine, and ACh at millimolar concentrations, which might contribute to the death of snails exposed to the oils in their aquarium water.

Lower concentrations of the oils, however, excited buccal ganglion neurons, appearing to produce the rhythmic pattern of fictive feeding. This did not correlate with their antifeedant actions, but such excitatory effects might be relatively short lasting or offset by other effects (e.g., on the ability of snails to detect appetitive signals), or the normal burst pattern that underlies feeding might be altered. The biphasic dose-dependent effects of citral and geraniol on impulse activity and esophageal contractions were similar to those in the nerve cord and foregut of cockroaches (Price and Berry 2006). The variability in response to eugenol (depression, sometimes preceded by transient excitation) might result from a lower concentration needed for inhibition of spiking, perhaps offsetting an excitatory effect in most cases.

In the GBCs (and some other neurons), octopamine produced a prolonged hyperpolarizing response when applied by perfusion and a short-lasting, relatively rapid

hyperpolarizing response when applied by iontophoresis, both of which were blocked by phentolamine. This suggests a direct effect of octopamine and the presence of octopamine receptors on these cells. Unlike octopamine, none of the oils hyperpolarized the GBCs or other neurons that appeared to possess inhibitory octopamine receptors, indicating that they did not activate this particular octopamine receptor subtype. They also showed no selectivity as receptor antagonists; they blocked inhibitory responses to octopamine (and dopamine) only at millimolar concentrations and were more effective at blocking excitatory ACh responses.

Citral, geraniol, and (on some occasions) eugenol did, however, mimic octopamine in initiating or modulating the presumed fictive feeding sequence in the buccal ganglia. In order to determine whether this was a result of octopamine receptor activation, we used receptor antagonists. In order to try to block excitatory and inhibitory responses to octopamine independently, phentolamine and yohimbine were chosen as potent antagonists of octopamine$_1$ receptors, and metoclopramide was selected as an example of a different (octopamine$_2$) receptor antagonist (Evans and Robb 1993; see Evans and Maqueira 2005, for new classification scheme in insects).

Phentolamine, which blocked hyperpolarizing responses to octopamine in the GBCs, did not affect the excitatory and burst-producing effects of octopamine or citral and geraniol (although phentolamine inhibits feeding when injected into *Lymnaea stagnalis*; Vehovszky et al. 1998). Since concentrations as high as 5×10^{-4} mol l^{-1} were ineffective on excitation, this shows a high specificity of the drug for octopamine receptors that mediate inhibition. Metoclopramide blocked the burst response to perfused octopamine but was ineffective on similar responses to geraniol and citral even when used at doses that were at least ten times higher. Metoclopramide also blocked the depolarizing responses to iontophoresis of octopamine in the GBCs, which occurred after treatment with phentolamine, without blocking the depolarizing responses to geraniol and citral (the change from octopamine-mediated inhibition to excitation following phentolamine suggests the presence of two receptor types on the GBCs). Care must be taken with interpretation because, unlike octopamine, the lipophilic oils are able to enter neurons and produce direct intracellular effects that could perhaps mask any octopamine-receptor-mediated actions (see Enan 2005). There also is the possibility that the oils couple an octopamine receptor to a different second messenger pathway from that of octopamine (Evans et al. 1995). Nevertheless, the fact that the effects of the oils were not blocked by high concentrations of three octopaminergic antagonists suggests that they do not produce their effects by activation of excitatory or inhibitory octopamine receptors.

Knowledge of the mechanism of action of essential oils is relevant to their allelochemical properties as attractants, repellents, and toxins, and also to their use as pesticides. Concerns over increasing costs, human and environmental safety, and resistance to currently used synthetic pesticides have increased interest in the use of plant-derived compounds such as essential oils to control pests (Clark and Appleton 1997; Ngoh et al. 1998; Lahlou and Berrada 2001). Overall, our results with the oils suggest nonspecific effects rather than direct receptor targeting. This is further supported by our unpublished findings that ethanol, high $[K^+]_o$, and a variety of other essential oils (eucalyptus, lavender, Tea Tree, and menthol) also produced depolarization, burst firing, and blocking of spike activity. Whatever the mechanism, the sensitivity of the feeding network may contribute to the antifeeding and lethal effects of the oils, which would be expected to enter the animals from the water (irrespective of feeding) because of their lipophilic nature.

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References

AMIN, G., SALEHI SOURMAGHI, M. H., ZAHEDI, M., KHANAVI, M., and SAMADI, N. 2005. Essential oil composition and antimicrobial activity of *Oliveria decumbens*. *Fitoterapia* 76:704–707.

ARSHAVSKY, Y., DELIAGINA, T. G., MEIZEROV, E. S., ORLOVSKY, G. N., and PANCHIN, Y. 1988. Control of feeding movements in the freshwater snail *Planorbis corneus*. I. Rhythmical neurons of buccal ganglia. *Exp. Brain Res.* 70:310–322.

BENJAMIN, P. R. 1983. Gastropod feeding: behavioural and neural analysis of a complex multicomponent system, pp. 159–193, in A. Roberts, and B. Roberts (eds.). Neural Origin of Rhythmic Movements. Cambridge University Press, Cambridge.

BERRY, M. S., and COTTRELL, G. A. 1975. Excitatory, inhibitory and biphasic synaptic potentials mediated by an identified dopamine-containing neuron. *J. Physiol. Lond.* 244:589–612.

BHAT, S. V., NAGASAMPAGI, B. A., and SIVAKUMAR, M. 2005. Chemistry of Natural Products. Narosa Publishing House, New Delhi.

BRODIN, P., and RØED, A. 1984. Effects of eugenol on rat phrenic nerve and phrenic nerve-diaphragm preparations. *Arch. Oral Biol.* 29:611–615.

CHADHA, M. S., EISNER, T., MONRO, A., and MEINWALD, J. 1962. Defense mechanisms of arthropods-VII. Citronellal and citral in the mandibular gland secretion of the ant *Acanthomyops claviger* (Roger). *J. Insect Physiol.* 8:175–176.

CHENG, S.-S., CHANG, H.-T., CHANG, S.-T., TSAI, K.-H., and CHEN, W.-J. 2003. Bioactivity of selected plant essential oils against the yellow fever mosquito *Aedes aegypti* larvae. *Biores. Technol.* 89:99–102.

CLARK, T. E., and APPLETON, C. C. 1997. The molluscicidal activity of *Apodytes dimidiata* E. Meyer ex Arn (Icacinaceae), *Gardenia thunbergia* L.f. (Rubiaceae) and *Warburgia salutaris* (Bertol. F.) Chiov. (Cannelaceae), three South African plants. *J. Ethnopharm.* 56:15–30.

COATS, J. R., KARR, L. L., and DREWES, C. D. 1991. Toxicity and neurotoxic effects of monoterpenoids in insects and earthworms, pp. 305–316, in P. A. Hedin (ed.). Naturally Occurring Pest Bioregulators. ACS Symposium Series 449. American Chemical Society, Washington DC.

DE SOUZA, C. P., DE OLIVEIRA, A. B., ARAUJO, N., and KATZ, N. 1991. Effects of eugenol and derivatives on *Biomphalaria glabrata*. *Rev. Bras. Biol.* 51:295–299.

DYRYNDA, P. E. J. 1986. Defensive strategies of modular organisms. *Phil. Trans. R. Soc. Lond. B* 313:227–243.

ELLIOTT, C. J. H., and VEHOVSZKY, Á. 2001. Octopamine is not just for arthropods. *Physiol. News* 45:17–19.

ENAN, E. E. 2001. Insecticidal activity of essential oils: octopaminergic sites of action. *Comp. Biochem. Physiol. C* 130:325–337.

ENAN, E. E. 2005. Molecular and pharmacological analysis of an octopamine receptor from American cockroach and fruit fly in response to plant essential oils. *Arch. Insect Biochem. Physiol.* 59:161–171.

EVANS, P. D., and ROBB, S. 1993. Octopamine receptor subtypes and their modes of action. *Neurochem. Res.* 18:869–874.

EVANS, P. D., and MAQUEIRA, B. 2005. Insect octopamine receptors: a new classification scheme based on studies of cloned *Drosophila* G-protein coupled receptors. *Invertebr. Neurosci.* 5:111–118.

EVANS, P. D., ROBB, S., CHEEK, T. R., REALE, V., HANNAN, F. L., SWALES, L. S., HALL, M., and MIDGLEY, J. M. 1995. Agonist-specific coupling of G-protein coupled receptors to second messenger systems. *Prog. Brain Res.* 106:259–268.

FINNEY, D. J. 1952. Probit Analysis. Cambridge University Press, Cambridge.

GHELARDINI, C., GALEOTTI, N., SALVATORE, G., and MAWWANTI, G. 1999. Local anaesthetic activity of the essential oil of *Lavandula angustifolia*. *Planta Medica* 65:700–703.

HIRIPI, L., VEHOVSZKY, Á., JUHOS, S., and ELEKES, K. 1998. An octopaminergic system in the CNS of the snails, *Lymnaea stagnalis* and *Helix pomatia*. *Phil. Trans. R. Soc. Lond. B* 353:1621–1629.

HUANG, Y., HO, S. H., LEE, H. -C., and YAP, Y. -L. 2002. Insecticidal properties of eugenol, isoeugenol and methyleugenol and their effects on nutrition of *Sitophilus zeamais* Motsch. (Coleoptera: Curculionidae) and *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae). *J. Stored Products Res.* 38:403–412.

ISMAN, M. B. 2000. Plant essential oils for pest and disease management. *Crop Prot.* 19:603–608.

KATERINOPOULOS, H. E., PAGONA, G., AFRATIS, A., and STRATIGAKIS, N. 2005. Composition and insect attracting activity of the essential oil of *Rosmarinus officinalis*. *J. Chem. Ecol.* 31:111–122.

KOSTYUKOVSKY, M., RAFAELI, A., GILEADI, C., DEMCHENKO, N., and SHAAYA, E. 2002. Activation of octopaminergic receptors by essential oil constituents isolated from aromatic plants: possible mode of action against insect pests. *Pest Manag. Sci.* 58:1101–1106.

LAHLOU, M., and BERRADA, R. 2001. Potential of essential oils in schistosomiasis control in Morocco. *Int. J. Aroma.* 11:87–96.

MIKICH, S. B., BIANCONI, G. V., MAIA, B. H. L. N. S., and TEIXEIRA, S. D. 2003. Attraction of the fruit-eating bat *Carollia perspicillata* to *Piper gaudichaudianum* essential oil. *J. Chem. Ecol.* 29:2379–2383.

NAKAMURA, Y., MIYAMOTO, M., MURAKAMI, A., OHIGASHI, H., OSAWA, T., and UCHIDA, K. 2003. A phase II detoxification enzyme inducer from lemongrass: identification of citral and involvement of electrophilic reaction in the enzyme induction. *Biochem. Biophys. Res. Commun.* 302:593–600.

NGOH, S. P., CHOO, L. E. W., PANG, F. Y., HUANG, Y., KINI, M. R., and HO, S. H. 1998. Insecticidal and repellent properties of nine volatile constituents of essential oils against the American cockroach, *Periplaneta americana*. *Pestic. Sci.* 54:261–268.

O'REILLY-WAPSTRA, J. M., POTTS, B. M., MCARTHUR, C., DAVIES, N. W., and TILYARD, P. 2005. Inheritance of resistance to mammalian herbivores and of plant defensive chemistry in a *Eucalyptus* species. *J. Chem. Ecol.* 31:519–537.

PAVELA, R. 2005. Insecticidal activity of some essential oils against larvae of *Spodoptera littoralis*. *Fitoterapia* 76:691–696.

PRICE, D. N., and BERRY, M. S. 2006. Comparison of effects of octopamine and insecticidal essential oils on activity in the nerve cord, foregut, and dorsal unpaired median neurons of cockroaches. *J. Insect Physiol.* 52:309–319.

SINGH, S., SINGH, V. K., and SINGH, D. K. 1997. Molluscicidal activity of some common spice plants. *Biol. Agric. Hortic.* 14:237–249.

SZABADICS, J., and ERDÉLYI, L. 2000. Pre- and postsynaptic effects of eugenol and related compounds on *Helix pomatia L. Acta Biol. Hung.* 51:265–273.

VASUDEVAN, P., KASHYAP, S., and SHARMA, S. 1997. Tagetes: a multipurpose plant. *Biores. Tech.* 62:29–35.

VEHOVSZKY, Á., and ELLIOTT, C. J. H. 2002. Heterosynaptic modulation by the octopaminergic OC interneurons increases the synaptic outputs of protraction phase interneurons (SO, N1L) in the feeding system of *Lymnaea stagnalis*. *Neurosci.* 115:483–494.

VEHOVSZKY, Á., ELLIOTT, C. J. H., VORONEZHSKAYA, E. E., HIRIPI, L., and ELEKES, K. 1998. Octopamine: a new feeding modulator in *Lymnaea*. *Phil. Trans. R. Soc. Lond. B* 353:1631–1643.

have developed mechanisms to downregulate inhibitor genes due to this mechanical wounding (Kessler and Baldwin 2002). Protease/amylase inhibitors also may be important to plant defense against insects with sucking mouthparts. For example, a proteinase inhibitor accumulated at a significantly higher level in barley leaves infested with aphids (Casaretto and Corcuera 1998). Elevated levels of protease inhibitors either through artificial diet or via transgenic plants resulted in the suppression of aphid growth and development (Rahbe et al. 2002, 2003; Ceci et al. 2003).

Despite this progress, little is known about the role and regulation of inhibitor genes during compatible (virulent parasites in susceptible hosts) and incompatible (avirulent parasites in resistant hosts) interactions in plant–insect systems that have a gene-for-gene interaction. The wheat–Hessian fly, *Mayetiola destructor* (Say) (Diptera: Cecidomyiidae), system follows a typical gene-for-gene interaction, and provides a good model for studying the scheme of attack and counter-attack between insects and plants (Harris et al. 2003). From a previous analysis of wheat gene expression with Affymetrix microarrays, we identified a group of inhibitor-like genes whose expression was affected by *M. destructor* attack (Liu et al. 2007). In this study, we investigated the dynamic expression of the inhibitor-like genes in wheat seedlings attacked by virulent or avirulent *M. destructor* larvae. We found that the expression of the inhibitor-like genes was suppressed strongly during compatible interactions. On the other hand, dramatic induction of these genes was observed during incompatible interactions.

Methods and Materials

Plants and Insects Two wheat, *Triticum aestivum*, cultivars, Molly and Karl-92, and two *M. destructor* biotypes, Great Plains (*GP*) and *vH13*, were used. Both *GP* and *vH13* were derived from the same population. Karl-92 carries no *M. destructor* resistance (*R*) genes, and was used for maintaining the population and for complementary DNA (cDNA) cloning. Molly carries the *M. destructor R*-gene *H13*, and is compatible to *vH13* but incompatible to *GP* insects (Patterson et al. 1994; Rider et al. 2002). Molly seedlings were susceptible plants (compatible interaction) when they were infested with *vH13*, and were resistant plants (incompatible interaction) when infested with *GP*. *GP* was maintained on two-leaf seedlings of Karl-92, and *vH13* was maintained on Molly seedlings in growth chambers at 20°C and 12:12 hr (L/D) photoperiod.

Cloning and Characterization of Inhibitor-like cDNAs Expressed sequence tags (ESTs) corresponding to specific inhibitor-like genes were identified from a previous microarray analysis (Liu et al. 2007). To clone full-length cDNAs, an individual EST was used to blast the GenBank EST database to identify other ESTs that share high sequence similarity. Contigs were then assembled with all similar EST sequences by using the CAP3 Program (http://pbil.univ-lyon1.fr/cap3.php, Huang and Madan 1999). A pair of primers was synthesized according to the contig sequence, and was used for polymerase chain reaction (PCR) amplification. DNA template for PCR amplification was derived by reverse transcription of total RNA extracted from Karl-92. The PCR fragments were either sequenced directly or ligated into the pPCR2.1-XL-TOPO plasmid by using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA). If ligated into the plasmid, individual clones were picked up and plasmid DNA samples for the individual clones were then sequenced. DNA sequencing was carried out either at Kansas State University, Manhattan, KS, USA or at the Jamie Whitten Delta States Research Center of USDA-ARS at Stoneville, MS, USA.

Sequence alignments were generated with ClustalW (www.ebi.ac.uk/Tools/clustalw/index.html). Secretion signal peptides were predicted using SignalP v. 1.1 (Center for Biological Sequence Analysis, Technical University of Denmark, www.cbs.dtu.dk/services/SignalP) or PSORT II (http://psort.nibb.ac.jp).

Experimental Treatments and RNA Extraction Wheat seedlings were grown and infested with different biotypes of *M. destructor* in a growth chamber set at 20±1°C (daytime) and 18±1°C (night) with a 14:10 hr (L/D) photoperiod. Seedlings were infested with a mean of 10 larvae at the two-leaf stage. Larval hatching time was monitored with additional infested plants under the same growth chamber conditions. Specifically, plants for monitoring hatching time were cut off at the base and were put into a test tube. Newly hatched larvae migrated along the leaves and between leaf sheathes. Initial attacking time was recorded when the larvae moved out of the leaf sheath into the test tube.

Wounding was accomplished with a dissecting pin (size 1, Benz Microscope, Ann Arbor, MI, USA) mounted onto a pin-vise (Cat#: 4845, Bioquip, Rancho Dominguez, CA, USA). Ten penetrations per plant were made above the base of a plant, where *M. destructor* larvae feed. For the combinational treatments of wounding plus larval attack, wounding was carried out immediately before larval migration to the feeding site. Unwounded *H13* plants at the same developmental stage were taken as controls.

Tissue samples from 20 plants per replicate were collected and pooled at 4, 12, 24, 48, 72, 96, and 120 hr after initial larval attack. Only those plants with 10 to 15 larvae per plant were chosen for tissue collection by using only the tissue at the feeding site. Approximately 1 cm of leaf-sheath tissue was collected for RNA extraction. The *H13* seedlings infested with v*H13* were taken as plants during compatible interactions, whereas the *H13* seedlings

infested with *GP* were taken as plants during incompatible interactions. Seedlings under the same conditions, but without infestation, were used as controls. Each experiment contained three independent sets of seedlings (biological replicates) that were treated in the same way at different times in different chambers.

Total RNA was extracted from wheat tissue by using TRI reagent™ according to the manufacturer's guidelines (Molecular Research Center, Inc. Cincinnati, OH, USA). The RNA samples were further purified through an RNease kit according to the manufacturer's instructions (QIAGEN Inc., Valencia, CA, USA). The RNA concentrations were measured by using a NanoDrop-1000 Spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA).

Real-Time PCR Analysis After removing potential DNA contamination by DNase I, total RNA was reverse-transcribed into cDNA by using superscript reverse transcriptase following the manufacturer's instructions (BD Biosciences, San Jose, CA, USA). After removing the RNA in the reaction by DNase-free RNase A, the cDNAs were used as template for real-time PCR (RT-PCR) analysis.

Real-time PCR was performed with iQ SYBR Green Supermix on a iCycler real time detection system (Bio-Rad, Hercules, CA, USA). Each reaction was carried out with 2 μl of a 1/40 (*v*/*v*) dilution of the first cDNA strand, and 0.5 μM of each primer in 25 μl total volume. The cycling conditions were: 95°C for 5 min followed by 45 cycles of denaturation at 95°C for 20 sec, annealing and extension at 62°C to 64.5°C, depending on the primer set, for 45 sec. At the end of the cycles, PCR specificity was verified by obtaining a dissociation curve, derived by cooling the denatured samples to 55°C and raising the temperature 0.5°C for 10 sec for each cycle, for a total of 80 cycles until reaching 95°C. The PCR products were analyzed on 1.5% agarose gels, and subsequently purified and sequenced to confirm specific amplification.

Primers (Table 1) were designed by using the Beacon Designer (v. 2.0) software from Biosoft International (Palo Alto, CA, USA). Plasmid DNA containing the corresponding insert was used to generate a calibration standard curve, where Cycle Threshold (Ct) values were plotted to serve as standard concentrations. The transcript concentration for each sample was calculated based on the standard concentrations. A negative control without template was always included for each primer set. Template concentrations of different samples were normalized against the ribosomal protein gene 21 (AF093630). For each sample analyzed, results represent the mean of values obtained from at least two independent PCR reactions and from at least three independent biological replicates.

Results

cDNAs Encoding Inhibitor-like Proteins Four groups of cDNAs were identified, and all the four groups encode small proteins with 76 to 131 amino acids (Fig. 1). All of the putative proteins contain a secretion signal peptide and have eight to 12 conserved cysteine residues in the mature protein region. However, there is no sequence similarity among members from different groups.

Group I was previously identified as defensins that were specifically induced in wheat during cold acclimation (*AI-L4*, *BAC10287*) (Koike et al. 2002). Sequence comparison revealed that proteins in Group I share high sequence similarity with a sorghum inhibitor (P21924) that inhibited α-amylases from two insect species, a locust, *Locusta migratoria*, and the American cockroach, *Periplaneta americana* (L.) (Fig. 1a) (Bloch and Richardson 1991). Because of this structural similarity, we refer to this group of cDNAs as α-amylase inhibitor-like cDNAs 1 to 4 (*AI-L1* to *AI-L4*). The specific functions of the inhibitors, however, need to be determined since protease and amylase inhibitors share structural similarities (Rawlings et al. 2005), and some can inhibit multiple types of enzymes (Odani and Ikenaka 1976).

Group II encodes proteins with >80% sequence similarity to a wheat Bowman–Birk type trypsin inhibitor (P81713) (Fig. 1b) (Poerio et al. 1994). Accordingly, members in Group II were named trypsin inhibitor-like protein 1 (*TI-L1*) to trypsin inhibitor-like protein 4 (*TI-L4*).

Unlike Groups I and II, which share sequence conservation with functionally defined proteins, Groups III and IV encode proteins that share sequence similarity with GenBank proteins whose functions have not been defined. Group III encodes proteins that belong to a large family referred to as "protease inhibitor/seed storage/LTP" (Fig. 1c) (Marchler-Bauer et al. 2007). This family is composed of protease and amylase inhi-

Table 1 Primers for real-time PCR analysis of wheat, *Triticum aestivum*, inhibitor-like genes

Gene	Sense Primer	Anti-Sense Primer
AI-L1	5′-GTCAGTCAGTGTACTTGTTC	5′-CAACGACCTTGTCACATTAC
AI-L2	5′-CCAAGAGCCACAAGTTCAAG	5′-TTACGGGACACACGATGC
AI-L3	5′-ACAGAGATGGGGACGATGAAGA	5′-AATTGTCAGTGGCATCGTT
AI-L4	5′-GTTCGTGTGGCTGTCTCC	5′-TCCGTCCATACTGAAACAAGG
TI-L1	5′-TGTAAGACCTGTGCCAAGC	5′-CTGAAAGAAGACCGAGAGAAAC
PI-L1	5′-CGTCCTCAACATCGTCCAG	5′-CCTCTCGCACCACTAATCC
TN-L1	5′-GATACACACAAACACAACGAAG	5′-GGCGAACAGGATGACCAG

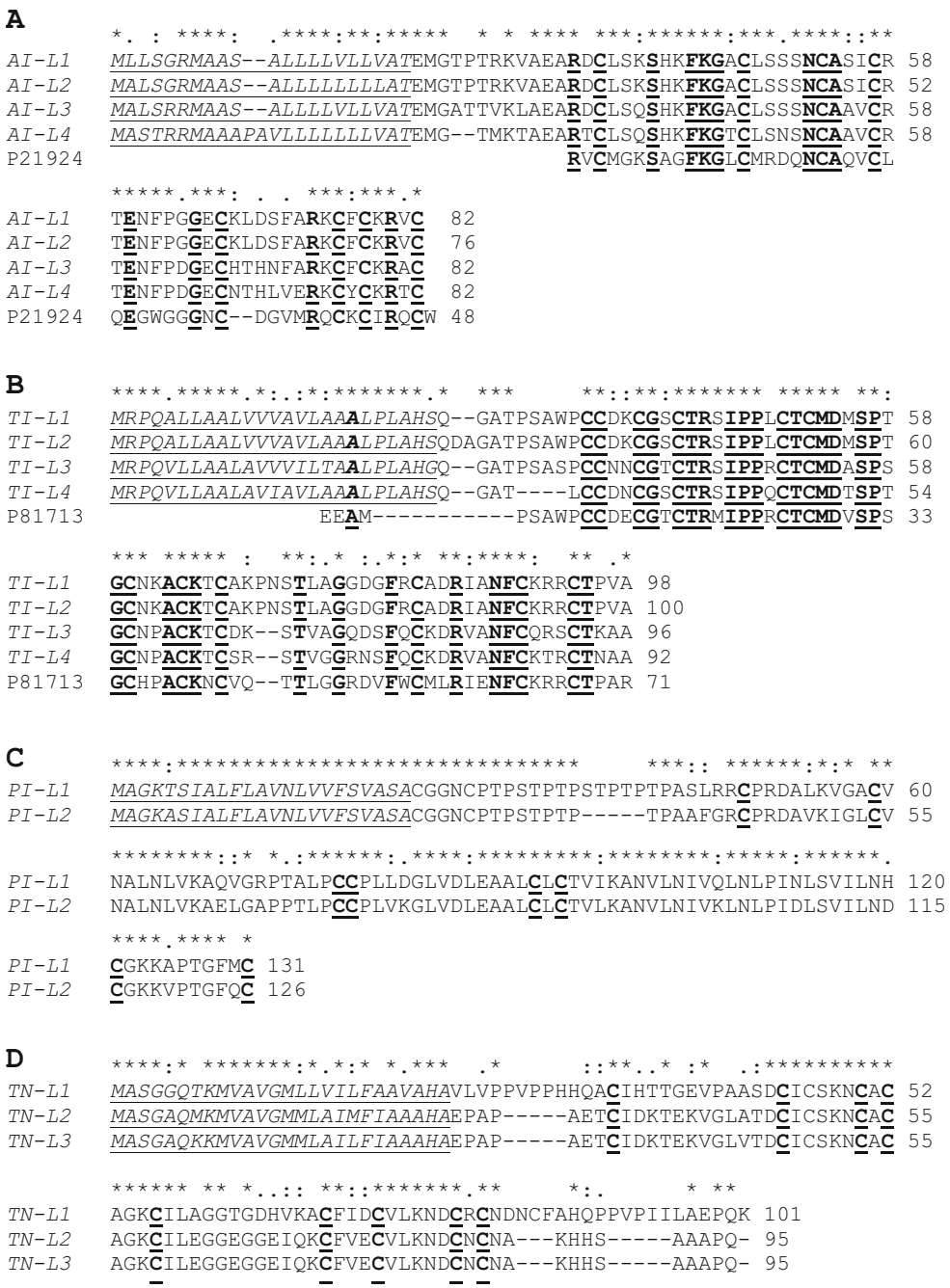

Fig. 1 Sequence alignments of inhibitor-like proteins from wheat, *Triticum aestivum*. The alignments were made by using the program *ClustalW* (see "Methods and Materials"). The *asterisk* represents conserved residues, the symbols ":" and "." represent conserved substitutions, whereas the dash (-) represents a deletion or insertion in the alignments. Bold and underlined letters represent residues that are either identical with a functionally defined protein or cysteines that are representative of the family. Predicted secretion signal peptides at the N-termini are italicized and underlined. (A) A group of proteins with sequence similarity to an inhibitor of insect amylases. (B) A group of proteins with sequence similarity to a characterized Bowman–Birk type trypsin inhibitor. (C) A group of proteins with sequence similarity to a conserved family named protease inhibitor/storage protein/lipid transfer protein (Marchler-Bauer et al. 2007). (D) A group of proteins with sequence similarity to thionins. The nucleotide GenBank accession numbers are EU293126 (*AI-L1*), EU293127 (*AI-L2*), EU293128 (*TI-L1*), EU293129 (*TI-L2*), EU293130 (*TI-L3*), EU293131 (*TI-L4*), EU293132 (*PI-L1*), EU293133 (*PI-L2*), and EU293134 (*TN-L1*). The Affymetrix identification numbers for the original ESTs are: Ta.14568.1 (*AI-L1*), Ta.28550.1 (*AI-L2*), Ta.7756.2 (*TI-L1*), and Ta.5208.1 (*TN-L1*). Other sequences previously published are nucleotide sequences AB089942 (*AI-L3*) and BT008925 (*AI-L4*), and protein sequences AAC49286 (*TN-L2*) and AAP12730 (*TN-L3*)

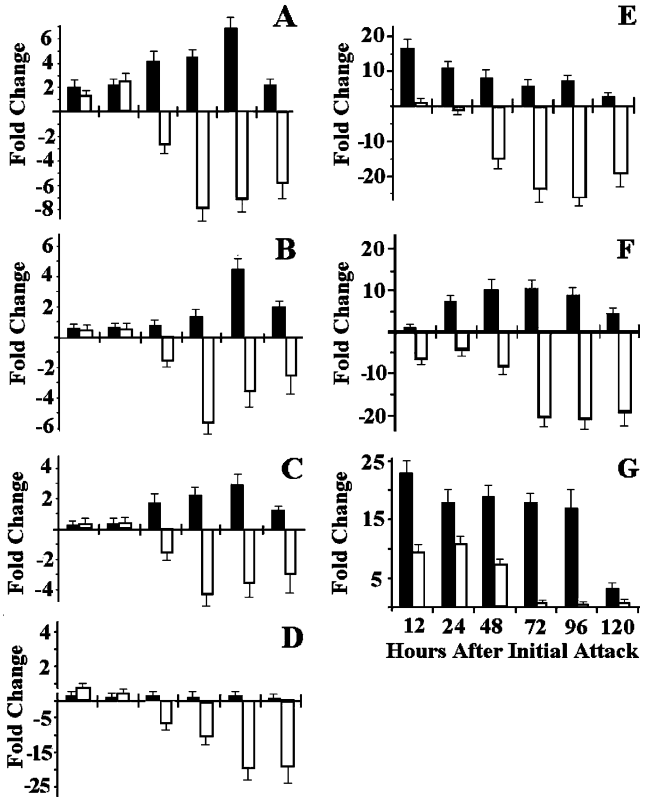

Fig. 2 Differential expression of inhibitor-like genes from wheat, *Triticum aestivum*, during incompatible and compatible interactions with Hessian fly, *Mayetiola destructor*. Solid bars represent fold changes during incompatible interactions, whereas open bars represent fold changes during compatible interactions. Samples were collected at 12, 24, 48, 96, and 120 hr after initial attack by larval *M. destructor*. A to G are for genes *AI-L1, AI-L2, AI-L3, AI-L4, TI-L1, PI-L1*, and *TN-L1*, respectively

bitors, seed storage proteins, and lipid transfer proteins from plants, but the specific function of this family has not been established. Many proteins in this family may have multiple functions such as serving both enzyme inhibitors and storage proteins.

Group IV encodes proteins referred to as thionin-like proteins because of their high cysteine content (Ray et al. 2003). This group of genes was rapidly induced in wheat upon attack by a fungal pathogen. Again, the specific functions of the thionin-like proteins remain to be determined. Many proteins previously characterized as thionins have been found to be protease inhibitors later (Bloch and Richardson 1991).

Upregulation during Incompatible Interactions (GP Insects in H13 Plants) To examine the expression patterns of these genes during incompatible interactions, RT-PCR was carried out with specific primer pairs (Table 1). During incompatible interactions, a similar pattern was observed for the four members of Group I (Fig. 2a–d), except for *AI-L4*, which was not significantly affected by *M. destructor* attacks during incompatible interactions. For the other three genes, significant upregulation began at 24 hr and maximum upregulation was reached at 96 hr. The upregulation declined at 120 hr and returned to normal at 144 hr (data not shown).

Because of high sequence similarity among members in Groups II to IV, primer pairs specific for each cDNA were not found. As a result, a common pair of primers was designed for each gene group. *TI-L1* was strongly (>15-fold) upregulated at 12 hr (Fig. 2e). Upregulation became weaker thereafter and returned to normal at 120 hr. In comparison, *PI-L1* was gradually upregulated beginning at 12 hr and reached maximum at 48 to 72 hr. *TN-L1*, on the other hand, was upregulated strongly (>20-fold) at 12 hr during incompatible and remained high up to 96 hr, followed by a sudden drop at 120 hr (Fig. 2g).

Downregulation during Compatible Interactions (vH13 Insects in H13 Plants) All inhibitor genes except *TN-L1* were strongly downregulated during compatible interactions (Fig. 2a–f). Downregulation began at 48 hr and reached maximum between 72 to 96 hr. *TN-L1* was the only gene that was upregulated during compatible and incompatible interactions (Fig. 2g). However, upregulation was much weaker during compatible interactions than during incompatible interactions, resulting in an incompatible/compatible expression ratio (I/C ratio) of 1.8 to 42 at different time points (Table 2).

Wound-induced Expression Since inhibitor genes responsive to insect feeding are also inducible by wounding (Ryan and

Table 2 Ratios between the expression levels of wheat, *Triticum aestivum*, inhibitor-like genes during incompatible and compatible interactions with Hessian fly, *Mayetiola destructor* (*I*/*C* ratio)

Gene	12 Hours	24 Hours	48 Hours	72 Hours	96 Hours	120 Hours
AI-L1	3.5±0.81	0.91±056	13±3.2	32±8.7	46±6.9	11±2.1
AI-L2	1.8±0.31	1.1±0.24	2.3±0.33	10±1.8	20±4.2	2.4±0.62
AI-L3	1.5±0.22	1.2±0.17	3.5±0.35	8.8±1.4	9.8±1.3	3.3±0.35
AI-L4	0.38±0.18	0.46±0.22	2.2±0.15	4.6±0.18	25±2.1	19±2.3
TI-L1	43±3.4	28±3.5	68±5.4	83±6.5	128±10	59±3.8
PI-L1	10±0.69	32±2.8	100±8.3	180±9.2	160±8.5	78±2.4
TN-L1	2.3±0.13	1.8±0.22	3.3±0.18	36±2.5	42±2.8	6±0.36

Moura 2002), we examined the effect of wounding on the expression of the five inhibitor-like genes, *AI-L3, AI-L4, TI-L1, PI-L1*, and *TN-L1*. All five genes exhibited higher expression levels at 12, 24, and 72 hr following wounding (Fig. 3). Induction by wounding reached maximum (1.5 to 3.5 fold) at 12 hr for *TI-L1* and *TN-L1*, and at 24 hr for *AI-L3, AI-L4*, and *PI-L1*. The wound effect on all five genes declined to baseline levels after 72 hr.

Since both wounding and avirulent larvae upregulated inhibitor-like genes, we then examined possible synergistic effect on gene upregulation. Gene upregulation by wounding and by avirulent larvae appeared additive at some time points (Fig. 3b), but no synergistic effect was observed between these two factors. During compatible interactions, the wound-induced upregulation was suppressed by virulent larvae (Fig. 3c).

Discussion

This research represents the first systematic analysis of the expression patterns of inhibitor-like genes in a plant–insect system that follows a typical gene-for-gene interaction. Several groups of diverse inhibitor-like genes were found to be strongly downregulated by insect feeding during compatible interactions, whereas the same genes were upregulated during incompatible interactions. The combination of upregulation during incompatible and downregulation during compatible interactions resulted in dramatic differences in gene expression between resistant and susceptible plants (Table 2), which strongly suggests inhibitor-like genes are part of defense mechanisms in wheat plants against *M. destructor* attacks. There are diverse proteases including various trypsins and chymotrypsins, cysteine proteases, and different carboxypeptidases in the *M. destructor* digestive system (Zhu et al. 2005; Liu et al. 2007). Elevated inhibitors along with other toxic substances may reduce nutrient uptake and absorption by *M. destructor* larvae.

For chewing insects, upregulation of inhibitor genes is achieved, at least in part, through wounding (Ryan and Moura 2002). For *M. destructor*, the mechanism for upregulation of the inhibitor-like genes appeared independent of the wounding pathway. First, wound-induced upregulation exhibited similar expression for all inhibitor-like genes examined (Fig. 3a), whereas avirulent larva-induced upregulation during incompatible interactions exhibited different patterns for different genes. Second, wound-induced upregulation was relatively weak (two- to three-fold) and for a short time. These differences suggest that the stronger upregulation for longer time is associated with avirulent larval attacks and might be mediated through an alternative pathway(s) mediated by the *R*-gene present in the wheat plants. Further research is required to delineate the mechanism(s) of the avirulent larva-induced upregulation.

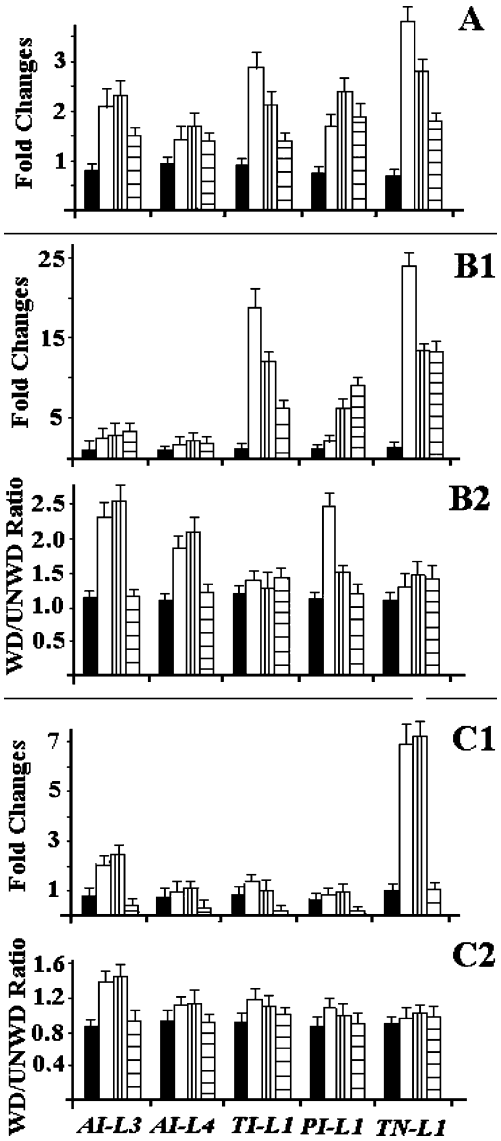

Fig. 3 Impact of wounding on the expression of inhibitor-like genes from wheat, *Triticum aestivum*. Solid, open, vertically striped, and horizontally striped bars represent data obtained from 4, 12, 24, and 72 hr after wounding or initial larval attacks by the Hessian fly, *Mayetiola destructor*. WD/UNWD represents ratios of expression levels between wounded and unwounded samples. (A) Fold changes of gene expression after wounding. (B, upper portion) Fold changes of gene expression after wounding and avirulent (biotype *GP*) larval attacks. (B, lower portion) Ratios between the expression levels of plants that were wounded mechanically and also attacked by avirulent larvae, and the corresponding expression levels of plants that were only attacked by avirulent larvae. (C, upper portion) Fold changes of gene expression after wounding and virulent (biotype *vH13*) larval attacks. (C, lower portion) Ratios between the expression levels of plants that were both wounded mechanically and attacked by virulent larvae, and the corresponding expression levels of plants that were only attacked by virulent larvae

In comparison with various patterns of gene upregulation during incompatible interactions, the downregulation of the inhibitor-like genes by virulent larvae during compatible interactions was similar for all the inhibitor-like genes (Fig. 2a–f).

Significant downregulation was at 48 hr, maximum downregulation at 72 hr, and it remained strong thereafter. The timeframe for the downregulation of the inhibitor-like genes was similar to that of the upregulation of nutrient metabolism genes during compatible interactions (Zhu et al. 2008). The same time frame for the downregulation of defense genes and the upregulation of nutrient genes indicated that gene regulation during compatible interactions was achieved by a common, coordinated mechanism(s). This coordinated mechanism might be related to the ability of virulent larvae to manipulate wheat plants for the creation of nutritive cells at the feeding site (Harris et al. 2006; Anderson and Harris 2006). Hessian fly larvae inject a large number of proteins and other substances into host plants during feeding (Haseman 1930; Hatchett et al. 1990; Chen et al. 2004, 2006, 2008). These injected proteins might target primary and secondary metabolic pathways, resulting in the suppression of plant defense and induction of nutrient supply. The suppression of defenses and formation of nutritive cells provides conditions favorable for a larva to grow and develop.

Host plant resistance is an effective way to control *M. destructor* damage. The challenge with the host plant resistance strategy is that *M. destructor* can develop new biotypes that can quickly overcome deployed resistance (Ratcliffe et al. 2000). The upregulation during incompatible interactions and downregulation during compatible interactions suggests strongly that the inhibitor-like genes are part of the mechanism of *R*-gene mediated defense against *M. destructor*. Further research on the regulatory pathways of defense genes may lead to a more durable resistance by by-passing the major dominant *R*-genes, which are easy to be overcome by new, virulent biotypes.

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References

AGRAWAL, A. A., and FORDYCE, J. A. 2000. Induced indirect defence in a lycaenid-ant association: the regulation of a resource in a mutualism. *Proc. R. Soc. Land. B* 267:1857–1861.

ANDERSON, K. G., and HARRIS, M. O. 2006. Does R gene resistance allow wheat to prevent plant growth effects associated with Hessian fly (Diptera: Cecidomyiidae) attack. *J. Econ. Entomol.* 99:1842–1853.

BLOCH, C. Jr., and RICHARDSON, M. 1991. A new family of small (5 kDa) protein inhibitors of insect alpha-amylases from seeds or sorghum (*Sorghum bicolar* (L) Moench) have sequence homologies with wheat gamma-purothionins. *FEBS Lett.* 279:101–104.

CASARETTO, J. A., and CORCUERA, L. J. 1998. Proteinase inhibitor accumulationin aphid-infested barley leaves. *Phytochemistry* 49:2279–2286.

CECI, L. R., VOLPICELLA, M., RAHBÉ, Y., GALLERANI, R., BEEKWILDER, J., and JONGSMA, M. A. 2003. Selection by phage display of a variant mustard trypsin inhibitor toxic against aphids. *Plant J.* 33:557–566.

CHEN, M.-S. 2008. Inducible direct plant defense against insect herbivores. *Insect Sci.* 15:101–114.

CHEN, M.-S., FELLERS, J. P., STUART, J. J., REESE, J. C., and LIU, X.-M. 2004. A group of related cDNAs encoding secreted proteins from Hessian fly [*Mayetiola destructor* (Say)] salivary glands. *Insect Mol. Biol.* 13:101–108.

CHEN, M.-S., FELLERS, J. P., ZHU, Y. C., STUART, J. J., HULBERT, S., El-BOUHSSINI, M., and LIU, X. -M. 2006. A super-family of genes coding for secreted salivary gland proteins from the Hessian fly, *Mayetiola destructor. J. Insect Sci.* 6:12 (http://insectscience.org/6.12/).

CHEN, M. -S., ZHAO, H. X., ZHU, Y. C., SCHEFFLER, B., LIU, X. M., LIU, X., HULBERT, S., and STUART, J. J. 2008. Analysis of transcripts and proteins expressed in the salivary glands of Hessian fly (*Mayetiola destructor*) larvae. *J. Insect Physiol.* 54:1–16.

HASEMAN, L. 1930. The Hessian fly larva and its method of taking food. *J. Econ. Entomol.* 23:316–319.

HARRIS, M. O., STUART, J. J., MOHAN, M., NAIR, S., LAMB, R. J., and ROHFRITSCH, O. 2003. Grasses and gall midges: plant defense and insect adaptation. *Annu. Rev. Entomol.* 48:549–577.

HARRIS, M. O., FREEMAN, T. P., ROHFRITSCH, O., ANDERSON, K. G., PAYNE, S. A., and MOORE, J. A. 2006. Virulent Hessian fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interactions with wheat. *Ann. Entomol. Soc. Am.* 99:305–316.

HATCHETT, J. H., KREITNER, G. L., and ELZINGA, R. J. 1990. Larval mouthparts and feeding mechanism of the Hessian fly (Diptera: Cecidomyiidae). *Ann. Entomol. Soc. Am.* 83:1137–1147.

HUANG, X., and MADAN, A. 1999. CAP3: a DNA sequence assembly program. *Genome Res.* 9:868–877.

KESSLER, A., and BALDWIN, I. T. 2002. Plant responses to insect herbivory. *Annu. Rev. Plant Biol.* 53:299–328.

KOIKE, M., OKAMOTO, T., TSUDA, S., and IMAI, R. 2002. A novel plant defensin-like gene of winter wheat is specifically induced during cold acclimation. *Biochem. Biophys. Res. Commun.* 298:46–53.

LIU, X. -M., BAI, J., HUANG, L., ZHU, L., LIU, X., WENG, N., REESE, J. C., HARRIS, M., STUART, J. J., and CHEN, M.-S. 2007. Gene expression of different wheat genotypes during attack by virulent and avirulent Hessian fly (*Mayetiola destructor*) larvae. *J. Chem. Ecol.* 33:2171–2194.

MARCHLER-BAUER, A., ANDERSON, J. B., DERBYSHIRE, M. K., DE WEESE-SCOTT, C., GONZALES, N. R., GWADZ, M., HAO, L., HE, S., HURWITZ, D. I., JACKSON, J. D., KE, Z., KRYLOV, D., LANCZYCKI, C. J., LIEBERT, C. A., LIU, C., LU, F., LU, S., MARCHLER, G. H., MULLOKANDOV, M., SONG, J. S., THANKI, N., YAMASHITA, R. A., YIN, J. J., ZHANG, D., and BRYANT, S. H. 2007. CDD: a conserved domain database for interactive domain family analysis. *Nucleic Acids Res.* 35:D237–D240.

ODANI, S., and IKENAKA, T. 1976. The amino acid sequences of two soybean double headed proteinase inhibitors and evolutionary consideration on the legume proteinase inhibitors. *J. Biochem.* 80:641–643.

PATTERSON, F. L., MASS, F. B. III, FOSTER, J. E., RATCLIFFE, R. H., CAMBRON, S., SAFANSKI, G., TAYLOR, P. L., and OHM, H. W. 1994. Registration of eight Hessian fly resistant common winter wheat germplasm lines (Carol, Erin, Flynn, Iris, Joy, Karen, Lola, and Molly). *Crop Sci.* 34:315–316.

POERIO, E., CAPORALE, C., CARRANO, L., CARUSO, C., VACCA, F., and BUONOCORE, V. 1994. The amino acid sequence and reactive

site of a single-headed trypsin inhibitor from wheat endosperm. *J. Protein Chem.* 13:187–194.

RAHBE, Y., FERRASSON, E., RABESONA, H., and QUILLIEN, L. 2002. Toxicity to the pea aphid *Acyrthosiphon pisum* of anti-chymotrypsin isoforms and fragments of Bowman–Birk protease inhibitors from pea seeds. *Insect Biochem. Mol. Biol.* 33:299–306.

RAHBE, Y., DERAISON, C., BONADE-BOTTINO, M., GIRARD, C., NARDON, C., and JOUANIN, L. 2003. Effects of the cysteine protease inhibitor oryzacystatin (OC-I) on different aphids and reduced performance of *Myzus persicae* on OC-I expressing transgenic oilseed rape. *Plant Sci.* 164:441–450.

RATCLIFFE, R. H., CAMBRON, S. E., FLANDERS, K. L., BOSQUE-PEREZ, N. A., CLEMENT, S. L., and OHM, H. W. 2000. Biotype composition of Hessian fly (Diptera: Cecidomyiidae) populations from the southeastern, midwestern, and northwestern United States and virulence to resistance genes in wheat. *J. Econ. Entomol.* 93:1319–1328.

RAWLINGS, N. D., TOLLE, D. P., and BARRETT, A. J. 2005. Evolutionary families of peptidase inhibitors. *Biochem. J.* 378:705–716.

RAY, S., ANDERSON, J. M., URMEEV, F. I., and GOODWIN, S. B. 2003. Rapid induction of a protein disulfide isomerase and defense-related genes in wheat in response to the hemibiotrophic fungal pathogen *Mycosphaerella graminicola*. *Plant Mol. Biol.* 53:701–714.

RIDER, S. D. Jr., SUN, W., RATCLIFFE, R. H., and STUART, J. J. 2002. Chromosome landing near avirulence gene vH13 in the Hessian fly. *Genome* 45:812–822.

RYAN, C. A., and MOURA, D. S. 2002. Systemic wound signaling in plants: A new perception. *Proc. Nat. Acad. Sci. U. S. A.* 99:6519–6520.

SCHNEE, C., KOLLNER, T. G., TURLINGS, T. C. J., GERSHENZON, J., and DEGENHARDT, J. 2006. The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proc. Nat. Acad. Sci. U. S. A.* 103:1129–1134.

ZHU, Y. C., LIU, X., MADDUR, M., OPPERT, B., and CHEN, M. S. 2005. Cloning and characterization of chymotrypsin- and trypsin-like cDNAs from the gut of the Hessian fly [*Mayetiola destructor* (say)]. *Insect Biochem. Mol.* 35:(1)23–32.

ZHU, L., LIU, X. M., LIU, X., JEANNOTTE, R., REESE, J. C., HARRIS, M., STUART, J. J., and CHEN, M. S. 2008. Hessian fly (*Mayetiola Destructor*) attack causes dramatic shift in carbon/nitrogen metabolism in wheat. *Mol. Plant-Microbe Interact.* 21:70–78.

sible for photosynthesis and general metabolism are usually repressed by insect infestation. This compromises plant growth and productivity.

One factor that appears to mediate interactions between an herbivorous insect and its plant host is the composition of the regurgitant or saliva produced by the insect. Specific elicitors isolated from the regurgitant of pest insects applied to mechanically wounded leaves often results in a plant response that more closely mimics the response to insect feeding. A variety of different elicitors have been characterized to date. For example, glucose oxidase from the salivary glands of *Helicoverpa zea* (Boddie) altered defense responses when applied to wounded leaves of tobacco (Eichenseer et al. 1999; Musser et al. 2002). Recently, a small peptide, inceptin, resulting from the proteolysis of the plant-derived enzyme cATP synthase, was identified as an elicitor from the caterpillar *Spodoptera frugiperda* (Schmelz et al. 2006). Elicitors can alter the composition of volatile defensive blends that are generated by the plant during the defense response, illustrating that the plant's response to insect herbivory at the molecular level is different from that to wounding alone. For instance, application of a β-glucosidase isolated from *Pieris brassicae* (L.) regurgitant to a wounded leaf of cabbage resulted in the generation of a volatile blend that is also produced by herbivory (Mattiacci et al. 1995).

Not all elicitors are peptides. Fatty acid–amino acid conjugates (FACs) are perhaps the best characterized elicitors of volatile production (e.g., Pohnert et al. 1999). Application of volicitin, a FAC component of *S. exigua* (Hübner) saliva, to a wounded leaf elicited volatiles similar to those produced by insect infestation rather than those resulting from mechanical injury alone (Alborn et al. 1997). Work from the Baldwin group (Halitschke et al. 2001, 2003; Schittko et al. 2001; Roda et al. 2004) has examined the effect of regurgitant from *Manduca sexta* (L.) on *Nicotiana attenuata* and has isolated FACs that mimic the effect of the regurgitant. Roda et al. (2004) found that regurgitant treatment led to differential expression of 138 genes, 63 of which were upregulated and 75 that were downregulated. FACs in the regurgitant were responsible for 53 upregulated and 56 downregulated genes. In a comparison of wounded poplar leaves versus application of regurgitant from forest tent caterpillar, *Malacosoma disstria* Hübner, to negligibly wounded leaves, Major and Constabel (2006) found that 38 genes were induced by both treatments, and these genes tended to be involved in stress and secondary metabolism. In contrast to the study in tobacco, only two genes were unique to regurgitant treatment, suggesting that regurgitant in this case only augmented the wound response. Clearly, a number of insect elicitors may affect the plant response to insect attack. However, it is less clear to what extent these observations of specific plant–insect interactions can be extended to interactions between other species.

Colorado potato beetle (CPB), *Leptinotarsa decemlineata* (Say) (Coleoptera: Chrysomelidae), is a serious pest of potato, *Solanum tuberosum*, and other solanaceous crops. This herbivorous insect results in hundreds of millions of dollars of crop losses annually. Despite the economic impact of CPB on solanaceous crops, little is known about the components of CPB regurgitant, or how CPB regurgitant affects the defense response of potato at the molecular level. We have recently begun to characterize CPB regurgitant produced from insects reared on tomato and have concluded that it contains a proteinaceous component that inhibits genes that encode proteinase inhibitors in tomato (Lawrence et al. 2007). Kruzmane et al. (2002) have shown that CPB regurgitant enhances peroxidase and polyphenol oxidase activity in potato; these studies also demonstrated that ethylene is increased in regurgitant-treated wounded leaves. It has been shown that potato plants produce a suite of volatiles—including 2-phenylethanol—upon attack by CPB that attract the CPB predator *Perillus bioculatus* (F.) (Heteroptera: Pentatomidae; Schütz et al. 1997; Weissbecker et al. 1999). However, a molecular examination of the specific CPB/potato interaction by examining the effect of regurgitant treatment has not been undertaken.

As part of our ongoing studies to characterize CPB–potato interactions at the molecular level, we have used an 11,421 EST Solanaceae microarray to identify additional genes in potato whose expression is altered by putative elicitors present in the regurgitant of CPB raised on potato. Experiments that compared gene expression profiles of mechanically wounded leaves to which CPB regurgitant was applied versus mechanically wounded leaves without regurgitant revealed that the plant's molecular response to wounding plus CPB regurgitant is not the same as that induced by wounding alone. This set of regurgitant-responsive genes likely comprises both genes that are responsive to regurgitant but not wounding, as well as those genes that are responsive to wounding, but for which this response is augmented by the presence of regurgitant. The addition of CPB regurgitant alters the expression of a suite of defense-associated genes compared to wounding alone, including a gene putatively involved in the synthesis of 2-phenylethanol. Our study provides evidence that CPB modulates the potato's defense response against this devastating herbivore potentially via elicitors present in its regurgitant.

Methods and Materials

Plant Material Potato tubers from *S. tuberosum* var. Kennebec were planted in individual 10-cm pots contain-

ing Metromix® (Scotts, Marysville, OH, USA) and Osmocote® (Scotts). Plants were grown for 4 weeks in a greenhouse, and only plants with at least eight leaves were used in the tests. For real-time polymerase chain reaction (PCR) experiments, plants were grown for 4 weeks in a growth chamber with a 16:8 (light to dark, L to D) cycle at 25°C during the light phase and 20°C during the dark phase.

CPB Rearing and Regurgitant Isolation CPB came from a colony at the USDA—ARS Insect Biocontrol Laboratory that originated from eggs provided by the New Jersey Department of Agriculture in 1996. Field-collected insects from potato fields at the USDA—ARS Beltsville Agricultural Research Center (Beltsville, MD, USA) were introduced annually to maintain genetic diversity. CPB were reared on *S. tuberosum* var. Kennebec and maintained in a laboratory under a 16:8 (L to D) cycle at approximately 25°C. Regurgitant was collected from the oral cavity of fourth instar CPB with a P200 Pipetteman (Gilson, Oakland, CA, USA). The regurgitant was centrifuged for 5 min at 10,000×*g* and the supernatant was collected and stored at −80°C.

Wounding, Regurgitant Treatment, and Infestation Two separate series of experiments were carried out—one for microarray analyses and a second for real-time PCR analyses. Mechanical wounding involved crushing the leaf from the sixth node from the bottom with pliers, while avoiding the major veins, at 1-cm intervals. One hundred microliter of a 1:3 aqueous dilution of the regurgitant or 100 µl of water (control) were added to wounded leaves. Plants were incubated for 4 h after which the wounded leaves were excised and frozen rapidly in liquid nitrogen. For microarray experiments, five plants were pooled for each sample, and the experiments were repeated three times, i.e., there were three biological replicates.

In the second series of experiments for real-time PCR analysis, five treatments were carried out: control (untreated) plants, water-treated wounded plants, regurgitant-treated wounded plants, mildly CPB-infested plants, and acutely CPB-infested plants. Water- and regurgitant-treated wounded plants were treated as described above. For infestation treatments, plants were divided into two groups; the sixth leaf from the bottom was covered with a fine mesh sleeve, and ten third- to fourth-instar CPB larvae were added. The first infestation level was achieved by leaving the ten CPB on the plant for 1 h and then removing them. The plants were harvested 3 h later. The second infestation level was achieved by leaving the ten CPB on the plant for 4 h, removing them, and then harvesting the plant immediately. For real-time PCR (Fig. 1), two plants were pooled for each sample, and experiments were repeated four times.

RNA Isolation for Microarray and Real-Time PCR For microarray analyses, RNA was isolated from *S. tuberosum* leaves with Qiagen's RNeasy kit by using the protocol recommended by the manufacturer (Qiagen, Valencia, CA, USA). The protocol is available at http://www.tigr.org/tdb/potato/microarray_SOPs.shtml. For real-time PCR, RNA was isolated by using Qiagen's RNeasy Plant Mini kit

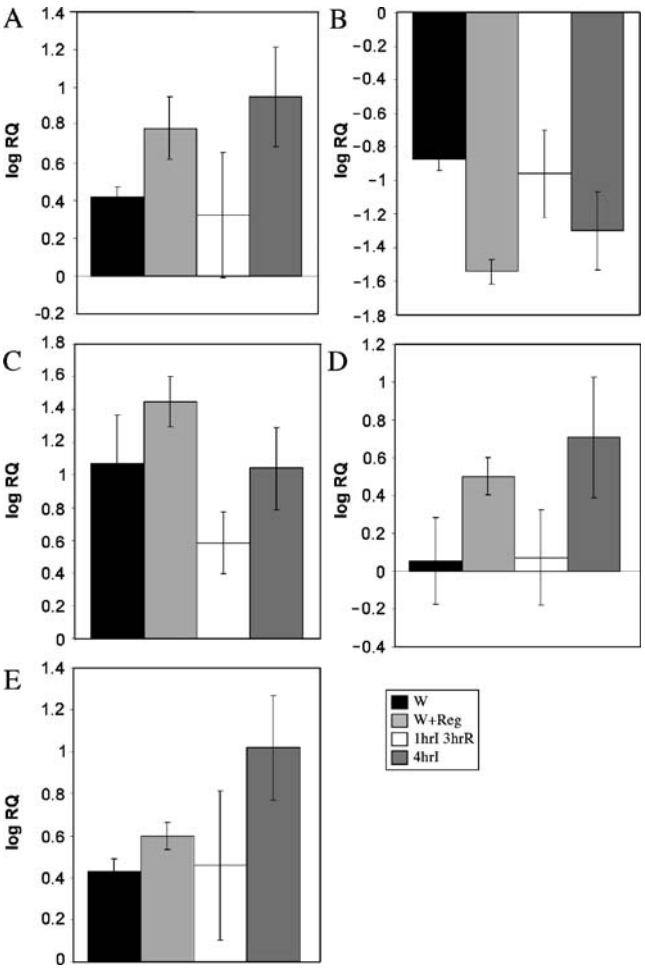

Fig. 1 Real-time PCR of five genes from *S. tuberosum* var. Kennebec that are differentially expressed by treatment of wounded leaves with regurgitant of *L. decemlineata*. *W*=wounded+water, *W+Reg*=wounded+regurgitant 1:3, *1hI3hR*=1 h infestation followed by 3 h recovery, *4hI*=4 h continuous infestation. **a** *STMEV47*, ZPT2–13 transcription factor; **b** *STMCK44*, carbonic anhydrase; **c** *STMFB59*, class IV chitinase; **d** *STMEP88*, aromatic amino acid decarboxylase; and **e** *STMDJ96*, Zim domain protein 1, JAZ 1, transcription factor. Fold change levels of gene expression were expressed as RQ. As such, an RQ of 1 indicates a sample exhibiting the same relative transcript abundance as the control. The data were $\log_{10}$ transformed prior to graphing; hence, equal relative transcript abundance in control and treatment samples result in a log RQ value of 0. Each *bar* represents four biological replicates with *error bars* representing standard error

adding an RNase free DNAse step using the manufacture's protocol (Qiagen).

Microarrays Two-color spotted cDNA microarrays were used to carry out gene expression profiling experiments. The TIGR 10K EST Solanaceae microarray contains 11,412 annotated cDNA clones spotted as randomized duplicates on the array. Because our primary objective was to identify regurgitant-responsive genes, hybridizations were carried out in which wounding plus regurgitant leaf samples were cohybridized (paired) with wounding plus water leaf samples. This direct comparison provides greater ability to identify genes differentially expressed in response to regurgitant. The variance is smaller in the direct comparison technique than in indirect comparisons where arrays pairing wounding plus water vs. control leaf samples are compared to arrays pairing wounding plus regurgitant vs. control leaf samples (Dobbin and Simon 2002). For each treatment–control comparison, three biological replicates were analyzed; for each biological replicate, a dye swap of technical replicates was performed. In total, six arrays for wound and wound plus regurgitant were carried out. The TIGR Solanaceae Expression Profiling Service performed all the microarray procedures including cDNA labeling, hybridization, data quantification, and data normalization by using LOWESS (protocols available at http://www.tigr.org/tdb/potato/microarray_SOPs.shtml). The data from the microarray experiments are available from the TIGR Solanaceae Gene Expression Database (http://www.tigr.org/tigr-scripts/tdb/potato/study/potato_study_hybs.pl?study=86&user=&pass=&sort=id&order=asc).

Exported data were analyzed in R (Ihaka and Gentleman 1996) with the BioConductor suite of packages (Gentleman et al. 2004). Quality assessment of the raw and background-corrected data was carried out by inspection of ratio–intensity plots (also known as minus–add (MA) plots)—pairwise correlations of ratio (M) values between slides; and distribution and density of intensity (A) values. Data were analyzed with the linear models for microarray data package (Smyth 2005) and exploratory analysis for two-color spotted microarray data (marray) package (Yang and Paquet 2005) by using methods described in Dudoit and Yang (2002), Smyth and Speed (2003), Smyth (2004), and Smyth et al. (2005). Within-array data were normalized by two-dimensional spatial loess and print-tip loess detrending procedure. Data were then scaled to have the same median-absolute-deviation across arrays. Nonspecific filtering was applied to reduce false discovery rate by removing invalid and low intensity genes. Intensity filtering was done with the genefilter package to remove genes whose A values were smaller than 7 in at least 75% of the samples. Linear models were fitted to the normalized data by using duplicate correlations, and empirical Bayes analysis was used to compute moderated *t*-statistics, which were then used to obtain *P*-values. For multiple testing, the *P*-value adjustment method of Benjamini and Hochberg (1995) was applied to control the false discovery rate (i.e., expected proportion of truly nondifferentially expressed genes among the rejected hypotheses). An adjusted *P*-value cutoff of 0.01 was used to generate differentially expressed gene lists. Differentially expressed genes were chosen if the fold change was >1.5 (induced) or <0.67 (repressed).

Real-time PCR TaqMan Reverse Transcription reagents (Applied Biosystems, Foster City, CA, USA) were used to synthesize cDNA. Reaction conditions were 1× TaqMan RT buffer, 5.5 mM $MgCl_2$, 500 μM dNTPs, 2.5 μM random hexamers, 0.4 U/μl RNase Inhibitor, and 1.25 U/μl multiscribe reverse transcriptase and incubated at 25°C 10 min, 48°C 30 min, and finally 95°C 5 min.

Real-time PCR was performed with a 7500 Real-Time PCR System (Applied Biosystems) by using the following parameters: 50°C 2 min, 95°C 10 min, followed by 40 cycles of 95°C 15 s, 60°C 1 min. *Power* SYBR Green PCR Master Mix (Applied Biosystems) was used in a final reaction volume of 25 μl. Target gene primers were used at a final concentration of 900 nM and 18S ribosomal endogenous control primers at 100 nM.

To utilize the comparative C_T method of relative quantitation of gene expression, validation experiments were performed on all target gene primers (primer pairs listed in Table 1). The primers used for 18S rRNA were as described in Nicot et al. (2005). All target gene primers had amplification efficiency similar to the 18S amplicon (absolute value of the slope of ΔC_T (target gene-18S) vs. log input RNA were all <0.1). Dissociation curves were performed for all primer pairs to check specificity of primers for the target gene. Fold change levels of gene expression were expressed as relative quantitation (RQ) values by using a "calibrator" sample as a reference with Sequence Detection Software version 1.4 (Applied Biosystems). As such, an RQ of 1 indicates a sample exhibiting the same relative transcript abundance as the control. The data were $\log_{10}$ transformed prior to graphing; hence, equal relative transcript abundance in control and treatment samples resulted in a log RQ value of 0.

Results and Discussion

Genes are Both Induced and Repressed by CPB Regurgitant As an important step towards our long-term goal of better understanding the contribution of CPB to the CPB–potato interaction, we set out to use microarray gene

Table 1 Primer pairs selected for real-time PCR

Clone ID	5′-3′ Sequence	3′-5′ Sequence
STMDJ96	CAAACAAAACCCCACAAACTACTTCACT	GCTGTGGCATTGACACTTGACACTT
STMFB59	GGAACTGTTGGTTCTAGTGATGATTC	TGTAGCACATATGTCCAGTTTCATGT
STMEP88	GGCAACTTTCATGCGTCAAA	GCACTAATTCGCTGATGAAATTGT
STMEV47	AAGAAGTCTAATAGCAGCAAGAGGAT	GGATTTAAAAAAAGCATCGCAAA
STMCK44	CCATTGAGTACGCTGTTCTTCATC	AAGCACTGTGGCCAATGACA
18S rRNA[a]	GGGCATTCGTATTTCATAGTCAGAG	CGGTTCTTGATTAATGAAAACATCCT

[a] The GenBank accession number for 18S rRNA is X67238

expression profiling to identify genes whose expression is modulated by the presence of CPB regurgitant. Regurgitant-responsive genes can serve as vital reporter genes for investigations of CPB–potato interactions and particularly for identification of components of CPB regurgitant that act as elicitors to induce or repress the expression of these genes. Accordingly, leaves were wounded and treated with either CPB regurgitant at a 1:3 dilution or with water. Two-colored microarrays were performed to compare these two conditions. This experimental design provided the most direct means to identify genes whose expression is modulated by CPB regurgitant, since our primary objective was to identify regurgitant-responsive genes rather than investigate the effect of regurgitant on the expression of wounding-responsive genes. Four hours following wounding was chosen as the most appropriate time point for these analyses, based on the rationale that most genes that are induced early in the defense response would still be upregulated at 4 h; genes that are induced only later in the defense response would be commencing upregulation at 4 h, and genes that are differentially expressed only as an indirect consequence of the wounding plus or minus regurgitant treatment would not yet have been upregulated at 4 h, i.e., allowing us to minimize secondary effects of the plant/insect interaction. Korth and Dixon (1997) have demonstrated that while the timing of maximum expression of genes in response to wounding or wounding plus regurgitant can differ over the course of the defense response, both treatments result in a local induction of gene expression over baseline levels within a few hours of treatment, and that this induction persists for many hours. This and other studies (e.g., Christopher et al. 2004; Delessert et al. 2004) suggest that while the timing of sample harvest is critical to detect *peak* levels of gene expression during the defense response, a relatively broad window of opportunity exists to detect *statistically different* levels of gene expression for the majority of genes differentially expressed during the defense response. Consequently, we decided to focus on a single time point following wounding of the leaves for our microarray analysis. A limitation to this approach is that the time course of the regurgitant-associated induction or repression of gene expression is not known, which affects to some extent our ability to make biological inferences from the data. It is also possible that the analysis failed to identify those regurgitant-responsive genes that are significantly induced or repressed very early or very late in response to the treatment but not at 4 h following wounding. Similarly, this analysis would not reveal regurgitant-responsive genes that are not present on the TIGR Solanaceae microarray.

Analyses of the microarray data revealed 127 genes whose transcript abundance was significantly different in plants treated with mechanical wounding plus regurgitant compared to plants that were treated with mechanical wounding alone. The genes selected as significantly differentially expressed had an adjusted P-value of <0.01 and a fold changed of >1.5 (induced) or <0.67 (repressed). The regurgitant-responsive gene set can include genes that are regurgitant but not wounding responsive, as well as genes that are wounding responsive but whose expression patterns are intensified by regurgitant. Annotations for these differentially expressed genes are based on similarities to annotated sequences at the National Center for Biotechnology Information queried using Basic Local Alignment Search Tool (BLAST)X (Altschul et al. 1997).

There were 73 genes found to be induced (adjusted P-value<0.01, fold change>1.5) by adding CPB regurgitant to wounded leaves compared to the addition of water to wounded leaves (Table 2). They include proteins involved in secondary metabolism, general metabolism, protein expression, transcriptional regulation, stress, and pathogen responses. Not unexpectedly, a number of genes that encode proteins of unknown function were also induced. This can be in part ascribed to the lack of complete coding sequence information for a number of cDNAs represented on the array, which reduces the probability of retrieving a significant match from a sequence similarity search.

Interestingly, a total of 54 genes were repressed (adjusted P-value<0.01, fold change<0.67) in wounded leaves in the presence of regurgitant (Table 3). Genes associated with photosynthesis and stress were the most prevalent among downregulated genes. Genes were considered repressed if, in addition to a P-value of ≤ 0.01, the fold change was <0.67. Genes of secondary metabolism were more abundant

Table 2 Genes of *S. tuberosum* var. Kennebec induced by treatment of wounded leaves with regurgitant of *L. decemlineata*

Clone ID	Description	Notes	FC		Adjusted *P*-value	
			1st spot	2nd spot	1st spot	2nd spot
	General metabolism					
STMER62	Copper-containing amine oxidase		1.75	1.76	0.006	0.006
STMCU61	Glyceraldehyde-3-phosphate dehydrogenase		1.63	1.61	0.003	0.004
STMHX09	Oxidoreductase family protein		1.77	1.74	0.001	0.001
STMEW21	Pyruvate decarboxylase		1.51	1.42	0.007	0.007
STMHV89	Pyruvate decarboxylase		1.52	1.50	0.005	0.006
STMIP08	UDP-glycosyltransferase 85A8		2.11	1.99	0.003	0.003
STMGA89	Phosphoribosylpyrophosphate synthetase		1.61	1.64	0.003	0.013
STMIS31	Dihydrodipicolinate reductase	aa metabolism	1.51	1.47	0.003	0.013
STMJC81	UDP-glucoronosyl and UDP-glucosyltransferase	Carbohydrate	1.36	1.62	0.004	0.004
STMIB34	Glycosyltransferase putative	Cell wall	1.64	1.53	0.003	0.003
STMFB08	Acyl-CoA synthetase putative	Fatty acids	2.10	2.04	0.004	0.004
STMIF62	Omega-6 desaturase	Fatty acids	1.77	1.69	0.003	0.007
STMGH41	Omega-6 fatty acid desaturase	Fatty acids	1.97	1.86	0.006	0.007
STMER74	Ferric-chelate reductase	Iron uptake	1.52	1.51	0.004	0.005
STMGA88	CXE carboxylesterase	Lipids	1.61	1.80	0.003	0.010
STMER46	Allantoinase	Nitrogen	1.79	1.79	0.007	0.010
STMCP60	Allantoinase putative	Nitrogen	1.73	1.71	0.010	0.011
STMDI05	Glutamine synthetase	Nitrogen	1.54	1.57	0.009	0.009
	Other					
STMIX86	Amino acid transporter family protein		1.52	1.55	0.003	0.003
STMEQ77	Auxin efflux carrier family protein		1.56	1.49	0.006	0.007
STMJG89	Late embryogenesis abundant protein 5		1.86	1.87	0.009	0.010
STMER95	Multidrug resistance-associated protein MRP1	Transport	1.47	1.53	0.003	0.004
STMEJ57	Glucose-6-phosphate/phosphate translocator 2	Transport	1.56	1.74	0.004	0.010
	Protein expression					
STMJG07	Syntaxin-related protein Nt-syr1		1.54	1.49	0.009	0.012
STMIO78	tolB protein-related protein		2.10	1.81	0.009	0.010
	Secondary metabolism			1.00		
STMIU03	2OG-Fe(II) oxygenase		1.64	1.52	0.003	0.004
STMIV90	Copper amine oxidase	Lignin	1.51	1.52	0.007	0.011
STMIS10	Cytochrome P450		1.67	1.68	0.003	0.004
STMJC24	Cytochrome P450		2.05	1.96	0.003	0.004
STMIZ25	Cytochrome P450		1.58	1.62	0.004	0.012
STMJF12	Cytochrome P450 71D10		1.75	1.59	0.003	0.003
STMJF13	Cytochrome P450 71D10		1.82	1.65	0.004	0.006
STMIZ06	Cytochrome P450 76A2 (CYPLXXVIA2)		1.53	1.53	0.005	0.010
STMEV43	Cytochrome P450 76A2 (CYPLXXVIA2)		1.55	1.57	0.009	0.009
STMHW65	Cytochrome P450 76A2 (CYPLXXVIA2)		1.51	1.51	0.007	0.010
STMIW91	Multicopper oxidase		1.51	1.50	0.003	0.004
STMEZ84	*N*-hydroxycinnamoyl-CoA: tyramine *N*-hydroxycinnamoyl transferase	Phenylpropanoids	1.72	1.75	0.007	0.007
STMIP12	Peroxidase	Lignin	2.01	1.97	0.006	0.007
STMIR86	Peroxidase chain A	Lignin	1.69	1.82	0.009	0.015
STMEP54	Rhizome secoisolariciresinol dehydrogenase	Lignin	1.90	1.88	0.001	0.003
STMFB13	Terpene synthase	Terpenes	1.81	1.89	0.003	0.004
STMHI44	Terpene cyclase	Terpenes	1.59	1.57	0.001	0.004
STMGR31	Transferase family protein		1.53	1.42	0.004	0.005
STMJE63	Tyramine hydroxycinnamoyl transferase	Phenylpropanoids	1.89	1.86	0.004	0.004
STMIP44	Tyramine hydroxycinnamoyl transferase	Phenylpropanoids	1.62	1.60	0.009	0.011
STMEP88	Aromatic amino acid decarboxylase 1A	Volatiles	1.96	1.83	0.010	0.010
	Stress					
STMIP26	Alternative oxidase 1b		1.58	1.76	0.003	0.004

Table 2 (continued)

Clone ID	Description	Notes	FC		Adjusted *P*-value	
			1st spot	2nd spot	1st spot	2nd spot
STMGP26	Avr9/Cf-9 rapidly elicited protein 284	Pathogen	1.72	1.74	0.007	0.011
STMEO91	Class II	Chitinase pathogen	1.51	1.46	0.003	0.005
STMFB59	Class IV	Chitinase pathogen	1.75	1.73	0.006	0.007
STMEH61	Extracellular dermal glycoprotein putative/EDGP	Wounding	1.58	1.51	0.006	0.011
STMEG50	Formate dehydrogenase		1.52	1.53	0.007	0.007
STMET22	Glutathione *S*-transferase		1.60	1.59	0.004	0.004
STMJA60	Hin1-like protein	Pathogen	1.60	1.57	0.006	0.012
STMDW19	Pathogenesis-related protein 10	Methy jasmonate	1.49	1.52	0.004	0.005
STMCF73	Pathogenesis-related protein 10	Methy jasmonate	1.58	1.54	0.003	0.007
STMIR88	Pathogenesis-related protein 10	Methy jasmonate	2.00	1.87	0.004	0.006
STMEY92	Universal stress protein		1.85	1.78	0.003	0.004
STMHY05	Unknown		1.73	1.61	0.006	0.010
STMHL75	Vacuolar processing enzyme-1b		1.51	1.51	0.006	0.008
STMEP50	Wound-induced protein 1	Wounding	1.51	1.48	0.005	0.006
STMEZ47	Pathogen-inducible alpha-dioxygenase	Herbivore	1.66	1.64	0.003	0.003
	Transcription factors					
STMGC50	Myb-related transcription factor		1.51	1.68	0.004	0.009
STMDJ96	ZIM domain protein 1		1.51	1.52	0.004	0.005
STMEV47	ZPT2–13		1.53	1.45	0.005	0.015
	Unknown					
STMEP34	Cys-rich domain		1.58	1.55	0.009	0.011
STMIX36	DC1 domain-containing protein		1.50	1.32	0.004	0.005
STMJA37	No current TC		1.84	1.85	0.008	0.010
STMEV12	No significant match		1.68	1.63	0.010	0.015

FC Fold change

when compared to genes repressed by regurgitant treatment (Tables 2 and 3).

Expression patterns of five genes found to be differentially expressed by CPB regurgitant with the microarray were confirmed with real-time PCR with independent experimental material (Fig. 1). The genes were chosen to represent different functional categories. These experiments further compared transcript abundance in water-treated and regurgitant-treated wounded leaves to that of control (unwounded) leaves as well as to two infestation treatments. The latter one provided an additional control to determine whether wounding plus regurgitant was a reasonable substitute for infestation in generating a defense response. All five genes showed expression patterns in regurgitant-treated wounded leaves and water-treated wounded leaves that were in agreement with the microarray data. For four genes—*STMEV47* (similar to ZPT2–13 zinc finger protein), *STMFB59* (similar to class IV chitinase), *STMDJ96* (similar to ZIM domain protein), and *STMEP88* (aromatic amino acid decarboxylase)—real-time PCR data demonstrated that transcript abundance in regurgitant-treated wounded leaves was significantly higher than in water-treated wounded leaves. With the exception of *STMEP88*, each of these genes was also upregulated by wounding alone relative to control untreated plants; for these three genes, the presence of regurgitant served to amplify the induction of gene expression over wounding alone. The fifth gene that was tested, *STMCK44* (similar to carbonic anhydrase), was downregulated by wounding and further downregulated by wounding plus regurgitant, consistent with the microarray data. Each of these five genes was also differentially expressed in response to CPB infestation of leaves in the same pattern produced by mechanical wounding plus regurgitant (Fig. 1), lending validation to the wounding/regurgitant method. Two different infestation treatments were carried out to contrast the effect with the addition of regurgitant to wounded leaves. Infestation consisted of either 4 h of continuous infestation (4hI) or the leaves were subjected to 1 h of infestation followed by 3 h of recovery (1hI3hR). Neither of these infestation treatments was specifically designed to mimic the wounding treatment in intensity or timing and, as such, comparison of the amplitudes of gene responses to mechanical wounding plus or minus regurgitant to those

Table 3 Genes of *S. tuberosum* var. Kennebec repressed by treatment of wounded leaves with regurgitant of *L. decemlineata*

Clone ID	Description	Notes	FC		Adjusted *P*-value	
			1st spot	2nd spot	1st spot	2nd spot
	General metabolism					
STMHE94	NADH:ubiquinone oxidoreductase		0.61	0.61	0.007	0.011
STMCL39	H-protein	Amino acids	0.65	0.64	0.003	0.003
STMCN66	Putative glucosyltransferase	Cell walls	0.66	0.73	0.003	0.012
STMGQ22	Endo-beta-1 4-D-glucanase	Cell walls	0.64	0.64	0.004	0.004
STMGB59	Expansin11 precursor	Cell walls	0.60	0.61	0.004	0.006
STMIR25	Glyceraldehyde-3-phosphate dehydrogenase	Glycolysis	0.59	0.59	0.004	0.004
STMCK16	GDSL-motif lipase/hydrolase family protein	Lipids	0.59	0.56	0.004	0.008
STMCG91	Nitrite reductase	Nitrogen	0.66	0.64	0.004	0.008
STMIX44	*N*-acetylglucosaminyl-transferase	Oligosaccharides	0.59	0.61	0.004	0.004
	Other					
STMIF38	Senescence-associated protein		0.66	0.65	0.003	0.004
STMIJ79	Fasciclin-like arabinogalactan-protein (FLA9)		0.61	0.68	0.004	0.004
STMJC16	GAST1 protein precursor	GA induced	0.57	0.55	0.003	0.003
	Photosynthesis					
STMCK44	Carbonic anhydrase		0.54	0.55	0.003	0.004
STMCV75	Carbonic anhydrase		0.55	0.55	0.003	0.003
STMCR16	Carbonic anhydrase		0.56	0.54	0.003	0.003
STMCL01	Carbonic anhydrase		0.57	0.55	0.003	0.003
STMIW27	Carbonic anhydrase		0.58	0.57	0.003	0.003
STMCP14	Glyceraldehyde-3-phosphate dehydrogenase A chloroplast		0.62	0.64	0.003	0.003
STMEW09	Glyceraldehyde-3-phosphate dehydrogenase A chloroplast		0.62	0.64	0.0023	0.00
STMCM20	Glyceraldehyde-3-phosphate dehydrogenase A chloroplast		0.63	0.64	0.003	0.003
STMHQ84	Photosystem II 10 kDa polypeptide		0.64	0.65	0.003	0.004
STMBB03	Rubisco		0.62	0.65	0.003	0.006
STMEO78	Thylakoid lumenal 16.5 kDa protein		0.65	0.64	0.003	0.003
	Protein expression					
STMJI48	Immunophilin putative/FKBP-type peptidyl–prolyl *cis–trans* isomerase		0.64	0.62	0.004	0.004
STMDS45	Ribosomal protein L14 putative		0.64	0.67	0.004	0.004
STMDS44	Ribosomal protein L14 putative		0.66	0.72	0.004	0.005
STMCQ24	Ribosomal protein S1-like RNA-binding domain		0.66	0.69	0.003	0.004
STMIO81	Trypsin putative		0.58	0.60	0.008	0.013
	Stress					
STMIX39	Chitin-binding lectin 1 precursor (PL-I)		0.49	0.52	0.007	0.010
STMCQ19	Chitin-binding lectin 1 precursor (PL-I)		0.56	0.60	0.008	0.010
STMET49	Cold-stress inducible protein		0.62	0.69	0.005	0.016
STMJI39	Proline-rich protein		0.56	0.57	0.003	0.003
STMGL17	Proline-rich protein		0.58	0.61	0.001	0.003
STMDO51	Proline-rich protein, putative		0.67	0.62	0.005	0.007
STMDB28	Proline-rich protein, putative		0.68	0.66	0.004	0.006
STMJM27	Protease inhibitor/seed storage/lipid transfer protein		0.61	0.61	0.010	0.019
STMGA57	Proteinase inhibitor type-2 TR8		0.60	0.60	0.005	0.008
STMGY25	Wound-induced proteinase inhibitor I		0.64	0.65	0.004	0.009
STMCM83	24K germin like protein	Herbivore	0.46	0.47	0.003	0.003
STMCX35	24K germin like protein precursor	Herbivore	0.49	0.46	0.003	0.003
STMFB56	Germin-like protein	Herbivore	0.53	0.54	0.004	0.004
STMFB14	Germin-like protein	Herbivore	0.55	0.55	0.006	0.009
STMIW53	Aspartic proteinase	Wound	0.64	0.65	0.006	0.007
	Transcription factor					
STMIZ44	Transcription regulator		0.64	0.63	0.001	0.004
	Unknown					
STMIW44	Expressed protein		0.61	0.64	0.003	0.003

Table 3 (continued)

Clone ID	Description	Notes	FC		Adjusted *P*-value	
			1st spot	2nd spot	1st spot	2nd spot
STMIV24	No current TC		0.65	0.63	0.003	0.003
STMBB26	No current TC		0.66	0.69	0.001	0.003
STMCB92	No significant match		0.52	0.62	0.004	0.004
STMJI40	No significant match		0.61	0.64	0.003	0.004
STMJM94	No significant match		0.64	0.68	0.004	0.004
STMII80	No significant match		0.65	0.66	0.005	0.007
STMIR33	No significant match		0.66	0.68	0.004	0.004
STMIP15	No significant match		0.69	0.66	0.003	0.003
STMJJ57	No significant match		0.61	0.80	0.004	0.040

FC Fold change

evoked by the infestation treatments should be interpreted with care. Each of the genes examined by real-time PCR showed a greater response to 4hI than to 1hI3hR; additionally, transcript abundance for most genes exhibited greater variance in the 1hI3hR treatment. Taken together, the quantitative real-time PCR experimental data demonstrated that four genes are differentially expressed by wounding, the expression is enhanced by regurgitant and infestation also results in differential expression of these genes.

Photosynthesis and Carbohydrate Metabolism Twenty percent of the genes repressed by regurgitant encoded photosynthetic proteins (Table 3). Examples include genes involved in photosynthetic electron transport such as a photosystem II reaction center protein and a thylakoid lumenal 16.5 kDa protein. This observed downregulation of photosynthetic genes is consistent with the findings of Hermsmeier et al. (2001). A number of genes associated with the utilization of carbon resources were repressed by regurgitant, including carbonic anhydrase, glyceraldehyde-3-phosphate dehydrogenase, and Rubisco. However, this was not an overriding pattern, as other genes involved in carbohydrate metabolism were induced when regurgitant was added to wounded leaves. For example, genes that encode pyruvate decarboxylase, glyceralydehyde-3-phosphate dehydrogenase, and uridine diphosphate (UDP)-glycosyltransferase were induced by regurgitant. Glyceraldehyde-3-phosphate dehydrogenase poses an interesting case in our study, as different genes that encode this enzyme showed upregulation or downregulation at 4 h after wounding plus regurgitant. Enzymes encoded by different members of this gene family are targeted to different cellular compartments and participate in different biochemical pathways: Chloroplastic glyceraldehyde-3-phosphate dehydrogenase catalyzes a step of the reduction phase of the Calvin cycle (photosynthetic carbon reduction cycle), while cytosolic glyceraldehyde-3-phosphate dehydrogenase is an enzyme of glycolysis. Furthermore, different forms of cytosolic glyceraldehyde-3-phosphate dehydrogenase exist that allow carbon flux through glycolysis even under non-favorable energy status conditions, such as when low concentrations of ATP are encountered (Dennis and Blakeley 2000). Clearly, CPB exerts an influence through its regurgitant not only on the plant's carbon acquisition via photosynthesis but also on the plant's utilization of its carbon resources. That some genes associated with carbon metabolism are upregulated by regurgitant while others are downregulated indicate that this modulation is not a wholesale induction or repression of resource utilization; rather, it would appear that the presence of CPB regurgitant signals changes to the plant that result in more subtle redirection of carbon flux through metabolic networks in the attacked leaves. It will be of interest to determine how CPB regurgitant influences carbon flux through the biochemical pathways associated with the genes shown to be regurgitant responsive in this study.

Protein Synthesis and Nitrogen Metabolism We detected a suite of differentially expressed genes associated with nitrogen-based biochemical pathways. Notably, genes that encode allantoinase were induced by CPB regurgitant in potato. Allantoinase is an enzyme of ureide metabolism that catalyzes the conversion of allantoin to allantoate. The ureides are nitrogen-rich organic compounds that are important for nitrogen transport and remobilization. Ureides are best characterized in nitrogen-fixing legumes, but recently enzymes implicated in ureide metabolism have been described in nonureide-type legume (Yang and Han 2004) and nonlegume species (Yang and Han 2004; Todd and Polacco 2006). The potato putative allantoinase genes, along with a putative allantoinase gene from tomato, show high sequence similarity to a functionally characterized

Arabidopsis allantoinase; these genes were distinct from genes that encode a phylogenetically related enzyme, dihydroorotase (Yang and Han 2004). Complementation studies with the Arabidopsis allantoinase gene confirmed the biochemical activity of the enzyme encoded by this gene, and mutagenesis studies were used to show that allantoinase allows the plant to utilize allantoin as a sole nitrogen source. In black locust, there is an upregulation of allantoinase in the fall and spring and, hence, is proposed to play a role in seasonal nitrogen cycling (Yang and Han 2004). Allantoinases have been linked previously to plant defense in potato. A putative allantoinase was induced in potato tuber by treatment with a crude elicitor made from hyphal wall components of the fungus *Phytophthora infestans* (Nakane et al. 2003).

Other genes associated with nitrogen utilization that were upregulated in the presence of regurgitant include glutamine synthetase (likely associated with nitrogen reassimilation and recycling) and a gene that encodes a protein similar to an amino acid transporter. Together, these results suggest that plants alter their nitrogen utilization strategies in response to elicitation by regurgitant over and beyond changes brought about in response to wounding. Our data suggest that these changes invoke enhanced recycling and remobilization of nitrogen resources in attacked leaves. While considerable attention has been paid to the effect of soil nitrogen availability on allocation of plant resources to defense and the effect of leaf nitrogen content on the performance of herbivorous insects (e.g., Stamp 2003 and references therein), much less is known about repartitioning and reallocation of nitrogen resources in plants upon infestation and how this may affect not only the insect's performance but also the plant's ability to resist attack to promote damage repair and to resume growth and development following attack. Clearly, nitrogen resource utilization in plants under attack by herbivorous insects requires further investigation.

Secondary Metabolism Genes involved in secondary metabolism were prominent in the list of upregulated genes of plants that had been wounded and treated with regurgitant. Eight different genes that encode cytochrome P450s were induced. Cytochrome P450s are a complex superfamily. In fact, 272 genes have been identified in the Arabidopsis genome (Schuler and Werck-Reichhart 2003). They are involved in biosynthetic reactions that produce such compounds as phenylpropanoids, alkaloids, and terpenoids. Terpenoids make up a portion of the volatiles created by infestation-induced plants, which could play a part in attraction of predator insects and parasitoids to the infested potato. Two genes encoding proteins involved in terpene synthesis are induced in these plants as well. In addition, several genes that may encode proteins involved in phenylpropanoid biosynthesis are induced: tyramine hydroxycinnamoyl transferase, copper amine oxidase, and peroxidase.

A gene that encodes aromatic amino acid decarboxylase was induced, and confirmation of this result by real time RT-PCR is presented in Fig. 1d. This enzyme is involved in the production of phenylalanine-derived volatile compounds important for insect attraction (Tieman et al. 2006). Phenylpropanoids are often anti-insecticidal compounds. Aromatic amino acid decarboxylase is the enzyme responsible for the precursor of 2-phenylethanol (Tieman et al. 2006), which is a volatile specific to CPB-damaged plants (Schütz et al. 1997) and is found to be particularly attractive to the CPB predator *P. bioculatis* (Weissbecker et al. 1999). This suggests that 2-phenylethanol is a volatile produced in response to regurgitant treatment of wounded potato plants. This plant response is part of a tritrophic interaction, which may indirectly result in plant defense.

Genes Implicated in Pathogen Defense and/or General Stress Response Many of the genes identified as differentially expressed in response to regurgitant application to wounded leaves have been associated either with defense against pathogens or as part of general stress response. Generally, pathogen-induced genes are induced by the phytohormone salicylic acid, while wounding-associated genes are induced by jasmonic acid. However, overlap in these pathways is well-documented, and it will be of interest to test the regulation of these genes by these compounds.

Chitinases were among those pathogen-associated genes affected by regurgitant application. Two chitinase genes were identified as regurgitant-induced (a class IV and class II). The class IV chitinase identified by BLAST search and the class II chitinase were found to be involved with the response to pathogen attack (Büchter et al. 1997; Shinya et al. 2007). The induction of the class IV chitinase is confirmed by real-time PCR data in Fig. 1c. Three genes with homology to pathogenesis protein 10 were also induced by regurgitant. The homolog of this gene is induced by methyl jasmonate and salicylic acid in *S. surattense* (Liu et al. 2006). This suggests that the protein may have a broad defense function.

A noteworthy category of repressed genes are those genes associated with stress. In tomato, we have found that proteinase inhibitors PinI and PinII are repressed by CPB regurgitant (Lawrence et al. 2007). There are 16 type 1 and type 2 proteinase inhibitor clones on the potato microarray. Three of these inhibitors are repressed by CPB regurgitant. Since proteinase inhibitors act by inhibiting digestion of proteins in the insect midgut, this repression may represent a mechanism by which the insect subverts the plant's defense mechanism.

Other potentially interesting genes that are repressed by regurgitant are the germins. Of the eight germin genes found on the potato microarray, four are repressed by CPB regurgitant. Germins are a large multigene family, which may contain superoxide dismutase activity, and some subfamilies may be induced by pathogens and herbivores (Lou and Baldwin 2006; Zimmermann et al. 2006). For example, the silencing of a germin from *N. attenuata* results in increased mass of *M. sexta* feeding on the plant and increases *Tupiocoris notatus* preference for the transgenic plant (Lou and Baldwin 2006). In Arabidopsis, 32 germins have been identified (Zimmermann et al. 2006). An extensive study of germins from barley examined members of each of the six identified subfamilies (Zimmermann et al. 2006). The barley sequence of HvGER2a showed the greatest similarity to the potato CPB repressed germin genes when examined by using BLASTX. The gene in barley is wound and cold-induced (Zimmermann et al. 2006) but the protein does not seem to have superoxide dismutase activity. Therefore, it is unclear how this protein or the potato germins might function. Perhaps knowing how CPB regurgitant inhibits germins in potato may offer insight into transgenic approaches that may circumvent this repression.

In summary, we have presented the first examination of transcriptional profiling of genes affected by CPB regurgitant. This is a valuable first step given the agronomic importance of this plant/insect interaction. It also affords the opportunity to characterize promoters from a number of regurgitant-induced genes. We found both induced and repressed genes due to regurgitant addition to wounded leaves. Characterization of the regurgitant may further elucidate the components involved in induction of potato genes by CPB regurgitant. CPB regurgitant affects the expression of a diverse set of genes: While many of these genes can be associated with classical stress and defense-associated mechanisms, including defensive chemistry biosynthesis, a number of these genes are related to the utilization of carbon and nitrogen resources. Interestingly, at least one of these regurgitant-responsive genes may be implicated in tritrophic interactions. As such, this study demonstrates that CPB regurgitant affects the expression of genes beyond those typically associated with the host's defensive arsenal and illustrates the complexity of the interactions that occur between herbivorous insects and host plants and potentially with other organisms.

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References

ALBORN, H. T., TURLINGS, T. C. J., JONES, T. H., STENHAGEN, G., LOUGHRIN, J. H., and TUMLINSON, J. H. 1997. An elicitor of plant volatiles from beet armyworm oral secretions. *Science* 276:945–949.

ALTSCHUL, S. F., MADDEN, T. L., SCHAFFER, A. A., ZHANG, J., ZHANG, Z., MILLER, W., and LIPMAN, D. J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* 25:3389–3402.

BENJAMINI, Y., and HOCHBERG, G. Y. 1995. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J. R. Stat. Soc. (Britain)* 57:289–300.

BÜCHTER, R., STRÖMBERG, A., SCHMELZER, E., and KOMBRINK, E. 1997. Primary structure and expression of acidic (class II) chitinase in potato. *Plant Mol. Biol.* 35:749–761.

CHRISTOPHER, M. E., MIRANDA, M., MAJOR, I. T., and CONSTABEL, C. P. 2004. Gene expression profiling of systemically wound-induced defenses in hybrid poplar. *Planta* 219:936–947.

DELESSERT, C., WILSON, I. W., VAN DER,D., STRAETEN, DENNIS, E. S., and DOLFERUS, R. 2004. Spatial and temporal analysis of the local response to wounding in *Arabidopsis* leaves. *Plant Mol. Biol.* 55:165–181.

DENNIS, D. T., and BLAKELEY, S. D. 2000. Carbohydrate metabolism, pp. 630–675, in B. Buchanan, W. Gruissem, and R. Jones (eds.). Biochemistry and Molecular Biology of Plants American Society of Plant Physiologists, Rockville, MD.

DE, VOS, M., VAN, OSTEN, V. R., VAN, POECKE, R. M., VAN, PELT, J. A., POZO, M. J., MUELLER, M. J., BUCHALA, A. J., MÉTRAUX, J. P., VAN, LOON, L. C., DICKE, M., and PIETERSE, C. M. 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Mol. Plant-Microb. Interact.* 18:923–937.

DOBBIN, K., and SIMON, R. 2002. Comparison of microarray designs for class comparison and class discovery. *Bioinformatics* 18:1438–1445.

DUDOIT, S., and YANG, J. Y. H. 2002. Bioconductor R packages for exploratory analysis and normalization of cDNA microarray data, pp. 73–101, in G. Parmigiani, E. S. Garrett, R. A. Irizarry, and S. L. Zeger (eds.). The Analysis of Gene Expression Data: Methods and SoftwareSpringer, New York.

EICHENSEER, H., MATHEWS, M. C., BI, J. L., MURPHY, J. B., and FELTON, G. W. 1999. Salivary glucose oxidase: multifunctional roles for Helicoverpa zea. *Arch. Insect Biochem. Physiol*. 42:99–109.

GENTLEMAN, R. C., CAREY, V. J., BATES, D. M., BOLSTAD, B., DETTLING, M., DUDROIT, S., ELLIS, B., GAUTIER, L., GE, Y., GENTRY, J., HORNIK, K., HOTHORN, T., HUBER, W., IACUS, S., IRIZARRY, R., LEISCH, F., LI, C., MAECHLER, M., ROSSINI, A. J., SAWITZKI, G., SMITH, C., SMYTH, G., TIERNEY, L., YANG, J. Y. H., and ZHANG, J. 2004. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol*. 5:R80.

HALITSCHKE, R., SCHITTKO, U., POHNERT, G., BOLAND, W., and BALDWIN, I. T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata* III. Fatty acid–amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol*. 125:711–717.

HALITSCHKE, R., GASE, K., HUI, D., SCHMIDT, D. D., and BALDWIN, I. T. 2003. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata* VI. Microarray analysis reveals that most herbivore-specific transcriptional changes are mediated by fatty acid–amino acid conjugates. *Plant Physiol*. 131:1894–1902.

HERMSMEIER, D., SCHITTKO, U., and BALDWIN, I. T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiol*. 125:683–700.

IHAKA, R., and GENTLEMAN, R. 1996. R: A language for data analysis and graphics. *J. Comp. Graph. Stat.* 5:299–314.

KORTH, K. L., and DIXON, R. A. 1997. Evidence for chewing insect-specific molecular events distinct from a general wound response in leaves. *Plant Physiol*. 115:1299–1305.

KRUZMANE, D., JANKEVICA, L., and IEVINSH, G. 2002. Effect of regurgitant from *Leptinotarsa decemlineata* on wound responses in *Solanum tuberosum* and *Phaseolus vulgaris*. *Physiol. Plant*. 115:577–584.

LAWRENCE, S. D., NOVAK, N. G., and BLACKBURN, M. B. 2007. Inhibition of proteinase inhibitor transcripts by *Leptinotarsa decemlineata* regurgitant in *Solanum lycopersicum*. *J. Chem. Ecol.* 33:1041–1048.

LIU, X., HUANG, B., LIN, J., FEI, J., CHEN, Z., PANG, Y., SUN, X., and TANG, K. 2006. A novel pathogenesis-related protein (SsPR10) from *Solanum surattense* with ribonucleolytic and antimicrobial activity is stress- and pathogen-inducible. *J. Plant Physiol.* 163:546–556.

LOU, Y., and BALDWIN, I. T. 2006. Silencing of a germin-like gene in *Nicotiana attenuata* improves performance of native herbivores. *Plant Physiol.* 140:1126–1136.

MAJOR, I. T., and CONSTABEL, P. C. 2006. Molecular analysis of poplar defense against herbivory: comparison of wound- and insect elicitor-induced gene expression. *New Phytol.* 172:617–635.

MATTIACCI, L., DICKE, M., and POSTHUMUS, M. A. 1995. Beta-glucosidase: an elicitor of herbivore-induced plant odor that attracts host-searching parasitic wasps. *Proc. Nat. Acad. Sci. U.S. A.* 92:2036–2040.

MUSSER, R. O., HUM-MUSSER, M. C., BI, J. L., MURPHY, J. B., and FELTON, G. W. 2002. Caterpillar saliva beats plant defences. *Nature* 416:599–600.

NAKANE, E., KAWAKITA, K., DOKE, N., and YOSHIOKA, H. 2003. Elicitation of primary and secondary metabolism during defense in the potato. *J. Gen. Plant Pathol.* 69:378–384.

NICOT, N., HAUSMAN, J. F., HOFFMANN, L., and EVERS, D. 2005. Housekeeping gene selection for real-time RT-PCR normalization in potato during biotic and abiotic stress. *J. Exp. Bot.* 56:2907–2914.

POHNERT, G., JUNG, V., HAUKIOJA, E., LEMPA, K., and BOLAND, W. 1999. New fatty acid amides from regurgitant of lepidopteran (Noctuidae, Geometridae) caterpillars. *Tetrahedron* 55:11275–11280.

RALPH, S., ODDY, C., COOPER, D., YUEH, H., JANCSIK, S., KOLOSOVA, N., PHILIPPE, R. N., AESCHLIMAN, D., WHITE, R., HUBER, D., RITLAND, C. E., BENOIT, F., RIGBY, T., NANTEL, S., BUTTERFIELD, Y. S. N., KIRKPATRICK, R., CHUN, E., LIU, J., PALMQUIST, D., WYNHOVEN, B., STOTT, J., YANG, G., BARBER, S., HOLT, R. A., SIDDIQUI, A., JONES, S. J. M., MARRA, M. A., ELLIS, B. E., DOUGLAS, C. J., RITLAND, K., and BOHLMANN, J. 2006a. Genomics of hybrid poplar (*Populus trichocarpa x deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and a cDNA microarray for the study of insect-induced defences in poplar. *Mol. Ecol.* 15:1275–1297.

RALPH, G. S., YUEH, H., FRIEDMANN, M., AESCHLIMAN, D., ZEZNIK, J. A., NELSON, C. C., BUTTERFIELD, Y. S. N., KIRKPATRICK, R., LIU, J., JONES, S. J. M., MARRA, M. A., DOUGLAS, C. J., RITLAND, K., and BOHLMANN, J. 2006b. Conifer defence against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell Environ*. 29:1545–1570.

REYMOND, P., WEBER, H., DAMOND, M., and FARMER, E. E. 2000. Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. *Plant Cell* 12:707–719.

REYMOND, P., BODENHAUSEN, N., VAN, POECKE, M. P., KRISHNAMURTHY, V., DICKE, M., and FARMER, E. E. 2004. A conserved transcript pattern in response to a specialist and a generalist herbivore. *Plant Cell* 16:3132–3147.

RODA, A., HALITSCHKE, R., STEPPUHN, A., and BALDWIN, I. T. 2004. Individual variability in herbivore-specific elicitors from the plant's perspective. *Mol. Ecol*. 13:2421–2433.

SCHITTKO, U., HERMSMEIER, D., and BALDWIN, I. T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. II Accumulation of plant mRNAs in response to insect-derived cues. *Plant Physiol*. 125:701–710.

SCHMELZ, E. A., CARROLL, M. J., LECLERE, S., PHIPPS, S. M., MEREDITH, J., CHOUREY, P. S., ALBORN, H. T., and TEAL, P. E. A. 2006. Fragments of ATP synthase mediate plant perception of insect attack. *Proc. Natl. Acad. Sci. U.S.A.* 103:8894–8899.

SCHULER, M. A., and WERCK-REICHHART, D. 2003. Functional genomics of P450s. *Annu. Rev. Plant Biol.* 54:629–667.

SCHÜTZ, S., WEISSBECKER, B., KLEIN, A., and HUMMEL, H. E. 1997. Host plant selection of the Colorado potato beetle as influenced by damage induced volatiles of the potato plant. *Naturwissenschaften* 84:212–217.

SHINYA, T., HANAI, K., GÁLIS, I., SUVUKI, K., MATSUOKA, K., MATSUOKA, H., and SAITO, M. 2007. Characterization of NtChitIV, a class IV chitinase induced by ß-1,3-,1,6-glucan elicitor from Alternaria alternata 102: antagonistic effect of salicylic acid and methyl jasmonate on the induction of NtChitIV. *Biochem. Biophys. Res. Commun*. 353:311–317.

SMYTH, G. K. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. *Stat. Appl. Genet. Mol. Biol.* 3:1–26.

SMYTH, G. K. 2005. Limma: linear models for microarray data, pp. 397–420, in R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber (eds.). Bioinformatics and Computational Biology Solutions using R and BioconductorSpringer, New York.

SMYTH, G. K., and SPEED, T. 2003. Normalization of cDNA microarray data. *Methods* 31:265–273.

SMYTH, G. K., MICHAUD, J., and SCOTT, H. S. 2005. Use of within-array replicate spots for assessing differential expression in microarray experiments. *Bioinformatics* 21:2067–2075.

STAMP, N. 2003. Out of the quagmire of plant defense hypotheses. *Q. Rev. Biol.* 78:23–55.

TIEMAN, D., TAYLOR, M., SCHAUER, N., FERNIE, A. R., HANSON, A. D., and KLEE, H. J. 2006. Tomato aromatic amino acid decarboxylases participate in synthesis of the flavor volatiles 2-phenylethanol and 2-phenylacetaldehyde. *Proc. Nat. Acad. Sci. U.S.A.* 103:8287–8292.

TODD, C. D., and POLACCO, J. C. 2006. AtAAH encodes a protein with allantoate amidohydrolase activity from Arabidopsis thaliana. *Planta* 223:1108–1113.

WEISSBECKER, B., VAN, LOON, J. J. A., and DICKE, M. 1999. Electroantennogram responses of a predator *Perillus bioculatus* and its prey *Leptinotarsa decemlineata*, to plant volatiles. *J. Chem. Ecol.* 25:2313–2325.

YANG, J., and HAN, K.-H. 2004. Functional characterization of Allantoinase genes from *Arabidopsis* and a nonureide-type legume black locust. *Plant Physiol.* 134:1039–1049.

YANG, Y. H., and PAQUET, A. C. 2005. Preprocessing two-color spotted arrays, pp. 49–69, in R. Gentleman, V. Carey, S. Dudoit, R. Irizarry, and W. Huber (eds.). Bioinformatics and Computational Biology Solutions using R and BioconductorSpringer, New York.

ZIMMERMANN, G., BÄUMLEIN, H., MOCK, H. -P., HIMMELBACH, A., and SCHWEIZER, P. 2006. The multigene family encoding germin-like proteins of barley. Regulation and function in basal host resistance. *Plant Physiol.* 142:181–192.

Glucosinolates are generally considered to be feeding and oviposition stimulants for specialist insect herbivores and deterrents for generalist insects (Mithen et al. 1995b; Stowe 1998). There is evidence from laboratory and field experiments that differences in glucosinolate quantity and quality can mediate plant–insect interactions by influencing insect feeding behavior and damage levels (Mauricio 1998; Li et al. 2000; Lambrix et al. 2001; Barth and Jander 2006; Zhang et al. 2006). However, there is no consistency regarding previously reported insect herbivore responses to glucosinolate variation. Herbivory by diverse specialized insects has been either positively, negatively, or not associated with glucosinolate concentrations (or with the presence/absence of certain types of glucosinolates; Nielsen et al. 2001). For example, in a previous experiment, *B. napus* lines with augmented glucosinolate concentrations attracted more cabbage butterflies (*Pieris rapae*) and had increased damage by flea beetles (*Psylliodes chrysocephala*) compared to low glucosinolate lines (Giamoustaris and Mithen 1995). Conversely, Mauricio (1998) found decreased damage by two specialist flea beetles (*P. convexior* and *Phyllotreta zimmermani*) when feeding on *A. thaliana* genetic lines with high levels of total glucosinolates. In addition, although glucosinolates or their by-products repel birds, rabbits, gastropod mollusks, and generalist insects (Mithen et al. 1995b), Lambrix et al. (2001) found that certain glucosinolate hydrolysis products, such as nitriles, are less defensive than isothiocyanates against feeding by the generalist cabbage looper *Trichoplusia ni*.

Contrasting selection pressures exerted by different herbivores of natural plant populations may explain the variation in glucosinolate profiles observed in the Brassicaceae (Rodman 1980; Rodman et al. 1981; Kliebenstein et al. 2001; Windsor et al. 2005; Lankau 2007). Within *A. thaliana*, there are at least 16 different glucosinolate chemotypes. Five of these are prevalent in wild *Arabidopsis*, and they are focused on here, as in previous studies (Lambrix et al. 2001; Kliebenstein et al. 2001). Glucosinolate variation among natural populations of *A. thaliana* has been explained by the combination of functional and nonfunctional (null) alleles at five specific loci. These include: *GS-ELONG*, which regulates the carbon side-chain elongation of aliphatic glucosinolates; *GS-ALK*, which controls the production of alkenyl glucosinolates; *GS-OHP*, which regulates the production of hydroxypropyl glucosinolate; *GS-OH*, responsible for the conversion of 3-butenyl to 2-hydroxy-3-butenyl glucosinolate; and *GS-OX*, which regulates the conversion of methythioalkyl to methylsulfinylalkyl glucosinolates (Mithen et al. 1995a; Kliebenstein et al. 2001). A flexible "modular genetic system" that involves polymorphisms at these five loci has been proposed as responsible for generating the substantial natural variation in glucosinolates found in *A. thaliana*, which may allow this plant to adjust rapidly (or respond by evolution) to a myriad of biotic and physical environmental challenges (Kliebenstein et al. 2001).

Although several studies have evaluated herbivore selectivity among different plants, little is known regarding the relationship between intraspecific variations in glucosinolates and herbivory in the field (Raybould and Moyes 2001). In a common garden experiment, we specifically assessed whether there is natural variation in glucosinolate quality and quantity in 20 *A. thaliana* genotypes and whether this variation is associated with patterns of distribution of insect herbivores among plants, and, thus, insect damage levels and plant fitness. Evaluation of the link between variation in secondary chemistry and both herbivore damage and plant fitness is important because differential levels of herbivory can impose selection for plant resistance or tolerance, which in turn may affect population structure and dynamics. Changes in frequency of chemical types or concentrations that result from herbivory-imposed selection may be dependent on the specific insect community associated with the natural plant populations. For example, while insect generalists may inflict selection for increased production of specialized defensive chemicals (Muller and Martens 2005), a specialist-dominated insect community like the one associated with the experimental plants in this study may select for low levels of specialized chemical defenses.

Materials and Methods

Study Species The annual plant *A. thaliana* (Capparales: Brassicaceae) originated in Eastern Europe and is widely distributed throughout the temperate regions of North America, with records of its introduction in the mid-1800s (Murren et al. 2005; Vander-Zwan et al. 2000). Little is known regarding the genetic structure of *A. thaliana* populations in nature (Vander-Zwan et al. 2000). However, due to a selfing mode of reproduction and short-distance seed dispersal, natural populations appear to consist mostly of single genotypes (Bergelson et al. 1998). For this study, we selected 20 *A. thaliana* natural accessions (provided by the Arabidopsis Biological Resource Center, Ohio State University) from Europe, Asia, and northwest (NW) of Africa (Table 1). None of these accessions has either glabrous or hairy trichome phenotypes (unpublished data). Seed stocks were propagated as a single seed descent from each population. Thus, populations/accessions are composed essentially of a single genotype (hereafter, referred as "plant genotypes"). Selection of these genotypes from a wide array of geographical locations allowed sampling a broad range of the natural variation in glucosinolates present in *A. thaliana* populations.

Table 1 *A. thaliana* genotypes selected for the common garden experiment and their places of origin

Genotype	ABRC ID	Place of origin
Sav-0	6856	Slavice, Czechoslovakia
Kel-1	6100	Kelsterbach, Germany
Jl-3	6745	Vranov U Brna, Czechoslovakia
Phw-3	6045	Koeln, Germany
Kl-5	6761	Koeln, Germany
Phw-17	6066	Kent, UK
Wt-5	1613	Wietze, Germany
Cvi-0	6675	Cape Verdi Islands
Jm-1	6749	Jamolice, Czechoslovakia
Zu-1	6903	Zurich, Switzerland
Edi-0	6688	Edinburgh, UK
Ms-0	6797	Moscow, Russia
Hh-0	6735	Hohenlieth, Germany
Ge-0	6717	Geneve, Switzerland
Es-0	6699	Espoo, Finland
Bu-21	6652	Burghaun, Germany
Zu-0	6902	Zurich, Switzerland
Can-0	6660	Canary Islands, Spain
Et-0	6702	Etraygues, France
Phw-10	6055	Kent, UK

ABRC ID—indicate accession numbers from the Arabidopsis Biological Resource Center

Experimental Design and Plant Growth Conditions A common garden experiment was performed in NW Ohio (Bowling Green State University Research Station, Bowling Green, OH, USA) to evaluate the potential link between plant secondary chemistry (i.e., quality and quantity of glucosinolates) and both natural levels of insect herbivory and plant fitness. The experimental design consisted of a nested randomized block design including three blocks (plots) and 20 genotypes nested within plots. In this location, insect herbivores generally become more abundant by mid-June (Bidart-Bouzat, personal observation), approximately 1 month after seedlings were transplanted to the field.

Twenty-four individuals from each of the 20 selected genotypes were randomly planted among three plots in the field accounting for a total of 480 plants (from which 338 survived to reproduction). Individual plants of each genotype were grown from seeds in standard flats (24×45 cm^2×7 cm deep), which underwent a cold treatment (at 4°C and under dark conditions) in a growth chamber for 1 week to eliminate dormancy and ensure uniform germination. Afterwards, seeds were allowed to germinate with progressively increasing temperatures and light periods up to 20°C and periods of 14/10 h (L:D), respectively. Germination date was recorded, and approximately 1-week-old plants were transplanted to the field. To minimize microenvironmental variation and interplant competition, each individual plant was planted directly in the field in a 750 cm^3 hole filled with a mixture of 1:3 sand/commercial potting mix (composed of compost, field soil, and sphagnum moss) 20 cm apart. Plants dying from transplant shock were replaced during the first week after transplantation. Plots were covered with mulch for weed control and manually weeded and watered as necessary. Since the purpose of this study was to evaluate natural insect herbivory, each experimental plot (18 m^2) was surrounded by wire mesh to prevent potential mammalian herbivory (Weinig et al. 2003).

Measurement of Phenotypic Traits Individual plants were examined daily to record the presence/absence of associated herbivores and to sample a few of these for identification at the species level (lepidopteran larvae, flea beetles, aphids, and thrips). Insect sampling was performed during daytime. Lepidopteran larvae collected from experimental plants were grown on *A. thaliana* plants in the laboratory for species identification. During the first 25 days, almost no insect activity was observed. Insects became more abundant as weather conditions tended to be warmer and dryer (mid-June). Damage by herbivory was evaluated on the rosette at bolting time, and on buds and flowers (aerial damage) approximately 10 days after the plants first flowered (on all 338 plants that survived to reproduction). Rosette damage was measured through a qualitative index of plant damage on rosette leaves (adapted from McCloud and Berenbaum 1999). This index was calculated by assigning leaves to four size categories (1=6–8 cm; 2=4–6 cm; 3=2–4 cm; 4=0–2 cm), and three damage classes dependent upon the amount of tissue removed (25% or less, 25–75%, or more than 75%). Aerial damage was determined by the ratio of the number of damaged to total buds and flowers. The average of both rosette and aerial damage was considered as an index of total herbivore damage on each plant. Three-to-four apparently undamaged plants (rosettes) per genotype were collected for glucosinolate analyses at early bolting (chemical analysis protocol explained below). Total number of fruits and seed number per fruit (estimated from 20 randomly chosen fruits per plant) were measured at harvesting. An index of fitness was calculated by multiplying total fruit number by seed number per fruit for each plant.

Glucosinolate Analysis A modification of Hogge et al. (1988) extraction and purification protocol was used to quantify glucosinolate concentrations in *A. thaliana* rosette leaves. Rosette leaves collected from the field were flash frozen immediately in liquid N and later freeze dried. For glucosinolate extraction, 50 mg freeze-dried leaf tissue of each plant were ground with 2 ml 90% methanol and three 2.3-mm ball bearings. Samples were mixed at high speed with a paint shaker. Samples were subsequently centrifuged at full speed for 15 min; 1 ml of the sample supernatant was

added to 250 μl of DEAE Sephadex solution and further incubated for 60 min at room temperature. Glucosinolate-containing pellets were washed twice (first with 1 ml of methanol and then with 1 ml of water) and incubated overnight at room temperature after adding 500 μl of water and 10 μl of sulfatase solution (Sigma-Aldrich). To identify and quantify glucosinolates, 150 μl of each sample were run on an Agilent 1100 high-performance liquid chromatography (HPLC) with diodearray detection as previously described (Kliebenstein et al. 2001). Identity and area of HPLC peaks were determined by comparing them with those of purified standards (Reichelt et al. 2002).

Glucosinolate content is presented as μmol g^1 of freeze-dried rosette leaves. The following glucosinolates were identified across the plant genotypes: alkenyl (2-propenyl and 3-butenyl), hydroxyalkyl/alkenyl (3-hydroxypropyl, 4-hydroxybutyl, 2-hydroxy-3-butenyl), methylsulfinyl (3-methylsulfinylpropyl, 4-methylsulfinylbutyl, 7-methylsulfinylheptyl, 8-methylsulfinyloctyl), methylthio (3-methylthiopropyl, 4-methylthiobutyl, 7-methylthioheptyl, 8-methylthiooctyl), and indolyl (indol-3-ylmethyl, 4-methoxyindol-3-ylmethyl, *N*-methoxyindol-3-ylmethyl, 4-hydroxyindol-3-ylmethyl) glucosinolates (Reichelt et al. 2002). Glucosinolate profiles in the studied genotypes were determined on the basis of allelic variation at two loci *GS-ELONG* and *GS-AOP*. The *GS-ELONG* locus controls the production of glucosinolates containing side chains with three or four carbons (3C and 4C, respectively). Three allelic variants at the *GS-AOP* locus: *GS-NULL*, *GS-ALK*, and *GS-OHP* control the production of methylsulfinylalkyl, alkenyl, and hydroxyalkyl glucosinolates, respectively (Kliebenstein et al. 2001).

Statistical Analyses All statistical analyses were performed with SAS software (SAS Institute 1999, Version 8e), and all phenotypic variables were rank transformed. Rank transformation stabilizes the variance of the data, thus, correcting or minimizing heteroscedasticy (Potvin and Roff 1993). This approach is particularly useful for analyzing glucosinolates, since commonly used transformations for parametric procedures often do not correct for heterogeneity of residual variances. To evaluate the effect of glucosinolate profile (fixed effect) and plant genotype nested in profile (random effect) on glucosinolate contents, a mixed model analysis of variance (Proc. Mixed), was performed for each of the detected individual glucosinolates as well as for total aliphatic and indolyl glucosinolates, and total glucosinolate contents. The same approach was used to evaluate the effect of profile and genotype on herbivore damage levels and plant fitness. Mean damage levels and fitness among profiles were compared by using protected least-significant difference (LSD) tests. In addition, an analysis of covariance was carried out to infer whether differential damage levels affected fitness and to evaluate genotypic variation in damage by using the following model: Fitness=damage+genotype+damage×genotype. The effect of blocks (field plots) was removed from the final model because it was only marginally significant (P=0.053). Regression analyses were performed to evaluate the association between individual glucosinolates and damage levels as well as between total glucosinolate concentrations and both damage and fitness. Finally, a cluster analysis that used the unweighted pair group method with arithmetic means (UPGMA) was carried out. This grouped *A. thaliana* genotypes on the basis of the distribution of insect herbivore species. This grouping was statistically corroborated by using the nonparametric analysis of variance (ANOVA)-type statistic (ATS), which is based on an approximated F distribution (Brunner et al. 2002). Analyses of variance were additionally performed to compare glucosinolate concentrations, damage levels, and fitness among the groups of genotypes identified by the cluster analysis.

Results

Natural Variation in Glucosinolates Selected genotypes from diverse geographical areas were highly variable in both glucosinolate composition and quantity (Fig. 1; Tables 1 and 2). We measured all glucosinolates, both aliphatic and indolic, prior to (apparent) herbivory. However, potential glucosinolate induction (resulting from herbivory and/or mechanical damage) was not measured, and, thus may account for part of the genotypic variation in these chemicals. We identified variation at three of the known naturally variable glucosinolate loci, *GS-ELONG*, *GS-AOP*, and *GS-OH*. We focused on five glucosinolate profiles generated by allelic variation at the two loci, *GS-ELONG* (3C and 4C) and *GS-AOP* (*NULL*, *ALK* and *OHP*; Fig. 1). Hereafter, we refer to these profiles with the following abbreviations: 3C OHP, 3C ALK, 4C OHP, 4C ALK, and 4C NULL. Genotypes with 3C OHP and 3C ALK profiles accumulated primarily 3-hydroxypropyl (93% of total aliphatic glucosinolates, TAG) and 2-propenyl (79% of TAG), respectively. The 4C NULL and the 4C OHP profiles produced predominantly 4-methylsulfinylbutyl glucosinolates (about 60% of TAG). In addition, 2-butenyl and 2-hydroxy-3-butenyl glucosinolates made up more than 70% of the TAG in genotypes with the 4C ALK profile. Although the effect of profile was significant for most individual aliphatic glucosinolates, both absolute and relative concentrations of indolyl as well as total glucosinolates did not differ significantly among profiles (see Table 2). Conversely, pronounced variation among genotypes was detected for all aliphatic, indolyl, and total glucosinolate concentrations, in terms of

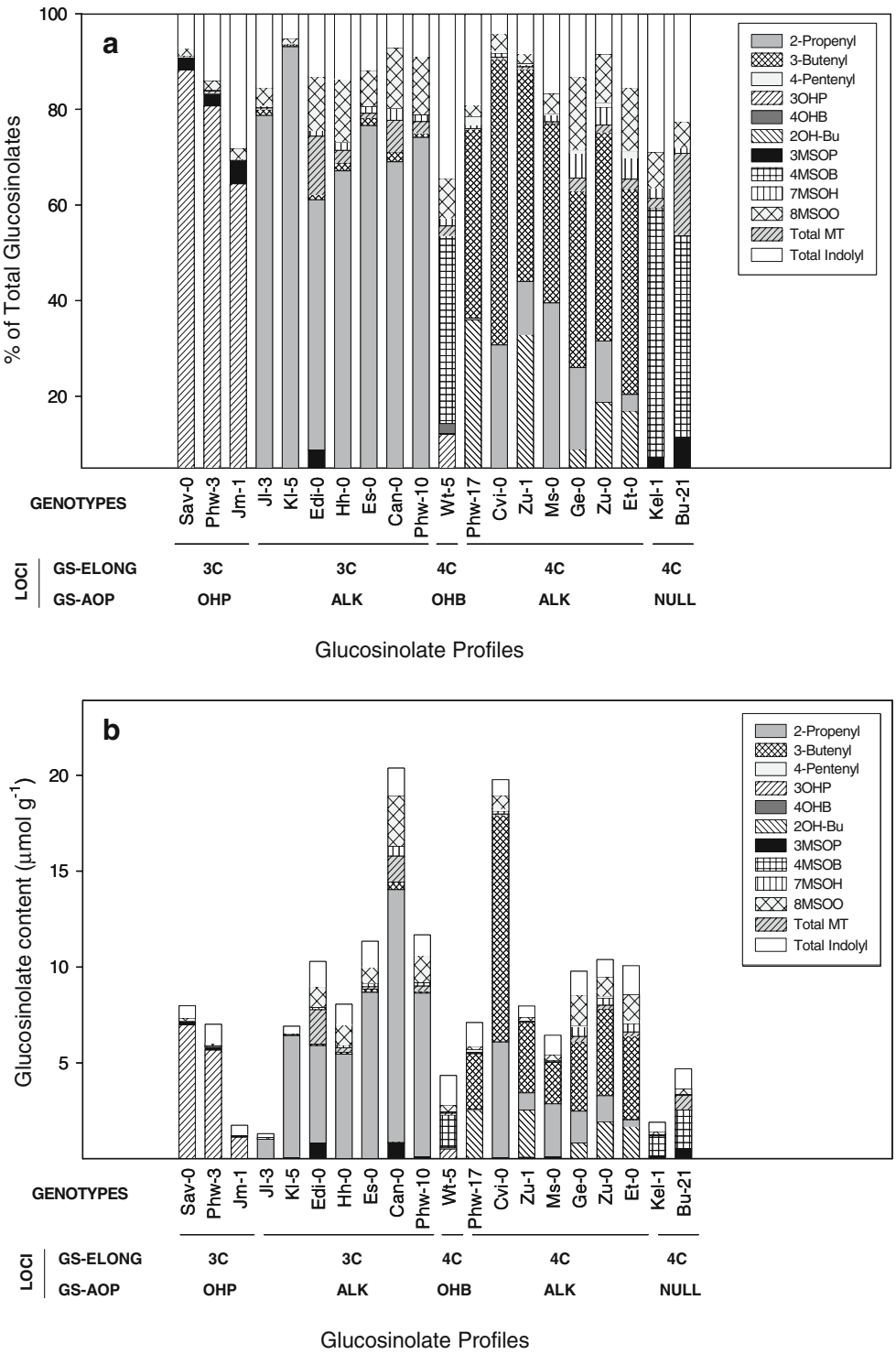

Fig. 1 Natural variation in glucosinolate profiles in *A. thaliana* determined on the basis of allelic variation at two loci, *GS-ELONG* (*3C* and *4C*) and *GS-AOP* (*NULL*, *ALK*, and *OHP*) and genotypic variation in the **a** relative and **b** absolute concentration of glucosinolates (µmol g^{-1} dry weight). Refer to Table 2 for glucosinolate abbreviations

both absolute and relative amounts (Table 2; Figs. 1 and 2). Although we tested indolyl glucosinolate type and content, we focused on the aliphatics, which showed stronger variation and because they are predominant in *A. thaliana* (in terms of concentrations). However, indolyl glucosinolates also have been shown to have a role in defense against insects (Kim and Jander 2007).

Glucosinolates, Insect Damage and Plant Fitness Herbivore damage levels as well as plant fitness were both apparently controlled by genetic variation among the *Arabidopsis* accessions (ANOVA $P<0.001$). As expected, higher levels of insect damage were associated with decreased fitness (Fig. 3). An analysis of covariance corroborated this pronounced relationship between fitness

Table 2 Variation in individual and total glucosinolate contents among chemical profiles and genotypes in *A. thaliana*

Source	Profile	Genotype (profile)	Profile	Genotype (profile)
Glucosinolates (N=69)	Absolute contents (μmol/g)		Relative contents (%)	
	$F_{4,15}$	$F_{15,49}$	$F_{4,15}$	$F_{15,49}$
2-Propenyl	13.33***	13.26***	31.92***	13.03***
3-Butenyl	45.55***	5.70***	53.86***	10.85***
4-Pentenyl	17.02***	6.18***	23.08***	7.60***
3OHP	10.64***	7.29***	9.96***	7.52***
4OHB	33.30***	–[a]	14352***	–[a]
2OH-Bu	5.55**	151.70***	5.76**	37.23***
3MSOP	5.30**	14.17***	10.48***	24.43***
4MSOB	4.46*	12.83***	4.28*	21.55***
7MSOH	2.51	26.48***	2.62	24.37***
8MSOO	1.89	16.04***	1.76	14.99***
Total MT	1.01	64.56***	1.97	36.00***
Total aliphatic	2.34	8.98***	–	–
I3M	0.89	3.94***	3.03*	6.59***
4MOI3M	1.47	6.11***	0.97	4.29***
NMOI3M	1.46	4.61***	0.43	5.02***
4OHI3M	0.75	6.27***	2.59	3.51***
Total indolyl	1.04	3.60***	–	–
Total glucosinolates	2.10	8.18***	–	–

3OHP 3-hydroxypropyl, *4OHB* 4-hydroxybutyl, *2OH-Bu* 2-hydroxy-3-butenyl, *3MSOP* 3-methylsulfinylpropyl, *4MSOB* 4-methylsulfinylbutyl, *7MSOH* 7-methylsulfinylheptyl, *8-MSOO* methylsulfinyloctyl, *Total MT* methylthio, *I3M* indol-3-ylmethyl, *4MOI3M* 4-methoxyindol-3-ylmethyl; *NMOI3M N*-methoxyindol-3-ylmethyl, *4OHI3M* 4-hydroxyindol-3-ylmethyl

*P<0.05; **P<0.01; ***P<0.001

[a] Only one genotype with this glucosinolate type

and damage (covariate) as well as a significant interaction between damage and genotype, which is indicative of genetic variation in the fitness response to insect herbivory (i.e., genetic variation for tolerance of herbivory damage; Fig. 3). The observed genotypic variation in plant damage and fitness was related to differences in both glucosinolate quantity and quality (Figs. 4 and 5). Glucosinolate levels were associated directly with damage (y=4.15+0.58x; Slope=0.58; P=0.003) and inversely related to fitness (y= 17.68–0.77x; Slope=−0.77; P<0.001; Fig. 4). The results are based on total glucosinolate concentrations since regression relationships between damage and either indi-

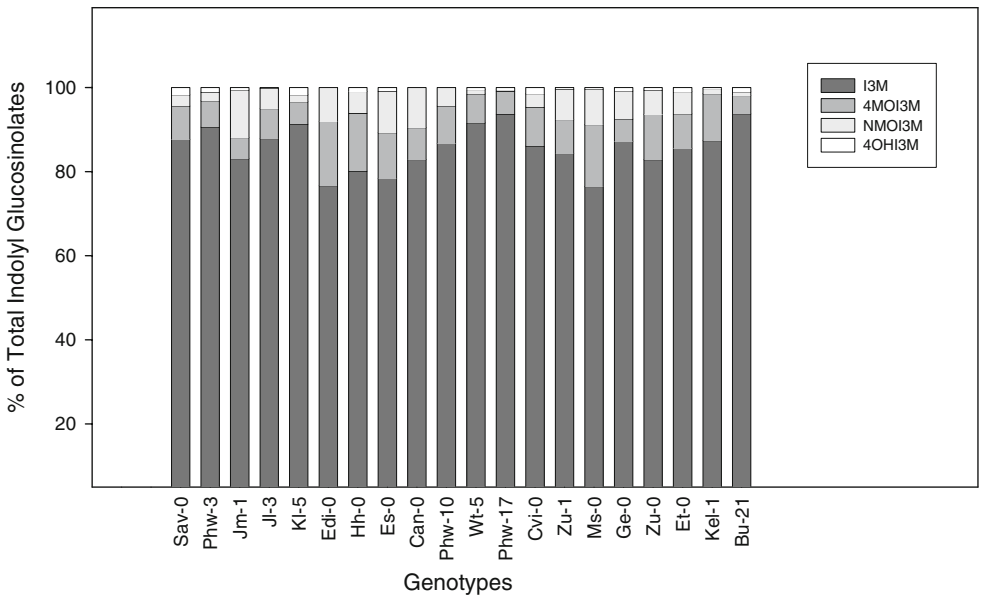

Fig. 2 Relative concentrations of individual indolyl glucosinolate in *A. thaliana* genotypes. Refer to Table 2 for glucosinolate abbreviations

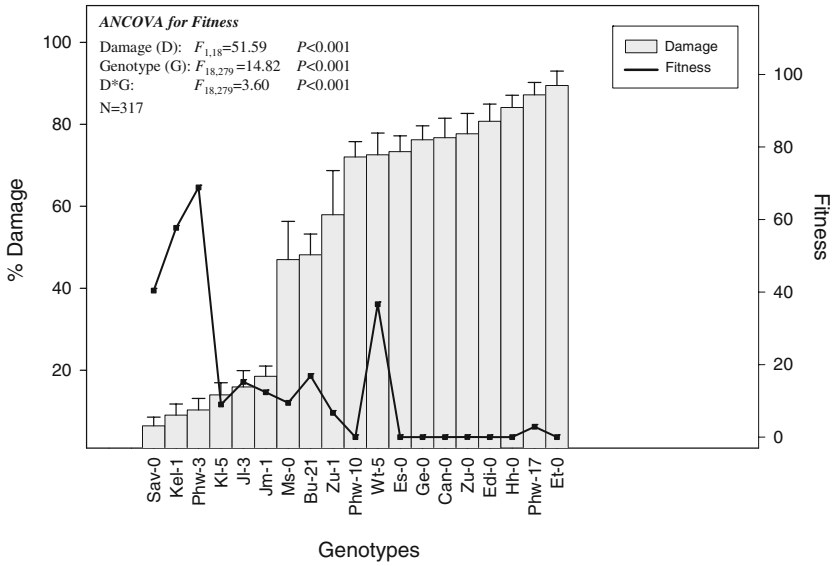

Fig. 3 Analysis of covariance estimating the effects of plant damage by natural levels of insect herbivory, genotype, and genotypic variation in damage on plant fitness (total number of seeds per plant $\times 10^2$)

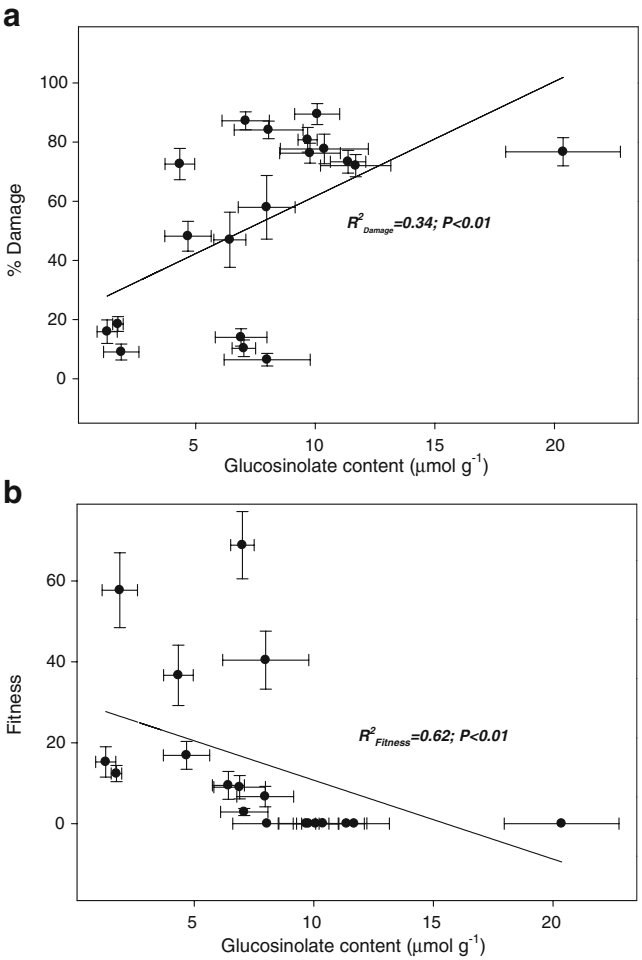

Fig. 4 Relationships between total glucosinolate concentrations (μmol g^{-1} dry weight) in *A. thaliana* and **a** damage and **b** fitness (total number of seeds per plant $\times 10^2$). Each *circle* denotes a genotypic mean

vidual aliphatic or indolyl concentrations were generally significant and consistent in the direction of their response; i.e., most glucosinolates revealed positive regression coefficients ranging from 2.20 to 8.57 and *P*-values ranging from <0.001 to 0.03. The exceptions were 3-hydroxypropyl and 3-methylsulfinylpropyl glucosinolates, which were inversely related to damage levels (both *P*-values <0.001). These associations between glucosinolates and both damage and fitness should be interpreted with caution because induction by subsequent herbivory and/or ontogenetic changes in glucosinolate levels might alter these relationships. The effect of glucosinolate profile was significant for mean damage levels ($P<0.001$, see Fig. 5) and plant fitness

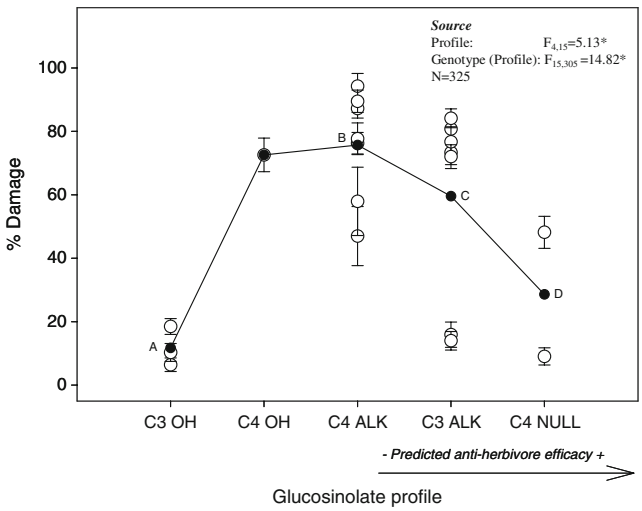

Fig. 5 Glucosinolate profiles in *A. thaliana* and damage by natural levels of insect herbivory. Chemical profiles in the *x* axis are ordered with increasing antiherbivore efficacy for the generalist cabbage looper (as previously described in Kliebenstein 2004). Means with *the same letter* are not significantly different at $P<0.05$ (based on least significant difference tests). *$P<0.01$

(P=0.002). Overall, genotypes that contained the *GS-ALK* allelic variant (regardless of the allelic status at *GS-ELONG*) had more damage and lower fitness than those that contained other alleles at the *GS-AOP* locus (Fitness: 3C/OHP=4C/OHP=4C/NULL >>> 3C/ALK=4C/ALK; significance based on protected LSD tests). However, variable outcomes were found for different plant genotypes (within profiles) in terms of both damage (P=0.008) and fitness (P<0.001). In other words, some genotypes differed in the amount of damage (and/or fitness) regardless of their specific chemotype. In addition, we compared the detected variation in natural insect herbivory with known preferences of the generalist cabbage looper (*T. ni*) for the same glucosinolate profiles (Lambrix et al. 2001; Kliebenstein 2004; Fig. 5). Results were in accordance with the previously ranked order preferences of the cabbage looper, in terms of increased feeding in genotypes with metabolites produced by 4C/ALK and 3C/ALK compared to 4C/NULL profiles. Nonetheless, results summarized in Fig. 5 (as well as the effect of profile on fitness) should be interpreted with caution because of the significant effect of genotype and because profile groups contained an unequal (and sometimes minimal) number of genotypes.

Glucosinolates: Patterns of Plant Utilization by Insect Herbivores in the Field Because there is potential for specialist and generalist insects to have different glucosinolate mediated interactions, we recorded the identity of insect herbivores that interacted with plants in the field. Several species were detected feeding on the plants, including diamondback moths (*Plutella xylostella*; Plutellidae, Lepidoptera), striped and western cabbage flea beetles (*P. striolata* and *P. pusilla*, respectively; Chrysomelidae, Coleoptera), cabbage aphids (*Brevicoryne brassicae*; Aphididae, Hemiptera), and onion thrips (*Thrips tabaci*; Thripidae, Thysanoptera). Although most of the insect damage was concentrated on the rosette leaves, which were attacked primarily by diamondback moths and flea beetles, aphids and thrips affected mostly plant aerial parts such as buds and flowers. Consequently, constitutive levels of glucosinolates (as well as quality), which were measured only on rosette leaves are likely more important for explaining selective feeding of diamondback moths and flea beetles rather than that of aphids and thrips. However, the overall glucosinolate chemotype within *Arabidopsis* has not been found to alter with tissue or herbivory (Kliebenstein et al. 2001). Four of these five herbivores are specialists on

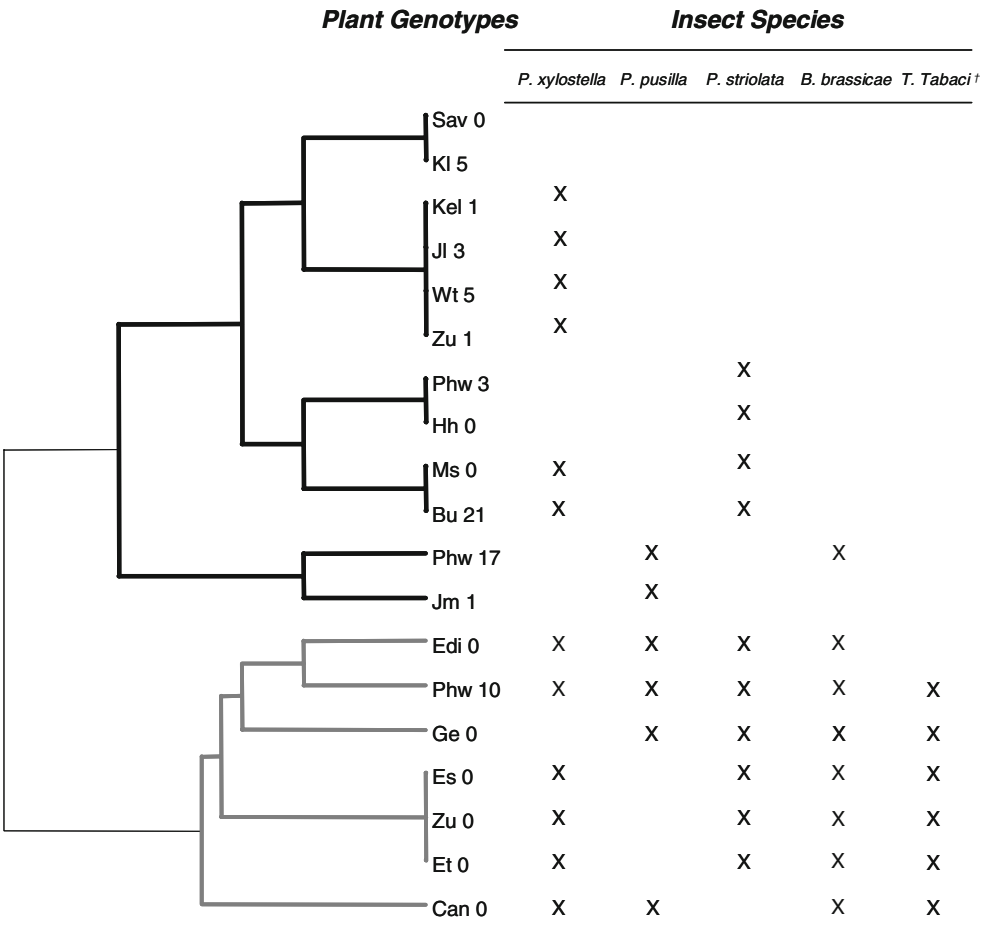

Fig. 6 UPGMA cluster analysis showing the association among *A. thaliana* genotypes with similar distribution of insect species feeding on the plants. The two identified clusters are indicated by different line colors (*black and gray*). *dagger* Only generalist insect

glucosinolate containing plants, while only *T. tabaci* is a generalist. As such, our herbivore community that attacked *Arabidopsis* in the field was composed predominantly of specialists. Only one genotype (Cvi-0) was heavily attacked by a downy mildew (Peranosporales), and all individuals in this genotype eventually died from fungal attack. The distribution of insect species among the studied plant genotypes was evaluated graphically by cluster analysis (Fig. 6). The Cvi-0 genotype was excluded from this analysis since it succumbed to a fungal attack rather than to insect herbivory. The cluster analysis revealed two groups of plant genotypes: one clustered genotypes with 1–2 associated insect species and the second clustered genotypes with 4–5 insect herbivores (Fig. 6). This grouping was corroborated by the nonparametric ATS test (F_{ATS}=29.37, P<0.001, N=19). It is interesting that these two groups of genotypes had nonoverlapping lower (1.3–8.05 μmol/g) and higher (9.68–20.36 μmol/g) total glucosinolate concentrations (significant differences in mean glucosinolate concentrations, P<0.001). These clusters also differed in their mean damage levels (P=0.003) and fitness responses. Specifically, the group of genotypes with lower glucosinolate levels was associated with lower numbers of insect species feeding on the plants and, consequently, lower mean damage levels (39% of the plant damaged) than genotypes with higher glucosinolates (mean damage index, 78%). In addition, only genotypes with lower glucosinolate concentrations contributed to fitness; all genotypes in the other group succumbed to insect herbivory. The outcome summarized in this section combined with results on the overall association between glucosinolate levels and both damage and fitness in this study suggest that glucosinolates might have been an important factor that influenced plant utilization patterns by insect herbivores in this experimental garden.

Discussion

Few studies have evaluated the potential link between intraspecific variation in secondary chemistry and patterns of plant use by insect herbivores in the field (Cronin and Abrahamson 2001). In an experimental garden, we demonstrated genotypic variation in the type and concentration of glucosinolates in *A. thaliana*, as well as significant relationships between glucosinolates and both herbivore damage and plant fitness. Even though correlational associations do no imply causation, this study suggests that genotypic variation in glucosinolates may be a factor that explains patterns of plant use by insect herbivores in the field (as estimated by plant damage and fitness). Specifically, genotypes with higher glucosinolate levels were associated with augmented damage levels and decreased fitness.

These results are contrary to the general expectation that enhanced chemical defense should result in decreased insect herbivory. Previous studies have shown that glucosinolate–insect relationships may be strongly influenced by the physical and biological context in which they develop. For example, Bidart-Bouzat et al. (2005) found that the performance of the diamondback moth, *P. xylostella*, was associated with glucosinolate type and concentration. However, this relationship was correlated with CO_2 levels and insect gender; that is, only performance of females (but not males) reared on elevated CO_2 grown plants was inversely related to 3-butenyl glucosinolate levels and positively associated to 2-propenyl, indolylmethyl, and total glucosinolates. Likewise, the interaction between secondary chemicals and insect herbivores also may be contingent on the type of insect herbivore community associated with the plants (Strauss and Irwin 2004). For example, Lankau (2007) provided evidence that high concentrations of the 2-propenyl glucosinolate resulted in increased damage by the specialist cabbage aphid but deterred attack by a generalist guild.

A specialist-dominated insect community, like the one associated with our experimental plants, may exert selection for decreased glucosinolate concentrations. As previously mentioned, *A. thaliana* genotypes with lower glucosinolate concentrations were associated with decreased damaged and, as a consequence, had higher fitness than those with higher amounts of these chemicals. Both herbivore damage and glucosinolate levels in this study were highly variable among plant genotypes as well as significantly correlated with fitness. This variation is a necessary condition for insect herbivory to impose selection on plant secondary chemistry (Marquis 1992). Thus, insect herbivory emerges as a potential selective factor that influences the type and concentration of glucosinolates in our common garden experiment. In contrast to our findings, a previous field study documented a reduction in damage levels by a suite of insect herbivores in plant genotypes with higher glucosinolate concentrations as well as directional selection favoring increased glucosinolate levels (Mauricio 1998; Mauricio and Rausher 1997). These authors reported the presence of specialist flea beetles as well as unidentified insect herbivores (lepidopteran larvae, leaf miners, beetles, and aphids), but it is not possible to ascertain if their herbivore community had a similar predominance of specialist herbivores. However, if their herbivore community had a predominance of generalists, this could explain the difference in genetic correlation between damage and glucosinolates in the two experiments. Since glucosinolates can stimulate feeding by specialist insects but deter generalist insect attacks (Bones and Rossiter 1996), the type and direction of selection that acts on these chemicals would depend on which herbivore type is more prevalent

and/or more detrimental to the plants and whether insect herbivory is maximal at lower, intermediate, or higher glucosinolate concentrations (Siemens and Mitchell-Olds 1996; Lankau 2007).

In addition to the total amount of a chemical class, individual constituents can have contrasting effects on insect herbivore behavior or performance. For example, while 2-propenyl glucosinolate is an effective feeding stimulant for the cabbage aphid, 4-pentenyl inhibits reproduction of this specialist, at least under laboratory conditions (Raybould and Moyes 2001). In our study, most individual glucosinolates (both aliphatic and indolyl) as well as their total amounts (total aliphatic, total indolyl, and the sum of these two) were positively associated with damage in the experimental population. The only two exceptions consisted of 3-hydroxypropyl and 3-methylsulfinylpropyl glucosinolates, which were negatively related to damage. It is interesting that 3C/OHP, the chemical profile of genotypes that accumulates 3-hydroxypropyl glucosinolates (see Fig. 5), has been considered one of the least effective profiles in deterring herbivory by the generalist *T. ni* in previous laboratory experiments (Lambrix et al. 2001; Kliebenstein et al. 2002; Kliebenstein 2004). Conversely, we found that the three genotypes with this chemical profile were minimally visited by insect herbivores and minimally damaged (Figs. 4 and 5). Thus, one could hypothesize that low herbivory levels in these genotypes might have resulted from the presence of the 3-hydroxypropyl glucosinolate, which may have not been an effective feeding or oviposition stimulant for the specialist insects attracted to the experimental plants.

In contrast to the 3C/OHP plants, genotypes that contain the *GS-ALK* allelic variant (regardless of the allelic status of *GS-ELONG*) had higher mean damage levels and lower fitness. Alkenyl glucosinolates, such as 2-propenyl and 3-butenyl glucosinolates, produced by the 3C/ALK and 4C/ALK profiles, respectively, are known for their ability to stimulate feeding and oviposition by numerous specialist insects such as cabbage aphids, cabbage seed weevils, brassica pod midges, cabbage root flies, turnip aphids, flea beetles, white cabbage butterflies, and diamondback moths (reviewed in Raybould and Moyes 2001). Hence, it is not surprising that the specialist insects attracted to our experimental garden preferentially attacked plant genotypes that contained higher amounts of these glucosinolate types.

Although secondary chemistry plays a major role in the evolution of plant–insect herbivore interactions (Zangerl and Berenbaum 1993), other physical properties or life history traits also influence herbivore distribution among plants (Jaenike 1990). For example, characteristics such as increased size or abundance (in addition to chemical properties) that make plants more "visible" to insects can affect host plant choice (Feeny 1976). In our study, plant size (i.e., aboveground vegetative biomass using damage as a covariate) was not associated with damage levels (data not shown). In addition, life history characteristics such as early flowering phenology and rapid growth may allow plants to escape damage in time, e.g., by flowering before insect herbivores become abundant later in their reproductive season (Pilson and Decker 2002). Genotypes of *A. thaliana* used in this experiment included both accessions considered to behave as early and late flowering as well as those with variable flowering phenology; however, all accessions flowered in the field within a relatively small 13-day interval. In addition, we found considerable overlap in the flowering time ranges of the two genotype groups identified by the cluster analysis. Thus, we believe flowering time was likely not a critical factor in this study.

Since our outdoor garden represented a novel environment for the plant genotypes used, the study also may have implications for the potential role of the genotype factor in the colonization success of nonnative habitats by invasive plant species. It is interesting that only half of the genotypes contributed to fitness and only four had considerably high fitness levels. Higher fitness in three of these genotypes (Sav-0, Kel-1, and Phw-3) could have resulted from their lower glucosinolate contents and/or low "attractant" property of the glucosinolate types that these genotypes produced, which apparently allowed them to "escape" herbivory by the associated insect specialist community. Genotype Wt-5 had the fourth highest mean fitness response among all genotypes studied, and it likely was able to compensate for the fairly high herbivore impact (approximately 69% of the plant damaged) mainly through its ability to tolerate herbivore damage by increasing branching and vegetative growth (data not shown). These results highlight the importance of intraspecific variation in plant resistance and tolerance to natural enemies when assessing the potential of alien species to become invasive in nonnative habitats.

In summary, although we do not discard the potential influence of other plant properties on herbivory levels in our study, the results suggest that variation in glucosinolate type and content is an important factor that affects insect selectivity among plant genotypes in the field. The observed association between variation in glucosinolate content, herbivore resistance, and fitness suggests that glucosinolate content is in part responsible for the observed variation in herbivore resistance. However, as shown by the percent of variation explained, it is not the only source of variation and other potential sources remain to be identified. For instance, variation in other herbivory resistance mechanisms, such as protease inhibitors, glucosinolate hydrolysis rate and specificity, other secondary metabolites, or inducibility, may influence herbivore variation in resistance and fitness. More field studies are needed to elucidate all the potential factors that control herbivore

resistance of *A. thaliana* in the field as well as to ascertain whether glucosinolate–insect herbivore relationships should be considered herbivore species specific, or to what extent they are dependent upon the degree of ecological specialization of the associated herbivore community.

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References

BARTH, C., and JANDER, G. 2006. Arabidopsis myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *Plant J*. 46:549–562.

BENDEROTH, M., TEXTOR, S., WINDSOR, A. J., MITCHELL-OLDS, T., GERSHENZON, J., and KROYMANN, J. 2006. Positive selection driving diversification in plant secondary metabolism. *Proc. Natl. Acad. Sci. U. S. A.* 103:9118–9123.

BERGELSON, J., STAHL, E., DUDEK, S., and KREITMAN, M. 1998. Genetic variation within and among populations of *Arabidopsis thaliana*. *Genetics* 148:1311–1323.

BIDART-BOUZAT, M. G., MITHEN, R., and BERENBAUM, M. R. 2005. Elevated CO_2 influences herbivory-induced defense responses of Arabidopsis thaliana. *Oecologia* 145:415–424.

BONES, A. M., ROSSITER, and J. T. 1996. The myrosinase–glucosinolate system, its organisation and biochemistry. *Physiol. Plant.* 97:194–208.

BRUNNER, E., DOMHOF, S., and LANGER, F. 2002. Nonparametric analysis of longitudinal data in factorial experiments. Wiley, New York, USA.

CRONIN, J. T., and ABRAHAMSON, W. G. 2001. Do parasitoids diversify in response to host-plant shifts by herbivorous insects. *Ecol. Entomol*. 26:347–355.

DETHIER, V. G. 1954. Evolution of feeding preferences in phytophagous insects. *Evolution* 8:33–54.

FEENY, P. 1976. Plant apparency and chemical defense, pp. 1–40, in J. W. Wallace, and R. L. Mansell (eds.). Biochemical interactions between plants and insectsPlenum, New York, USA..

FRAENKEL, G. S. 1959. The raison d'etre of secondary plant substances. *Science* 129:1466–1470.

GIAMOUSTARIS, A., and MITHEN, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp. *oleifera*) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126:347–363.

HOGGE, L. R., REED, D. W., UNDERHILL, E. W., and HAUGHN, G. W. 1988. HPLC separation of glucosinolates from leaves and seeds of *Arabidopsis thaliana* and their identification using thermospray liquid chromatography-mass spectrometry. *J. Chromatogr. Sci.* 26:551–556.

JAENIKE, J. 1990. Host specialization in phytophagous insects. *Annu. Rev. Ecolog. Syst.* 21:243–273.

KIM, J. H., and JANDER, G. 2007. *Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolate. *Plant J.* 49:1008–1019.

KLIEBENSTEIN, D. J. 2004. Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant Cell Environ.* 27:675–684.

KLIEBENSTEIN, D. J., KROYMANN, J., BROWN, P., FIGUTH, A., PEDERSEN, D., GERSHENZON, J., and MITCHELL-OLDS, T. 2001. Genetic control of natural variation in *Arabidopsis* glucosinolate accumulation. *Plant Physiol.* 126:811–825.

KLIEBENSTEIN, D. J., PEDERSEN, D., and MITCHELL-OLDS, T. 2002. Comparative analysis of insect resistance QTL and QTL controlling the myrosinase/glucosinolate system in *Arabidopsis thaliana*. *Genetics* 161:325–332.

LAMBRIX, V., REICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D. J., and GERSHENZON, J. 2001. The Arabidopsis epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13:2793–2807.

LANKAU, R. A. 2007. Specialist and generalist herbivores exert opposing selection on a chemical defense. *New Phytol.* 175:176–184.

LI, Q., EIGENBRODE, S. D., STRINGAM, G. R., and THIAGARAJAH, M. R. 2000. Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J. Chem. Ecol.* 26:2401–2419.

LOUDA, A., RODMAN, and J. E. 1983. Ecological patterns in the glucosinolate content of a native mustard *Cardamine cordifolia* in the rocky mountains USA. *J. Chem. Ecol.* 9:397–422.

MARQUIS, R. J. 1992. The selective impact of herbivores, pp. 301–325, in R. S. Fritz, and E. L. Simms (eds.). Plant Resistance to Herbivores and PathogensUniversity of Chicago Press, Chicago, Illinois, USA..

MAURICIO, R. 1998. Costs of resistance to natural enemies in field populations of the annual plant Arabidopsis thaliana. *Am. Nat.* 151:20–28.

MAURICIO, R., and RAUSHER, M. D. 1997. Experimental manipulation of putative selective agents provides evidence for the role of natural enemies in the evolution of plant defense. *Evolution* 51:1435–1444.

MCCLOUD, E. S., and BERENBAUM, M. R. 1999. Effects of enhanced UV-B radiation on a weedy forb (*Plantago lanceolata*) and its interactions with a generalist and specialist herbivore. *Entomol. Exp. Appl.* 93:233–247.

MITHEN, R., CLARKE, J., LISTER, C., and DEAN, D. C. 1995a. Genetics of aliphatic glucosinolates. III. Side chain structure of aliphatic glucosinolates in *Arabidopsis thaliana*. *Heredity* 74:210–215.

MITHEN, R., RAYBOULD, A. F., and GIAMOUSTARIS, A. 1995b. Divergent selection for secondary metabolites between wild populations of *Brassica oleracea* and its implications for plant-herbivore interactions. *Heredity* 75:472–484.

MULLER, C., and MARTENS, N. 2005. Testing predictions of the 'evolution of increased competitive ability' hypothesis for an invasive crucifer. *Evolutionary Ecol.* 19:533–550.

MURREN, C. J., DENNING, W., and PIGLIUCCI, M. 2005. Relationships between vegetative and life history traits and fitness in a novel field environment: impacts of herbivores. *Evol. Ecol.* 19:583–601.

NIELSEN, J. K., HANSEN, M. L., AGERBIRK, N., PETERSEN, B. L., and HALKIER, B. A. 2001. Responses of the flea beetles *Phyllotreta nemorum* and *P. cruciferae* to metabolically engineered *Arabidopsis thaliana* with an altered glucosinolate profile. *Chemoecology* 11:75–83.

PILSON, D., and DECKER, K. L. 2002. Compensation for herbivory in wild sunflower: response to simulated damage by the head-clipping weevil. *Ecology* 83:3097–3107.

POTVIN, C., and ROFF, D. A. 1993. Distribution-free and robust statistical methods: viable alternatives to parametric statistics. *Ecology* 74:1617–1628.

RAYBOULD, A. F., and MOYES, C. L. 2001. The ecological genetics of aliphatic glucosinolates. *Heredity* 87:383–391.

REICHELT, M., BROWN, P. D., SCHNEIDER, B., OLDHAM, N. J., STAUBER, E., TOKUHISA, J., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and GERSHENZON, J. 2002. Benzoic acid glucosinolate esters and other glucosinolates from *Arabidopsis thaliana*. *Phytochemistry* 59:663–671.

RODMAN, J. 1980. Population variation and hybridization in sea-rockets (*Cakile*, Cruciferae): seed glucosinolate characters. *Am. J. Bot.* 67:1145–1159.

RODMAN, J. E., KRUCKEBERG, A. R., and ALSHEHBAZ, I. A. 1981. Chemotaxonomic Diversity and Complexity in Seed Glucosinolates of Caulanthus and Streptanthus (Cruciferae). *Syst. Bot.* 6:197–222.

SAS Institute 1999. Statistical Software Version 8e. Cary, North Carolina, USA.

SIEMENS, D. H., and MITCHELL-OLDS, T. 1996. Glucosinolates and herbivory by specialists (Coleoptera: Chrysomelidae, Lepidoptera: Plutellidae): consequences of concentration and induced resistance. *Environ. Entomol.* 25:1344–1353.

STOWE, K. A. 1998. Realized defense of artificially selected lines of *Brassica rapa*: effects of quantitative genetic variation in foliar glucosinolate concentration. *Environ. Entomol.* 27:1166–1174.

STRAUSS, S. Y., IRWIN, and R. E. 2004. Ecological and evolutionary consequences of multispecies plant–animal interactions. *Annu. Rev. Ecol. Evol.* 17:278–284.

VANDER-ZWAN, C., BRODIE, S. A., and CAMPANELLA, J. J. 2000. The intraspecific phylogenetics of *Arabidopsis thaliana* in worldwide populations. *Syst. Bot.* 25:47–59.

WEINIG, C., STINCHCOMBE, J. R., and SCHMITT, J. 2003. Evolutionary genetics of resistance and tolerance to natural herbivory in Arabidopsis thaliana. *Evolution* 57:1270–1280.

WINDSOR, A. J., REICHELT, M., FIGUTH, A., SVATOS, A., KROYMANN, J., KLIEBENSTEIN, D. J., GERSHENZON, J., and MITCHELL-OLDS, T. 2005. Geographic and evolutionary diversification of glucosinolates among near relatives of *Arabidopsis thaliana* (Brassicaceae). *Phytochemistry* 66:1321–1333.

ZANGERL, A. R., and BERENBAUM, M. R. 1993. Plant chemistry, insect adaptations to plant chemistry, and host plant utilization patterns. *Ecology* 74:47–54.

ZHANG, Z. Y., OBER, J. A., and KLIEBENSTEIN, D. J. 2006. The gene controlling the quantitative trait locus *epithiospecifier modifier1* alters glucosinolate hydrolysis and insect resistance in Arabidopsis. *Plant Cell* 18:1524–1536.

be of importance for seed development (Vogel-Mikuš et al. 2007). Nevertheless, the possible role of differing GS profiles in AM formation during the life cycle of *T. praecox* or other AM-host plant species is not yet understood.

Several Brassicaceae species are hyperaccumulators that take up exceptionally high metal concentrations in their above-ground biomass without visible toxicity symptoms (Brooks et al. 1977). *T. praecox* is a recently reported Cd-hyperaccumulating species (Vogel-Mikuš et al. 2005), with the highest concentrations found during the vegetative phase of development (Pongrac et al. 2007). An evolutionary advantage of metal hyperaccumulation has been proposed. It may be an effective plant defense against pathogen and herbivore attack, with possibly no additional expensive organic defense compound formation being necessary (Boyd and Martens 1998; Poschenrieder et al. 2006b). However, a Cd-induced increase of total GS concentration (especially of sinalbin) observed in hydroponics of *T. praecox* has somewhat questioned the idea that metal accumulation leads to a decrease of investment in organic defense compound biosynthesis (Tolrà et al. 2006).

The relationships between metal and organic defenses in plants are complex (Hartl and Baldwin 2006; Poschenrieder et al. 2006a) and rarely have been studied simultaneously under field conditions (Noret et al. 2007). In the present study, three relationships were investigated in field-collected *T. praecox*: (1) the dynamics of total and individual GS in relation to plant developmental phases, (2) GS profiles in relation to AM formation in plant roots, and (3) GS and Cd concentrations in rosette leaves and their possible relation to plant defense.

Methods and Materials

Sampling and Plant Material Preparation Plants were collected from April to September 2005 in Žerjav, Northern Slovenia on a plot that is located on the rim of the valley, about 500 m from the main source of pollution, with closed vegetation and *Sesleria caerulea* L. and *T. praecox* as dominant plant species. The location is heavily polluted with Cd, Zn, and Pb, a result of centuries of Pb mining and smelting activities (Regvar et al. 2006). The rhizosphere soil of *T. praecox* was highly enriched in total Cd (84.3±7.6 mg kg^{-1}), with on average 28% Cd in available metal fractions in soil (Pongrac et al. 2007).

Five developmental phases during *T. praecox* life cycle were distinguished: (1) vegetative phase (VP) collected in April, (2) flower induction phase (FI) collected in April, (3) flowering phase (FP) collected in May, (4) seeding phase (SP) collected in June, and (5) senescence phase (SC) collected in September. Five plants per plot were collected and analyzed in each phase, with the exception of the vegetative and flower induction phase. During these latter phases, plants were very small. Therefore, three (VP) and two (FI) plants had to be pooled to provide a single sample; three sample pools per plot were collected in these phases. On average, 748±45 mg Cd kg^{-1} were found in roots, 738±103 mg Cd kg^{-1} in shoots, 496±76 mg Cd kg^{-1} in stalks (Pongrac et al. 2007), and 623±78 mg Cd kg^{-1} in seeds (Vogel-Mikuš et al. 2007) of the collected *T. praecox* plants.

Estimation of Arbuscular Mycorrhizal Colonization AM colonization was determined by vital staining of fresh root fragments by using the nitro blue tetrazolium chloride succinate method, with deposition of formazan as the viability indicator and counterstaining with acid fuchsin (Schaffer and Peterson 1993). Roots were mounted in lactoglycerol and immediately examined. The portions of vital AM colonization were estimated on 30 root fragments per plant according to Trouvelot et al. (1986): Mycorrhizal frequency (F%) describes frequency of root fragments colonized with fungus, global mycorrhizal intensity (M%) is the intensity of colonization in the root system, and mycorrhizal intensity in colonized fragments (m%) is the intensity of colonization in the fragments. Vital colonization represents only the colonization in which formazan deposition was observed.

Analysis of Plant Material Plant material was freeze-dried after a careful wash in tap and distilled water. Total GS were extracted from all plant samples (from roots N=21, rosette leaves N=21, flowering/seeding stalks N=10, and seeds N=1) with 70% (v/v) aqueous methanol in a boiling water bath for 5 min. After cooling and centrifugation, the pellet was re-extracted. The joint liquid phases were loaded onto a DEAE-Sephadex A-25 column and treated with myrosinase (thioglucoside glucohydrolase, EC 3.2.1.147; Sigma, St. Louis, MO, USA). The released D-glucose was quantified by using an enzyme-based analytical kit (Boehringer, Mannheim, Germany). The effectiveness of the extraction method was validated by using certified reference rapeseeds (CRM 367) from the European Community Bureau of Reference (BCR, Brussels, Belgium).

Extraction of individual GS was done as previously described (Tolrà et al. 2000) from two samples per developmental phase and plant tissue. Separation and quantification was performed with a liquid chromatograph equipped with a diode array detector Model HP 1090 (Hewlett Packard, CA, USA). Samples of 50 µl were injected onto a Lichospher 60RP Select B column. The mobile phases used for elution were acetonitrile and water containing 0.5% acetic acid. The gradient elution was from 0% to 60% acetonitrile for 74 min at a flow rate of 1 ml min^{-1}, at 30°C and the diode array set at 230 nm. Sinigrin

(2-propenyl-GS) from horseradish (Sigma-Aldrich, Quimica SA, Madrid) was used as an internal standard. Gluconapin (3-butenyl-GS) and glucotropaeolin (benzyl-GS; both from Merck) were used as external standards.

Statistical Analyses To test the overall effect of developmental phases on the total and individual GS concentrations and mycorrhizal colonization, the non-parametric Kruskal–Wallis test was applied, and when significant, Mann–Whitney *U* test with a Bonferroni correction was used to determine the significance of differences among developmental phases at $P<0.05$ (means±standard error). Spearman's correlation coefficient (r_s) was used when calculating correlation between AM colonization parameters and individual GS at $P<0.05$. Statistical tests were performed with Statistica Statsoft® (version 6.0) software.

Results

Total Glucosinolates in Different Organs The highest average total GS concentrations (μmol g^{-1} dry weight) were found in roots (64±9.1), followed by rosette leaves (36.6±6.8), flowering/seeding stalks (16.6±2.04), and seeds (14.3). There was a statistically significant influence of developmental phases on total GS in roots and shoots (Table 1). In roots, a significant decline occurred after the flower induction phase, while it increased again during senescence phase (Fig. 1a). The highest total GS concentrations in rosette leaves were found in the vegetative phase, with a decrease during the flowering, seeding, and senescence phases (Fig. 1a).

Individual Glucosinolates in Different Organs The highest number of individual GS with different aliphatic-, aromatic-, and indolyl-side chains in field collected samples of *T. praecox* was found in roots (eight), followed by seeds (seven), rosette leaves, and stalks (five) (Table 2). The prominent GS in roots and shoots was dependent on the developmental phase (Table 1). Glucobrassicanapin and sinalbin were prominent in rosette leaves (Fig. 1b). The proportion of indolyl-GS in rosette leaves declined with age until the flowering phase and resulted in predominance of aliphatic GS (Fig. 2a). On average, 10% of the total GS in rosette leaves was represented by aromatic GS, and this remained constant during development. Glucobrassicanapin, sinalbin, and 4-OH-glucobrassicin in rosette leaves were found in all developmental phases (Table 2).

The highest proportion of GS type in flowering/seeding stalks belonged to indolyl GS (Fig. 2b). Glucobrassicanapin, sinalbin, and 4-OH-glucobrassicin were found in flowering and seeding stalks, whereas sinigrin and traces of 4-methoxy-glucobrassicin were found only in seeding stalks (Table 2). The GS profile in *T. praecox* seeds was characterized by a higher proportion of aliphatic GS (Fig. 2b) than in stalks and by the presence of the aliphatic-GS, progoitrin, that was not found in other plant parts.

In roots, glucobrassicanapin was not found in the flower induction or flowering phases, whereas glucotropaeolin was found only in these two phases (Fig. 1c; Table 3). A decrease in the relative content of indolyl GS and a simultaneous increase of aliphatic- and aromatic GS after the vegetative phase was observed in roots, with aromatic GS being the highest in the flowering and seeding phase (Fig. 2c). Of the individual GS found in roots, sinigrin, gluconapin, and sinalbin were present in all phases (Table 2).

Mycorrhizal Colonization Significant variation in vital mycorrhizal colonization frequency (F%), global intensity (M%), and intensity in the colonized fragments (m%) was observed during the life cycle of *T. praecox* (Table 3), with peaks during flowering. A positive correlation between AM parameters and root glucotropaeolin concentration was found (Table 3). No other correlation was found between AM parameters and root individual GS.

Table 1 Significance levels of *h-test* (*Kruskal–Wallis*) for developmental phase effects on total and prominent individual glucosinolates in the life cycle of field collected *Thlaspi praecox*

	Roots			Rosette leaves		
	df	*H*	*P*	*df*	*H*	*P*
Total glucosinolates	4	13.1	0.01	4	9.8	0.043
Glucobrassicanapin	4	13.7	0.008	4	10.8	0.03
Sinalbin	4	3.2	>0.05	4	9.89	0.04
Glucotropaeolin	4	13.7	0.008	4	/	/
4-OH-glucobrassicin	4	12.6	0.013	4	8.2	>0.05

df Degrees of freedom

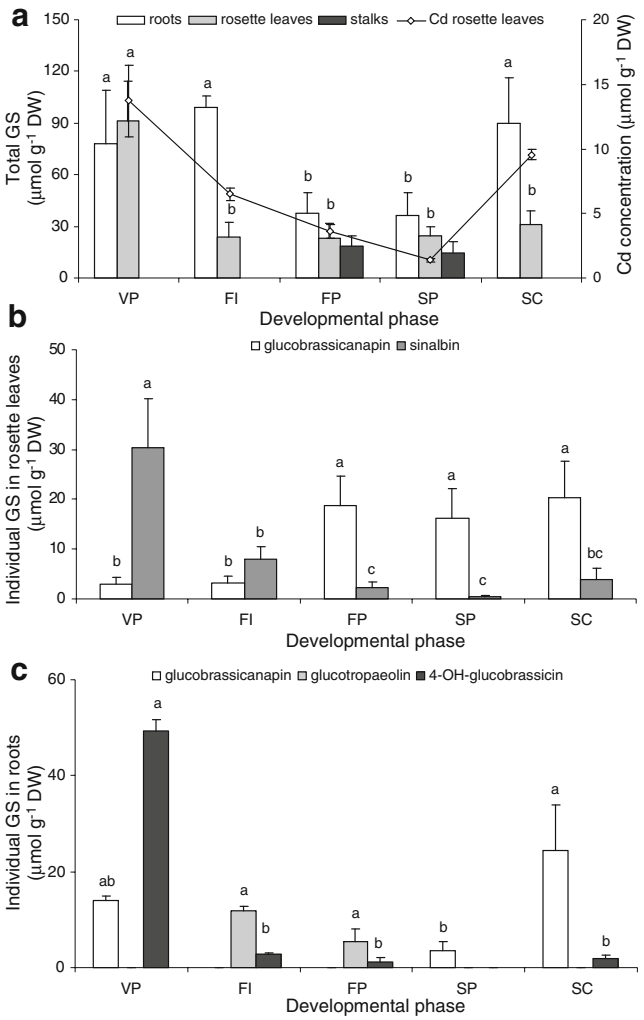

Fig. 1 Concentrations of **a** total glucosinolates (*GS*) in roots, rosette leaves, and stalks and Cd concentration in rosette leaves, **b** prominent glucosinolates in rosette leaves, and **c** prominent lucosinolates in roots in the life cycle of *Thlaspi praecox* (means ± standard error; N=3–5). *VP* Vegetative, *FI* flower induction, *FP* flowering, *SP* seeding, *SC* senescence phase, *DW* dry weight. Different letters above columns represent statistically significant difference for roots, rosette leaves and stalks separately (**a**) and for a given glucosinolate (**b** and **c**; Mann–Whitney U test, P<0.05)

Discussion

Organic defense compounds in plants can be influenced by several external factors and also by intrinsic signals (e.g., leaf age), as has been observed for glucosinolates (GS; Rosa et al. 1996; Petersen et al. 2002; Rangkadilok et al. 2002; Brown et al. 2003). The highest total GS concentrations in *Arabidopsis thaliana* have been reported in seeds, followed by flowers and fruits, leaves, and the lowest in roots (Petersen et al. 2002; Brown et al. 2003). Reproductive organs are expected to have the highest concentrations of defense compounds, as they contribute most to survival and fitness of the offspring, as described

Table 2 Semi-systematic and trivial names of glucosinolates found in different plant parts in the life cycle of field collected *Thlaspi praecox*

Type of side chain	Trivial name	Rosette leaves					Stalks			Seeds	Roots				
		VP	FI	FP	SP	SC	FP	SP	SC	SP	VP	FI	FP	SP	SC
Aliphatic-															
2-Hydroxy-3-butenyl-	Progoitrin*									x					
2-Propenyl-	Sinigrin							x		x	x	x	x	x	x
3-Butenyl-	Gluconapin	x		x	x					x	x	x	x	x	x
4-pentenyl-	Glucobrassicanapin	x	x	x	x	x	x	x	x	x	x			x	x
Aromatic															
p-hydroxybenzyl-	Sinalbin	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Benzyl-	Glucotropaeolin									x		x	x		
Indolyl-															
3-Indolylmethyl-	Glucobrassicin	x		x	x	x						x	x	x	x
4-Hydroxy-3-indolylmethyl-	4-OH-glucobrassicin	x	x	x	x	x	x	x	x	x	x	x	x		x
4-Methoxy-3-indolylmethyl-	4-Metoxy-glucobrassicin[a]							x					x		

VP Vegetative, *FI* flower induction, *FP* flowering, *SP* seeding, *SC* senescence phase
[a] Traces only

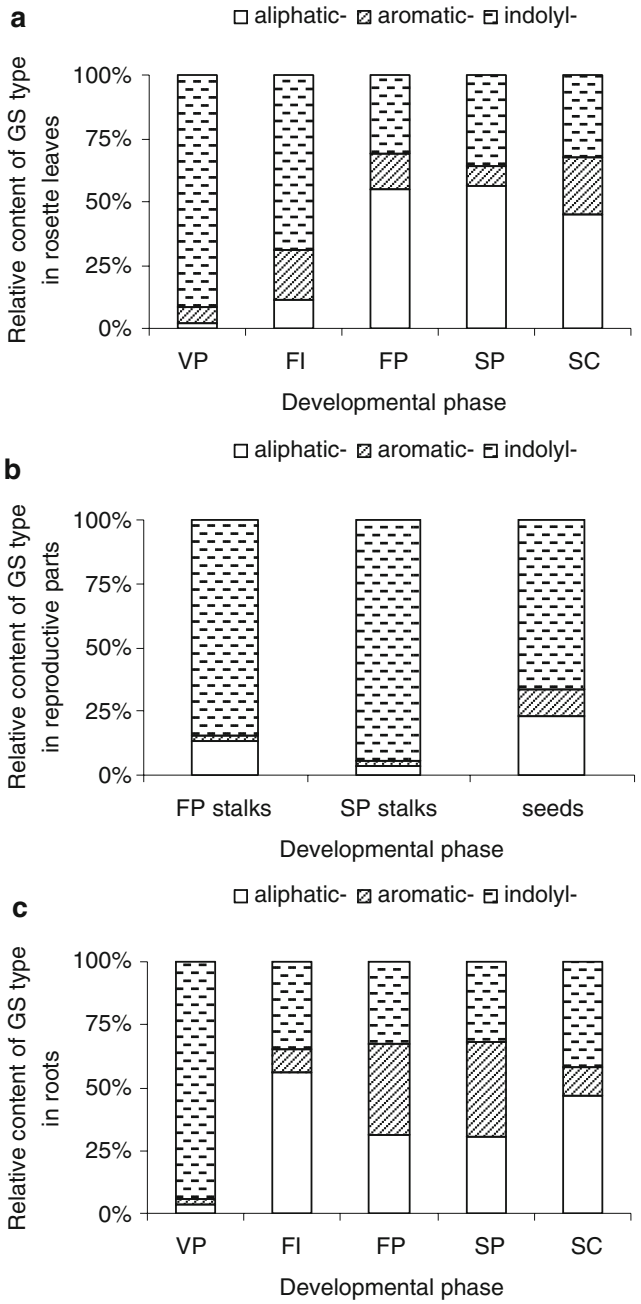

Fig. 2 Relative content of glucosinolate (*GS*) type (aliphatic-, aromatic- and indolyl-) in the life cycle of field collected *Thlaspi praecox* in **a** rosette leaves, **b** stalks and seeds and **c** roots (N=2; 100% presents the sum of total glucosinolate fractions). *VP* Vegetative, *FI* flower induction, *FP* flowering, *SP* seeding, *SC* senescence phase

by optimal defense theory (Wallace and Eigenbrode 2002). In contrast, the lowest GS concentrations were found in seeds of field collected metal-hyperaccumulating *T. praecox* (compared to other plant parts), with indolyl GS as the major GS type and the addition of a GS (progoitrin) that was not detected in any other plant parts. It seems likely that specific enzymes for the biosynthesis of this GS may be present only in seeds and that some of the seed GS were synthesized de novo in the silique as previously reported (Petersen et al. 2002).

An increase in the relative content of aliphatic GS at the expense of indolyl GS in rosette leaves was observed up to the flowering phase in field collected *T. praecox*. In contrast, the proportion of indolyl GS in leaves increased with age at the expense of aliphatic GS in *A. thaliana* (Brown et al. 2003). The concentration of the aliphatic GS, sinigrin, the major GS in *Brassica nigra*, decreased from seedling to early flowering phase, but increased in the late flowering phase, and decreased again during seed maturation (Rangkadilok et al. 2002). The observed differences in GS metabolism may be due to differences in genetics, differences in life cycle length (annuals *A. thaliana* and *B. nigra* vs. perennial *T. praecox*), and/or different growth conditions.

Root indolyl GS of *T. praecox* were found to prevail only in the vegetative phase, whereas in the other phases, they comprised a constant proportion averaging 33% of the total GS. Indolyl GS produce isothiocyanates that are unstable and decompose spontaneously (Fahey et al. 2001). They are, therefore, believed to be less effective deterrents or toxins. The restoration of high root total GS concentrations and aliphatic GS, particularly glucobrassicanapin, in the senescence phase compared to the flowering and seeding phases suggests involvement in root protection of the perennial *T. praecox* during overwintering. However, a targeted study is needed to examine defensive roles during specific developmental phases.

The presence of novel GS (progoitrin, glucotropaeolin, and glucobrassicin) in field collected *T. praecox* compared to hydroponically grown plants that were analyzed only in the vegetative phase (Tolrà et al. 2006) was related to specific developmental phases, mainly to flowering and

Table 3 Minimal–maximal values of vital arbuscular mycorrhizal (AM) colonization parameters In the life cycle of field collected *Thlaspi praecox* and Spearman's correlation coefficient (r_s) between AM colonization parameters and root glucotropaeolin concentration

Developmental phase	Vital AM root colonization		
	F%	M%	m%
VP	45–73	0.96–5	2.9–7
FI	50–60	2.0–8	4–22.5
FP	47–77	2.8–15	3.7–19
SP	0–23	0–2.1	0–11
SC	0–56	0–1.8	0–3.2
Root glucotropaeolin	r_s=0.67	r_s=0.76	r_s=0.76
	P=0.03	P=0.01	P=0.01

F% Colonization frequency, *M%* global intensity of colonization, *m%* intensity in the colonized fragments; *VP* vegetative, *FI* flower induction, *FP* flowering, *SP* seeding, *SC* senescence phase

seed production. In addition, among all detected individual GS, sinalbin was the only one present in all plant organs and in all developmental phases of field collected *T. praecox*. Its highest concentrations in rosette leaves were, however, measured in the vegetative phase, which also contained the highest Cd concentrations (Fig. 1) as previously reported by Pongrac et al. (2007). The presence of sinalbin also increased in hyperaccumulating *T. praecox* after Cd treatment in hydroponics, whereas it was not detected in the Cd-sensitive non-hyperaccumulator *Thlaspi arvense* (Tolrà et al. 2006). Thus, the synthesis of sinalbin may be linked with Cd metabolism in *T. praecox*. Additionally, the simultaneous presence of the highest Cd and total GS concentration in rosette leaves in the vegetative phase does not support the "trade-off" hypothesis as proposed by Boyd and Martens (1998) and Poschenrieder et al. (2006b) in this hyperaccumulating species. Rather, plants in the vegetative phase may profit by being protected by both high metal and high GS concentration.

The non-mycorrhizal status of plants is attributed to in situ root GS hydrolysis products, the presence or absence of lectins, and/or the structural-chemical properties of the root cell wall that hinder or inhibit fungal growth (Glenn et al. 1988). Increase of GS concentration and a distinct change of GS profile after inoculation have been observed in several GS-containing plant species regardless of whether colonization occurred or not (Vierheilig et al. 2000). Similarly, in *Tropaeolum majus* and *Carica papaya*, reduced AM colonization could not be correlated with induced concentrations of GS (Ludwig-Müller et al. 2002). As most of the non-AM colonizing plants contained gluconasturtiin (2-phenylethyl GS) in roots, this GS, but not others, has been proposed to act as a general AM inhibitory factor (Vierheilig et al. 2000). In our study, no gluconasturtiin was found, consistent with other GS-containing plants susceptible to AM colonization. However, a distinct change of GS profile and a decrease of total GS concentration in roots coincided with the highest AM colonization in *T. praecox*. The presence of glucotropaeolin and absence of glucobrassicanapin was observed during the reproductive phases of *T. praecox*. Quantities of glucotropaeolin, the main GS of the AM-host *T. majus*, have been shown to increase during AM colonization (Vierheilig et al. 2000; Ludwig-Müller et al. 2002). Glucotropaeolin is an important metabolite for root growth regulation and a potential precursor of phenylacetic acid. Similarly, indol-3-acetic acid is formed from indolyl GS in species of Brassicaceae (Ludwig-Müller 1999; Ludwig-Müller and Cohen 2002). Glucobrassicanapin, on the other hand, has been found only in AM non-host *Brassica napus* (Vierheilig et al. 2000). Thus, the pattern of GS in *T. praecox* roots, i.e., the presence and absence of specific GS, is likely to affect AM formation, as some GS may inhibit while others may contribute to or are of little consequence in AM formation. In addition to GS profiles and/or content, plant mineral demand, seasonal dynamics of mycorrhization (Pongrac et al. 2007), and/or diverse signaling molecules (Akiyama et al. 2005) may all contribute to AM colonization in *T. praecox*.

In summary, this study showed that GS concentrations and profiles change considerably in the life cycle of Cd/Zn hyperaccumulating *T. praecox*. The highest Cd and GS concentrations in rosette leaves are found in the vegetative phase, with indolyl GS the predominate form, presumably providing protection of young, palatable leaves. The presence of glucotropaeolin and absence of glucobrassicanapin in *T. praecox* roots during flower induction and the flowering phases coincide with the highest AM colonization levels during plant development. AM formation is accompanied by an overall absence of gluconasturtiin. In addition, the increase of total GS in roots in the senescence phase may contribute to effective protection of roots during overwintering and in early spring, with the aliphatic and indolyl GS as predominant GS forms.

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References

AGRAWAL, A. A., and KURASHIGE, N. S. 2003. A role for isothiocyanates in plant resistance against specialist herbivore *Pieris rapae*. *J. Chem. Ecol.* 29:1403–1415.

AKIYAMA, K., MATSUZAKI, K., and HAYASHI, H. 2005. Plant sesquiterpene induce hyphal branching in arbuscular mycorrhizal fungi. *Nature* 435:824–827.

BENNETT, R. N., and WALLSGROVE, R. M. 1994. Secondary metabolites in plant defense mechanisms. *New Phytol.* 127:617–633.

BOYD, R. S., and MARTENS, S. N. 1998. The significance of metal hyperaccumulation for biotic interactions. *Chemoecology* 8:1–7.

BROOKS, R. R., LEE, J., REEVES, R. D., and JAFFRÈ, T. 1977. Detection of nickeliferous rocks by analysis of herbarium specimens of indicator plants. *J. Geochem. Explor.* 7:49–57.

BROWN, P. D., TOKUHISA, J. G., REICHELT, M., and GERSHENZON, J. 2003. Variation of glucosinolate accumulation among different organs and developmental stages of *Arabidopsis thaliana*. *Phytochemistry* 62:471–481.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

GLENN, M. G., CHEW, F. S., and WILLIAMS, P. H. 1988. Influence of glucosinolate content of *Brassica* (Cruciferae) roots on growth of vesicular-arbuscular mycorrhizal fungi. *New Phytol.* 110:217–225.

HARTL, M., and BALDWIN, I. T. 2006. Evolution: the ecological reverberation of toxic trace elements. *Curr. Biol.* 16:R958–R960.

LUDWIG-MÜLLER, J. 1999. The biosynthesis of auxins. *Curr. Topics Plant Biol.* 1:77–88.

LUDWIG-MÜLLER, J., and COHEN, J. D. 2002. Identification and quantification of three active auxins in different tissues of *Tropaeolum majus*. *Physiol. Plant.* 115:320–329.

LUDWIG-MÜLLER, J., BENNETT, R. N., GARCIA-GARRIDO, J. M., PICHÉ, Y., and VIERHEILIG, H. 2002. Reduced arbuscular mycorrhizal root colonization in *Tropaeolum majus* and *Carica papaya* after jasmonic acid application cannot be attributed to increased glucosinolate levels. *J. Plant Physiol.* 159:517–523.

NORET, N., MEERTS, P., TOLRÀ, R., POSCHENRIEDER, C., BARCELÓ, J., and ESCARRÉ, J. 2005. Palatability of *Thlaspi caerulescens* for snails: influence of zinc and glucosinolates. *New Phytol.* 165:763–772.

NORET, N., MEERTS, P., VANHAELEN, M., DOS SANTOS, A., and ESCARRÉ, J. 2007. Do metal-rich plants deter herbivores? A field study of the defense hypothesis. *Oecologia* 152:1432–1439.

ORŁOWSKA, E., ZUBEK, S. Z., JURKIEWITCZ, A., SZAREK-LUKASZEEWSKA, G., and TURNAU, K. 2002. Influence of restoration on arbuscular mycorrhiza of *Biscutella leavigata* L. (Brassicaceae) and *Plantago lanceolata* (Plantaginaceae) from calamine spoil mounds. *Mycorrhiza* 12:153–160.

PETERSEN, B. L., CHEN, S., HANSEN, C. H., OLSEN, C. E., and HALKIER, B. A. 2002. Composition and content of glucosinolates in developing *Arabidopsis thaliana*. *Planta* 214:562–571.

PONGRAC, P., VOGEL-MIKUŠ, K., KUMP, P., NEČEMER, M., TOLRÀ, R., POSCHENRIEDER, C., BARCELÓ, J., and REGVAR, M. 2007. Changes in elemental uptake and arbuscular mycorrhizal colonization during the life cycle of *Thlaspi praecox* Wulfen. *Chemosphere* 69:1602–1609.

POSCHENRIEDER, C., TOLRÀ, R., and BARCELÓ, J. 2006a. Interactions between metal ion toxicity and defense against biotic stress: glucosinolates and benzoxazionids as case study. *For. Snow Landsc. Res.* 80:149–160.

POSCHENRIEDER, C., TOLRÀ, R., and BARCELÓ, J. 2006b. Can metals defend plants against biotic stress? *Trends Plant Sci.* 11:288–295.

RANGKADILOK, N., NICOLAS, M. E., BENNET, R. N., PREMIER, R. R., EAGLIG, D. R., and TAYLOR, P. W. J. 2002. Developmental changes in sinigrin and glucoraphanin in three *Brassica* species (*Brassica nigra*, *Brassica juncea* and *Brassica oleracea* var. *italica*). *Sci. Hortic.-Amsterdam* 96:11–26.

RASK, L., ANDREASSON, E., EKBOM, B., ERIKSSON, S., PONTOPPIDAN, B., and MEIJER, J. 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant. Mol. Biol.* 42:93–113.

REGVAR, M., VOGEL-MIKUŠ, K., KUGONIČ, N., TURK, B., and BATIČ, F. 2006. Vegetational and mycorrhizal successions at a metal polluted site—indications for the direction of phytostabilisation? *Environ. Pollut.* 144:976–984.

ROSA, E. A. S., HEANEY, R. K., PORTAS, C. A. M., and FENWICK, G. R. 1996. Changes in glucosinolate concentrations in *Brassica* crops (*B. oleracea* and *B. napus*) throughout growing seasons. *J. Sci. Food Agric.* 71:237–244.

SCHAFFER, G. F., and PETERSON, R. L. 1993. Modifications to clearing methods used in combinations with vital staining of roots colonized with vesicular-arbuscular mycorrhizal fungi. *Mycorrhiza* 4:29–35.

TOLRÀ, R. P., ALONSO, R., POSCHENRIEDER, C., BARCELÓ, D., and BARCELÓ, J. 2000. Determination of glucosinolates in rapeseed and *Thlaspi caerulescens* plants by liquid chromatography–atmospheric pressure chemical ionization mass spectrometry. *J. Chromatogr. A.* 889:75–81.

TOLRÀ, R., PONGRAC, P., POSCHENRIEDER, C., VOGEL-MIKUŠ, K., REGVAR, M., and BARCELÓ, J. 2006. Distinctive effects of cadmium on glucosinolate profiles in Cd hyperaccumulator *Thlaspi praecox* and non-hyperaccumulator *Thlaspi arvense*. *Plant Soil* 288:333–341.

TROUVELOT, A., KOUGH, J. L., and GIANINAZZI-PEARSON, V. 1986. Estimation of VA mycorrhizal infection levels. Research for methods having a functional significance (in French), pp. 217–221, in V. Gianinazzi-Pearson, and S. Gianinazzi (eds.). Physiological and Genetical Aspects of MycorrhizaeService des Publications, INRA Press, Paris, France.

VIERHEILIG, H., BENNETT, R., KIDDLE, G., KALDORF, M., and LUDWIG-MÜLLER, J. 2000. Differences in glucosinolate patterns and arbuscular mycorrhizal status of glucosinolate-containing plant species. *New Phytol.* 146:343–352.

VOGEL-MIKUŠ, K., DROBNE, D., and REGVAR, M. 2005. Zn, Cd and Pb accumulation and arbuscular mycorrhizal colonization of pennycress *Thlaspi praecox* Wulf. (Brassicaceae) from the vicinity of a lead mine and smelter in Slovenia. *Environ. Pollut.* 133:233–242.

VOGEL-MIKUŠ, K., PONGRAC, P., KUMP, P., NEČEMER, M., and REGVAR, M. 2006. Colonization of a Zn, Cd and Pb hyper-accumulator *Thlaspi praecox* Wulfen with indigenous arbuscular mycorrhizal fungal mixture induces changes in heavy metal and nutrient uptake. *Environ. Pollut.* 139:362–371.

VOGEL-MIKUŠ, K., PONGRAC, P., KUMP, P., NEČEMER, M., SIMČIČ, J., PELICON, P., BUDNAR, M., POVH, B., and REGVAR, M. 2007. Localisation and quantification of elements within seeds of Cd/Zn hyperaccumulator *Thlaspi praecox* by micro-PIXE. *Environ. Pollut.* 147:50–59.

WALLACE, S. K., and EIGENBRODE, S. D. 2002. Changes in the glucosinolate-myrosinase defense system in *Brassica juncea* cotyledons during seedling development. *J. Chem. Ecol.* 28:243–256.

Ca. P. mali by grafting shoots of already infected apple trees (from the previous years experiments) or left untreated as uninfected controls. All trees were checked for apple proliferation prior to experiments by extracting deoxyribonucleic acid (DNA) of the rootstock's phloem followed by PCR with specific primers (fAT/rAS) as described below. Trees were transferred from a cooling chamber to a greenhouse chamber 5 wk before the headspace sampling was started. The trees were maintained under natural light and temperature conditions (day/night: 16/8 hr, 20/15°C, 60% relative humidity [RH]) until flowering was completed (phenology stage 68). Subsequently, trees were transferred into a climate chamber (day/night: 12/12 hr, 20°C constant, 60% RH) for headspace sampling (phenology stages 69–79).

Rearing of Insects for Bioassays Mature adults of *C. picta* were collected from apple trees (*M. domestica*) in early spring after having returned from their overwintering host plants. In rearing cages (60×60×90 cm), they were maintained on uninfected or *Ca.* P. mali-infected apple plants where they could feed and oviposit. The rearing cages were located in a tempered greenhouse chamber under natural light conditions and a day/night temperature program (20/15°C). For behavioral trials, test insects were collected from the boxes 1 d before testing in groups of ten in Eppendorf vials at 4°C. All insects were tested within 2 wk after emergence.

Behavioral Bioassays Behavioral tests were carried out by using a dynamic olfactometer consisting of a Y-shaped glass tube (entrance arm: 12.5 cm, test arms=21.0 cm, inner diameter=0.6 cm) mounted on an angular board. Charcoal cleaned air (granulated 4–8 mm, Applichem GmbH, Darmstadt, Germany) was pumped (75 ml/min) through two glass jars (vol=2 l) containing the volatile sources. A twig of uninfected or phytoplasma infected apple plants (length 10–15 cm, phenology stage 69–71) was placed in a water-filled glass vial in each jar. The odors of infected and uninfected twigs were offered simultaneously. According to the method of Soroker et al. (2004), for each test, ten females were put together into the entrance arm. Every individual that passed a final marking (10.0 cm after the branching) on one of the test arms within 15 min was counted and placed into separate vials filled with ethanol (70%) for later determination of their infection status (see below). Both experiments were repeated 29 times. The numbers of psyllids were analyzed statistically after log (x+0.5) transformation by dependent paired t test using Statistica 5.5.

DNA Extraction: Plant Material Phloem tissue from cut roots was abraded. Between 1.0 and 1.5 g of phloem tissue was subjected to DNA extraction following the procedure described by Doyle and Doyle (1990). Instead of 0.2% β-mercaptoethanol, 2% sodium metabisulfide was used. The resulting DNA pellet was resuspended in 50 µl of sterile water and stored at −20°C.

Insect Material Psyllids that were used in bioassays (see above) were subjected to DNA extraction by using the same protocol as described above for plant material. Single insects were homogenized with a conical pestle (polypropylene, 1.5 ml, Eppendorf, Hamburg, Germany) in a 1.5-ml tube containing 150 µl extraction buffer and a pinch of silicon carbide (carborundum, Sigma-Aldrich, Munich, Germany) as grinding additive. The resulting DNA pellet was resuspended in 20 µl sterile water and stored at −20°C.

PCR Analysis DNA was purified by using the primer pair fAT/rAS specific for *Ca.* P. mali (Smart et al. 1996) and amplifying a 400-bp sequence in the 16S–23S ribosomal ribonucleic acid spacer region. PCR reactions were performed in a thermal cycler (Robocycler 96, Stratagene, La Jolla, CA, USA) with a reaction volume of 25 µl containing 2.5 µl extracted DNA, 0.5 pM of each primer, 250 µM of each nucleotide, 0.5 U polymerase, and 1× polymerase buffer (Invitrogen, Karlsruhe, Germany). Samples were subjected to 35 cycles that consisted of 1 min denaturation at 95°C, 1 min annealing at 51°C, and 1.5 min at 70°C. The products of each PCR were electrophoresed on a 1% agarose gel containing ethidium bromide (0.3 µg/ml) in 1× TAE buffer (40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 8.0). DNA was visualized and photographed while exposed to UV light (302 nm).

Headspace Sampling Single branches of apple plants (see above) were carefully wrapped in polyethylene terephthalate bags (20 cm diameter, Melitta, Minden, Germany). A stream of purified air (250 ml/min; controlled by a flowmeter) was pumped through the bag for 4 hr. For each treatment, two trees were sampled every second or third day over a period of 45 d. Volatiles from plant headspace were trapped in collection filters (charcoal 5 mg, Gränicher+Quartero, Daumazan, France) and eluted by rinsing the filter with 25 µl of dichloromethane containing 50 ng/µl of tridecane (Sigma-Aldrich, Munich, Germany) as internal standard (IS).

Analysis of Headspace Samples by GC/MS Each sample (0.5 µl) was injected splitless into a gas chromatograph (Shimadzu GC 17A, injector temperature 300°C) equipped with a 30 m×0.32 mm×0.25 µm HP-5 column (J & W, Santa Clara, CA, USA). The temperature program started at 40°C, was held for 3 min, and then

raised by 10°C/min to 250°C. The final temperature was held for 6 min. Helium (Air Liquide, Germany) was used as carrier gas (inlet pressure 3.2 kPa). The gas chromatograph was coupled to a quadrupole mass spectrometer (Shimadzu QP 5000). Electron ionization mass spectra were recorded at 70 eV with scanning from mass 35 to 500 at 0.5 scan/sec. Solvent delay was adjusted to 5 min. Identification of volatiles was determined by comparing retention times with commercially available standards (Sigma-Aldrich, Munich, Germany) and by comparison with the NIST 02 mass spectral database and NIST MS search 2.0 (National Institute of Standards and Technology, USA). The relative amounts of volatiles compared to IS were calculated as previously described by Gross et al. (2008).

Results and Discussion

Phytoplasmas infecting apple trees alter the odor of this host plant that lures their vector. *C. picta*, reared on uninfected plants without any contact with the phytoplasma during their ontogenesis (inexperienced), was attracted by the odor of infected plants (AP^+; Fig. 1a; dependent paired *t* test, $P<0.05$). By contrast, *C. picta* that were reared on infected plants (experienced) showed the opposite behavior depending on their infection status when exposed to the different odor sources. The odor of infected apple was highly attractive for psyllids that had been infected by phytoplasma ($P<0.001$), while adults of *C. picta* that had not been infected were attracted by the odor of uninfected plants or repelled by the odor of infected plants, respectively ($P<0.05$). The motivation of *C. picta* females to enter an olfactometer arm was 51% for experienced and 67% for inexperienced individuals. These are high activities for psyllids in olfactometer bioassays (Soroker et al. 2004). Interestingly, breeding of *C. picta* on infected apple trees resulted only in 29% infection of the potential vectors by phytoplasmas, as shown by PCR analysis of whole-body extracts, using specific primers for detection of *Ca.* P. mali. As all infected trees used for conducting experiments and rearing purposes showed apple proliferation symptoms, we assume that the phytoplasmas were present in the tissue of all parts of these plants. Thus, a much higher infection rate of *C. picta* reared on these trees is expected. Why some females of *C. picta* are susceptible to phytoplasma infection while others are not remains to be elucidated. The psyllids that apparently were not able to transmit the disease (experienced, but uninfected) were attracted by the odor of uninfected apple trees.

Analysis of the plants' headspace by GC/MS revealed about 25 different volatile compounds during a sampling period of 45 d. While there were no significant differences among all other volatiles emitted by uninfected and infected plants (data not shown), only apple plants infected by *Ca.* P. mali emitted the sesquiterpene β-caryophyllene in higher amounts (Fig. 1b). Emission was highest from the beginning of headspace sampling (phenology stage 69; BBCH scale) and decreased during 29 d to 0 (phenology stage 72). After this time, nearly no β-caryophyllene was emitted until the end of the sampling time (45th day, phenology stage 79).

C. picta spends only a short period of its life (2–3 mo) exclusively on apple for reproduction (phenology stages 53–59) and juvenile development (reproduction host). Soon after hatching (phenology stages 69–71), newly emerged adults migrate onto conifers, spending the next 8–9 mo including winter time there (overwintering host). During the relatively short period on apple, the newly hatched adults of *C. picta* are able to ingest *Ca.* P. mali from infected plant phloem (acquisition feeding). Following a latent period, ingested phytoplasmas leave the intestinal tract of their vector, invade the hemolymph, multiply, and infect further tissues of the insect (Christensen et al. 2005). Finally, they intrude into the salivary glands, from which they can infect a new host plant (transmission feeding). Interestingly, the phytoplasma induces emission of β-caryophyllene from apple plants exactly between hatching and migration of its vector (Fig. 1b). According to our behavioral studies, vector insects that developed on uninfected plants were attracted by the odor of infected plants. The phytoplasma-induced change in odor composition may be the attracting signal for its vector to change orientation from uninfected to infected plants, and promote the uptake of phytoplasmas from the phloem. Additionally, infected psyllids are also attracted by infected plants, and that may ensure a longer insect feeding period from these plants, thus acquiring a higher titer of *Ca*. P. mali before shifting to their overwintering host.

In summary, the presented results support our hypothesis that the apple proliferation phytoplasma manipulates both odor of infected plants and behavior of vector insects to promote its propagation. Because experienced but uninfected psyllids show the opposite behavior to infected psyllids, the phytoplasma within the insect may affect behavior as well. These abilities are certainly of epidemiological relevance and will be studied further to understand the complex interactions between pathogens reproducing in distinct hosts. Thus, both laboratory and field experiments are in progress in order to confirm a direct attraction of β-caryophyllene to *C. picta*.

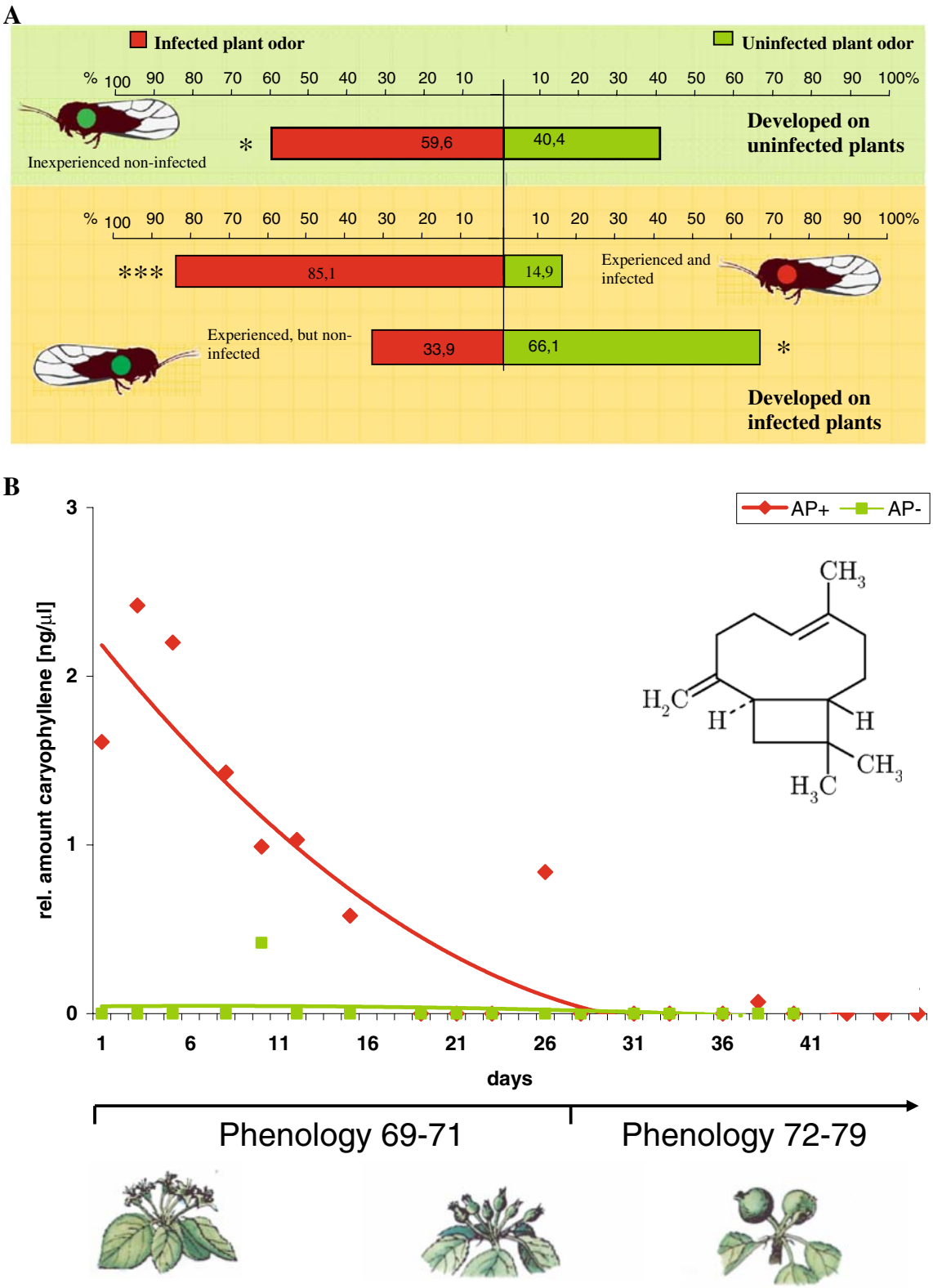

Fig. 1 **a** Olfactory preferences (%) of young adult females of *C. picta* to odors of phytoplasma infected apple vs. odors of uninfected apple. All preferences are statistically significant (dependent paired *t* test, *triple asterisk*, $P<0.001$; *asterisk*, $P<0.05$). **b** Means of relative amounts of β-caryophyllene emitted by infected plants (*red rhombs–darker gray*) compared to uninfected plants (*green squares—lighter gray*). The main emission occurs during the phenology stages 69–71 (BBCH scale: end of blooming [stage 69] until fruit diameter is about 10 mm [stage 71]) until day 28. *AP+* Apple infected by *Ca.* P. mali, *AP−* uninfected apple plant

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References

CHRISTENSEN, N. M., AXELSEN, K. B., NICOLAISEN, M., and SCHULZ, A 2005. Phytoplasmas and their interactions with hosts. *Trends Plant Sci.* 10:526–535.

DOYLE, J. J., and DOYLE, J. L. 1990. Isolation of plant DNA from fresh tissue. *Focus* 12:13–15.

FRISINGHELLI, C., DELAITI, L., GRANDO, M. S., FORTI, D., and VINDIMIAN, M. E 2000. *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. *J. Phytopathol.* 148:425–431.

GROSS, J., and MEKONEN, N. 2005. Plant odours influence the host finding behaviour of apple psyllids (*Cacopsylla picta*; *C. melanoneura*). *IOBC/WPRS Bull.* 28:351–355.

GROSS, J., SCHUMACHER, K., SCHMIDTBERG, H., and VILCINSKAS, A. 2008. Protected by fumigants: beetle perfumes in antimicrobial defense. *J. Chem. Ecol.* 34:179–188.

SEEMÜLLER, E., and SCHNEIDER, B. 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *Int. J. Syst. Evol. Microbiol.* 53:1217–1226.

SEEMÜLLER, E., GARNIER, M., and SCHNEIDER, B. 2002. Mycoplasmas of plants and insects, pp. 91–116, in S. Razin, and R. Herrmann (eds.). Molecular Biology and Pathology of MycoplasmasKluwer, London.

SMART, C. D., SCHNEIDER, B., BLOMQUIST, C. L., GUERRA, L. J., HARRISON, N. A., AHRENS, U., LORENZ, K. H., SEEMÜLLER, E., and KIRKPATRICK, B. C. 1996. Phytoplasma-specific PCR primers based on sequences of the 16S-23S rRNA spacer region. *Appl. Environ. Microbiol.* 62:2988–2993.

SOROKER, V., TALEBAEV, S., HARARI, A. R., and WESLEY, S. D. 2004. The role of chemical cues in host and mate location in the pear psylla *Cacopsylla bidens* (Homoptera: Psyllidae). *J. Insect Behav.* 17:613–626.

WEINTRAUB, P. G., and BEANLAND, L. 2006. Insect vectors of phytoplasmas. *Annu. Rev. Entomol.* 51:91–111.

plant species (Lesica and Miles 2004). Whereas beaver-caused mortality to poplar was extensive in this study (as high as 80% at some sites), beaver use of tamarisk was not observed at any of the ten study sites.

Tamarisk is considered undesirable forage because of low nutritive content and the presence of numerous polyphenolic compounds, including flavonoids, hydrolyzable and condensed tannins (Sharma and Parmar 1998). In addition, deciduous leaves of *Tamarix* species have elevated levels of sodium and salts of other alkali and alkaline earth metals present on the surface of the leaves in specialized glands (Kleinkopf and Wallace 1974). Sodium is one of many micronutrients required by herbivores. However, these nutrients are avoided when they are in excess (Villalba and Provenza 1996).

Mammalian species limit tannin intake when they lack the salivary proteins to bind tannins and render them innocuous (Hagerman and Robbins 1993). Beavers produce a single protein that binds to linear condensed tannins found in plants common to their diet (such as willow and poplar species) but not to branched condensed tannins (quebracho) or hydrolyzable tannins (Hagerman and Robbins 1993). Despite the presence of polyphenolics and highly elevated sodium chloride (NaCl) concentrations, a few mammalian herbivores are known to consume tamarisk. For example, goats can be enticed to consume tamarisk plants, and thus may represent a component of an integrated management plan for tamarisk removal (Richards and Whitesides 2006). Valley pocket gophers (*Thomomys bottae*) have been observed to forage tamarisk roots (Manning et al. 1996), and there have been anecdotal reports of beaver feeding on localized populations of tamarisk (Bergman, personal communication).

Diet preferences are strongly impacted by the post-ingestive consequences of food consumption (Provenza 1995). Phytochemicals that act on the sensory and gut systems of herbivores influence what plants or plant parts are eaten, when they are eaten, and how they are eaten. The poor palatability of tamarisk may be related, in part, to the nutritional status of the animal, hedonic qualities of the plant, and postingestive consequences that result from tannin binding of beneficial proteins. Supplements, analgesics, and masking agents can be used to minimize the antifeedant effects of tamarisk phytochemicals. For example, in humans, salty taste is reduced by addition of equimolar sucrose to salt solutions (Bartoshuk 1975). Astringency and postingestive effects of tannins can be reduced with polyethylene glycol (PEG), which irreversibly binds tannins and promotes intake of high tannin-containing plants by goats (Titus et al. 2001) and lambs (Titus et al. 2000). Accordingly, by alleviating or masking the negative attributes of tamarisk, one could promote its consumption by beavers.

Diet selection is influenced by the availability of food alternatives. The mere presence of multiple sources of the same food can drastically impact what foods are consumed and how much (Tordoff and Bachmanov 2003). Availability and nutritive quality of alternative foods influence the persistence of learned preferences (Kimball et al. 2002). Conversely, the limiting of foraging alternatives can be used to promote consumption of targeted food sources. In managed ecosystems, availability of desirable resources can be limited by exclusion (e.g., fences) and/or deterrent application (Nolte 1999). Thus, the application of an herbivore deterrent to desirable forage plants such as willow and poplar could promote tamarisk consumption by beavers.

A series of experiments was conducted with captive beavers to assess the feasibility of promoting consumption of tamarisk by altering its palatability with topical application of PEG and sugar and/or treatment of native plants with a deterrent.

Methods and Materials

Subjects Beavers were trapped in watersheds located near Olympia, WA, USA, and maintained in individual 3×5 m pens. The 1.5-m tall pen walls were clad with sheet metal. Each pen contained a 1,000-l corrugated steel water tank and insulated den box. Fresh water and a pelleted ration (Lab Diet 5012; PMI Nutrition International, Richmond, IN, USA) were provided in stainless steel food bowls. Diet enrichment consisted of apples, carrots, and dried corn on the cob. Animal procedures were approved by the National Wildlife Research Center (NWRC) Institutional Animal Care and Use Committee. All experiments were conducted during the period of June–August 2007.

Test diets Test and control diets were prepared commercially by Dyets (Bethlehem, PA, USA) with Purina Prolab RMH-1000, a commercial rat/mouse/hamster diet made from wheat, corn, and bone meal (as well as many other minor constituents, vitamins, and minerals). Maltodextrin and gum Arabic were added to both diets to confer integrity to the pellets in the wet environment anticipated in the pens. The control pellet was prepared with 15% cellulose (Dyets) (Table 1). All test pellets (approximately 45×12 mm diameter) were offered to the captive subjects in stainless steel food bowls.

Tamarisk leaves are high in tannins (Bailey et al. 2001), and sodium concentrations can far exceed 10% in plants growing in highly saline conditions (Kleinkopf and Wallace 1974). Leaf litter tannin content from *T. ramosissima* collected in Nevada, USA, was greater than 10% (Kennedy and Hobbie 2004). Thus, the tamarisk diet pellets contained

Table 1 Pelleted test diets used in two-choice tests with beavers

Ingredient	Control diet (%)	Tamarisk diet (%)
Purina RMH-1000	81	81
Cellulose	15	–
Sodium chloride (NaCl)	–	5
Quebracho tannin	–	10
Maltodextrin	2	2
Gum Arabic	2	2

10% quebracho tannin (Tannin, Peabody, MA, USA) and 5% NaCl (Aldrich Chemical, Milwaukee, WI, USA). Quebracho tannin was used in the formulation of the test diet for this study because beavers are physiologically incapable of binding branched condensed tannins with salivary proteins (Hagerman and Robbins 1993).

Plant cuttings Cuttings of black poplar (*Populus nigra*) and Scouler's willow (*Salix scouleriana*) were obtained locally near the Olympia, WA, USA, pen facilities. Tamarisk (*Tamarix ramosissima*) cuttings were collected from an invasive population located on the Saddle Mountain National Wildlife Refuge in Central Washington (USA). One meter cuttings were made from lateral branches such that the diameter at the base of the branch did not exceed 10 mm.

Cuttings were offered to captive beavers by placing them in specially constructed racks attached to the sides of each test pen. Each rack consisted of two galvanized fence top-rail tubes (5 cm diameter). The base rail was mounted 20 cm from the floor of the pen, and the upper rail 80 cm above the base rail. Twelve holes were drilled in both rails at identical locations such that the holes were spaced every 40 cm. Short lengths (7 cm) of 20 mm conduit were tack welded in the holes. One end of each cutting was placed into the conduit in the lower rail and threaded through the conduit in the upper rail. Wood dowels (15 mm diameter) were wedged into the conduit of the top rails to hold the cuttings securely in place.

Treatments Polyethylene glycol 3350 (VWR International, West Chester, PA, USA), fructose (Aldrich Chemical), and casein hydrolysate (HCA-411, American Casein, Burlington, NJ, USA) were used to treat pellets in multiple experiments. Pellets were soaked briefly in a 1.0% (*v*/*v*) solution of a latex sticker (Tactic®; Loveland Industries, Greeley, CO, USA), drained of excess sticker solution, and dusted with either casein hydrolysate (deterrent treatment) or 1:1 PEG/fructose (tamarisk treatment) at an application rate of 120 g treatment per kg pellet. Treated pellets were allowed to dry before offering to the test subjects. Plant cuttings were treated by spraying the leaves with the sticker solution and dusting with casein hydrolysate powder by hand.

Bioassay procedures Acclimation to the bioassay procedures was achieved by offering all subjects the training diet (Purina 5012 pellets) in single-choice tests commencing at 0800 hours daily. At the end of the 24-hr test period, test diets were removed and weighed to determine intake by difference and replacement made with fresh diets. Ten subjects (of 15 initially captured) that successfully acclimated to captive conditions and readily consumed the training diet for at least four consecutive days were retained for experiments 1–4. Two additional subjects were subsequently added for experiment 5.

Experiments 1–4 were each conducted for four consecutive days with position of diets (right, left) randomly assigned and alternated daily. Water was provided *ad libitum.* During intermission periods between experiments, subjects were provided *ad libitum* access to the basal diet (Lab Diets 5012) supplemented with apple, carrot, and/or dried corn. Experiments were conducted sequentially with the same test subjects.

In experiment 1, all subjects were offered the control (cellulose) and tamarisk (tannin and NaCl) diets in separate containers, whereas all subjects in experiment 2 were offered control and treated tamarisk (PEG and fructose treatment) diets in a similar two-choice test (Table 2). Daily intake (24-hr) of each diet was recorded, and preference scores were calculated to describe the proportion of tamarisk diet consumed in the two-choice tests (tamarisk diet intake divided by total intake).

All subjects in experiment 3 were offered control and deterrent-treated control (casein hydrolysate) diets while experiment 4 consisted of tamarisk (untreated) and deterrent-treated control (casein hydrolysate) diets in two-choice tests (Table 2). Preference scores were calculated from the intake data to describe the proportion of deterrent-treated control diet consumed. Mean tamarisk diet intake (g) in experiments 1, 2, and 4 was also calculated for graphical evaluation.

In experiment 5, subjects were assigned to one of two treatment groups such that training diet intake was similar between treatment groups. Two naïve subjects were included in experiment 5 (one per treatment group) for a total of 12 beavers. Subjects in the control group were

Table 2 Test diets and treatments offered to captive beavers in two-choice experiments

Experiment	Diet A	Diet B
1	Tamarisk diet	Control diet
2	PEG/fructose-treated tamarisk diet	Control diet
3	Deterrent-treated control diet	Control diet
4	Deterrent-treated control diet	Tamarisk diet

PEG: polyethylene glycol, *deterrent*: casein hydrolysate

offered four cuttings each of black poplar, Scouler's willow, and tamarisk. The 12 cuttings were randomly placed in the 12 rack locations. Subjects in the treatment group were similarly offered cuttings of the three plant species except that the black poplar and Scouler's willow cuttings were treated with casein hydrolysate. Consumption of plant cuttings was scored according to the following ordinal scale: 1, no evidence of browse; 2, sampled; 3, moderate browse; 4, completely browsed. Plant cuttings were offered for 24 hr on four consecutive days. Cuttings were replaced at the end of each 24-hr period regardless of browse activity, and new random positions were assigned daily.

Statistical analyses For each of the two-choice tests, preference scores were rank transformed within subjects so that day and diet position (right or left position of the diet referred as the proportion) were analyzed as fixed effects by analysis of variance (ANOVA). Mean preference scores for each subject were compared to values of 0.5 (indifference) for each experiment by *t*-test according to the procedures described by Willink (2005) for skewed data.

Browse scores from experiment 5 were subjected to a three-way ANOVA with treatment (control or deterrent treatment), species (willow, poplar, or tamarisk), and day considered fixed effects. Multiple comparisons of least-square means were made by controlling false discovery rate (Benjamini and Hochberg 1995). All statistical analyses were performed with SAS (Cary, NC, USA).

Results

ANOVA results from all two-choice tests indicated that neither test day nor diet position impacted diet preferences. Therefore, mean preference scores were examined to determine preferences for the test diets in experiments 1–4.

In experiment 1, the mean proportion of tamarisk diet consumed by the ten beavers was 0.46, indicating that tamarisk diet intake did not significantly differ from the control (P=0.38; Fig. 1). Treatment of the tamarisk diet with fructose and PEG did not increase the proportion of tamarisk diet consumed in experiment 2. The proportion (relative to the control diet) of treated tamarisk diet consumed was 0.39 (P=0.21; Fig. 1).

Avoidance of casein hydrolysate was demonstrated in experiment 3. The proportion (relative to the untreated control alternative) of casein hydrolysate-treated control diet was 0.024, which was significantly less than 0.5 (P< 0.001; Fig. 1). However, when the alternative food choice was tamarisk diet in experiment 4, the proportion of deterrent-treated diet consumption increased to 0.44, indicating that tamarisk diet intake did not significantly differ from the casein hydrolysate diet (P=0.19; Fig. 1). The tamarisk diet intake data from experiments 1, 2, and 4 suggest a maximum intake that could be tolerated in a 24-hr feeding period, regardless of treatment or the alternative food choice (Fig. 2).

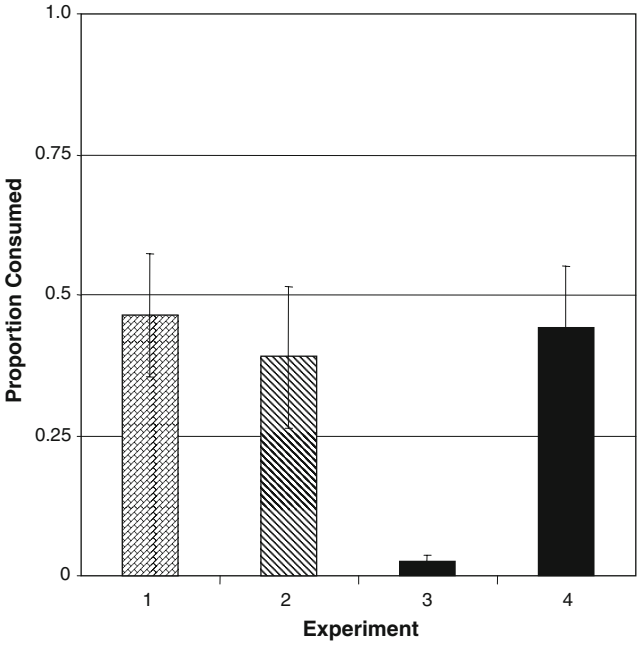

Fig. 1 Preference scores (mean+SE; N=10) for experiments 1–4 describing the proportion of test diet consumed (*crosshatched bars* untreated tamarisk diet, *diagonally hatched bars* sugar/PEG-treated tamarisk diet, *black bars* deterrent-treated control diet) in two-choice tests. The alternative diet for experiments 1–3 was untreated control. The alternative for experiment 4 was untreated tamarisk diet. A preference score of 0.5 indicates indifference. Results of *t*-tests indicated that only the casein hydrolysate preference score in experiment 3 was significantly less than 0.5 (P<0.001)

ANOVA results from experiment 5 demonstrated that browse score (i.e., preference for plant cuttings) was a function of test day (P=0.018), plant species (P<0.0001), and the species×treatment interaction (P=0.001). Browse scores were lower on day 4 of the test vs. days 1 or 3. Among the control group, foraging preference for the plants followed the order: willow>poplar>tamarisk (Fig. 3). However, deterrent treatment of willow and poplar promoted increased browsing of tamarisk and resulted in no significant preferences among the three species (Fig. 3).

Discussion

Herbivore preferences for various plant species are a function of maximizing nutritional benefits while at the same time limiting intake of plant secondary metabolites. For example, beavers strongly preferred aspen (*Populus tremuloides*) over red maple (*Acer rubrum*) in cafeteria tests despite having similar energy, protein, and fiber values (Doucet and Fryxell 1993). Differences in the palatability

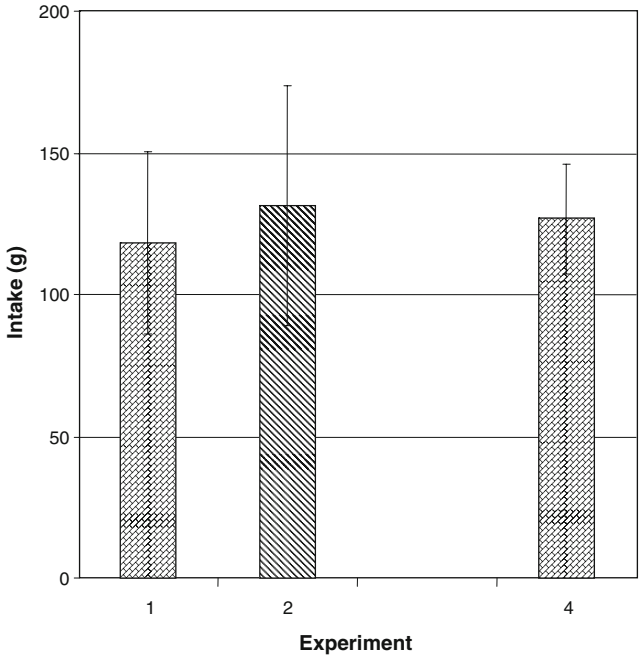

Fig. 2 Tamarisk diet intake (g) from experiments 1, 2, and 4 (mean+SE; N=10). The alternative diet for experiments 1 and 2 was untreated control diet. The alternative diet in experiment 4 was deterrent (casein hydrolysate)-treated control diet (*crosshatched bars* untreated tamarisk diet, *diagonally hatched bars* sugar/PEG-treated tamarisk diet)

of these species were a consequence of phenolic content, not absolute nutritional quality (Muller-Schwarze et al. 1994). Tamarisk can contribute to the nutritional needs of herbivores capable of tolerating the phenolics and NaCl present in the foliage. On a dry matter basis, tamarisk foliage contains approximately 13 kJ/g digestible energy, 21% crude protein, and 16% crude fiber (Kimball, unpublished data).

Tamarisk diet constituted one-half of the total consumption by beavers in experiment 1. Treatment with fructose (to suppress salt flavor) and PEG (to bind tannins) did not promote greater consumption of tamarisk diet in experiment 2. The lack of day effect in these experiments indicates that tamarisk diet intake limitation was not a function of conditioned aversion. It is possible that the high osmolality of the tamarisk diet contributed to fixing a maximum allowable intake (Fig. 2). Although mammals are well-equipped to excrete excess sodium with sufficient water consumption, NaCl satiety can result from hyperosmolality and/or dehydration (Stricker and Verbalis 1991).

It is unclear why treatment with PEG in experiment 2 did not promote greater consumption of the tamarisk diet via tannin binding. It is possible that the PEG concentration was not sufficient for the tannin content of the tamarisk diet. Beavers demonstrated the ability to consume significant quantities of tamarisk diet in the absence of PEG treatment despite lacking salivary proteins to bind quebracho tannin (Hagerman and Robbins 1993). Similarly, goats (also incapable of binding quebracho tannins) consumed significant quantities of tannin-containing foods when given experience with the food (Distel and Provenza 1991). In another study, the alteration of exposure to tannin-containing foods with nontannin foods on an every-other-day basis permitted goats to consume greater quantities of tannin diets vs. subjects offered tannin diet exclusively (Kimball and Nolte 2005). Thus, the presence of a tannin-free alternative food in the two-choice tests may have allowed beavers to maximize tannin intake.

In addition to physiological processes for tolerating phytochemicals (e.g., salivary proteins for tannin binding), herbivores can employ a variety of behavioral approaches to manipulate forage palatability. For example, beavers have been observed to soak branches in water, thus leaching phenolics that contribute to poor palatability (Muller-Schwarze et al. 2001). Diet mixing is another method herbivores employ that minimizes consumption of specific plant toxins and antifeedants (Freeland and Janzen 1974). This is a particularly efficient strategy when the various phytochemicals encountered are complimentary (Burritt and Provenza 2000). Toxic phytochemicals are considered complimentary when they act on different physiological systems in the herbivore—allowing the animal to consume greater quantities of the different foods in total than they could of any individual food item. Limiting intake of a

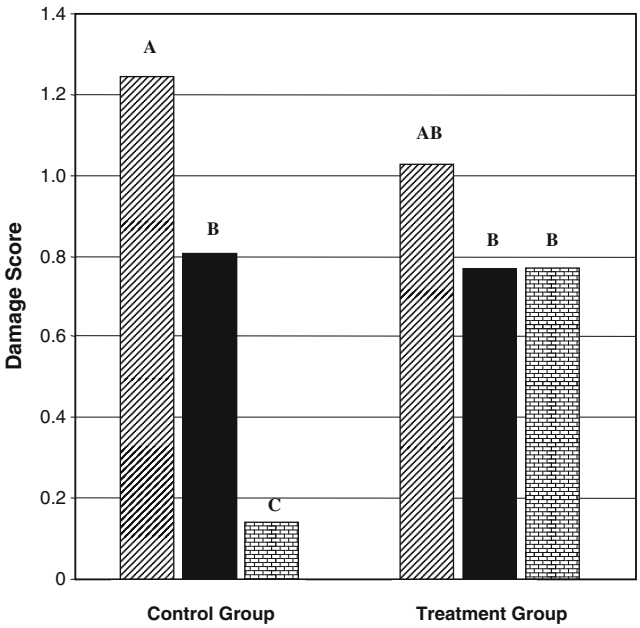

Fig. 3 Species×treatment interaction from the three-way ANOVA of browse scores demonstrates treatment effect on preferences of beavers (N=12) for plant cuttings in cafeteria tests (P=0.001). Casein hydrolysate was applied topically to willow and poplar cuttings in the treatment group. *Different letters* indicate significant differences among damage scores (multiple comparisons) made according to the method of Benjamini and Hochberg 1995) (*diagonally hatched bars S. scouleriana*, *black bars P. nigra*, *crosshatched bars T. ramosissima*)

particular food or phytochemical is not the only regulatory process used by herbivores while foraging. Although few studies have specifically monitored meal patterns, a recent study with ringtail possum demonstrated that intake rate and feeding time are altered in addition to total intake, thus regulating the consumption of deleterious phytochemicals (Wiggins et al. 2006).

Beavers strongly avoided the casein hydrolysate treatment when the control diet was the alternative food (experiment 3; Fig. 1). Avoidance of casein hydrolysate is predictable for most herbivores. Deer (Kimball et al. 2005), mountain beavers, pocket gophers (Figueroa et al. 2008), and guinea pigs (Field, personal communication) have shown strong avoidance responses to casein hydrolysate in single-choice tests. Beavers also avoid plants treated with commercial herbivore deterrents that contain egg or blood products (DuBow 2000).

When tamarisk diet was the alternative food, beavers no longer avoided the casein hydrolysate treatment (experiment 4, Fig. 1). Ironically, protein binding may have contributed to the reduction of casein hydrolysate repellency via diet mixing. Although PEG–tannin binding had no influence on tamarisk diet palatability in experiment 2, tannin–protein binding may have rendered casein hydrolysate more palatable in experiment 4. Self-medicating behavior (in this case, consumption of tannin-containing tamarisk diet that renders the deterrent-treated control diet more palatable) is common among herbivores. In fact, self-regulated PEG intake is the mechanism that makes its use practical in arid rangelands to promote consumption of high-tannin forages (Titus et al. 2000, 2001).

The cafeteria test with plant cuttings (experiment 5) demonstrated that willow was the preferred tree species tested (Fig. 3). In the absence of a deterrent treatment, there was little damage recorded to tamarisk cuttings. Because plant cuttings were not available *ad libitum*, use of tamarisk by the control group was likely influenced by unavailability of the preferred plants as they were exhausted. Deterrent treatment of willow and cottonwood with casein hydrolysate similarly reduced the availability of preferred plants, resulting in a significant increase of tamarisk use (Fig. 3).

Results of this study suggest that deterrent treatment of desirable plant species in wetland areas will facilitate foraging of invasive plants by beavers, including tamarisk. However, maximum intake of tamarisk may be regulated by processes that cannot be circumvented by PEG treatment or supplementation. It is important to emphasize that increased tamarisk consumption by beavers in natural areas holds no promise of tamarisk eradication. Rather, significant use of tamarisk by beavers may decrease the removal of native species and allow natural resources managers to meet management goals in the presence of these efficient ecosystem engineers.

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References

BAILEY, J. K., SCHWEITZER, J. A., and WHITHAM, T. G. 2001. Salt cedar negatively affects biodiversity of aquatic macroinvertebrates. *Wetlands* 21:442–447.

BARTOSHUK, L. M. 1975. Taste mixtures—Is mixture suppression related to compression. *Physiol. Behav.* 14:643–649.

BENJAMINI, Y., and HOCHBERG, Y. 1995. Controlling the false discovery rate—A practical and powerful approach to multiple testing. *J. R. Stat. Soc. B Stat. Methodol.* 57:289–300.

BURRITT, E. A., and PROVENZA, F. D. 2000. Role of toxins in intake of varied diets by sheep. *J. Chem. Ecol.* 26:1991–2005.

BUSCH, D. E., and SMITH, S. D. 1995. Mechanisms associated with decline of woody species in riparian ecosystems of the Southwestern US. *Ecol. Monogr.* 65:347–370.

DISTEL, R. A., and PROVENZA, F. D. 1991. Experience early in life affects voluntary intake of blackbrush by goats. *J. Chem. Ecol.* 17:431–450.

DOUCET, C. M., and FRYXELL, J. M. 1993. The effect of nutritional quality on forage preference by beavers. *Oikos* 67:201–208.

DUBOW, T. J. 2000. Reducing beaver damage to habitat restoration sites using less palatable tree species and repellents. M.S. thesis. Utah State University, Logan, UT.

FIGUEROA, J. A., KIMBALL, B. A., and PERRY, K. R. 2008. Lagomorph and rodent responses to two protein hydrolysates. *Crop Prot.* 27:851–854.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory by mammals—Role of plant secondary compounds. *Am. Nat.* 108:269–289.

HAGERMAN, A. E., and ROBBINS, C. T. 1993. Specificity of tannin-binding salivary proteins relative to diet selection by mammals. *Can. J. Zool.* 71:628–633.

KENNEDY, T. A., and HOBBIE, S. E. 2004. Saltcedar (*Tamarix ramosissima*) invasion alters organic matter dynamics in a desert stream. *Freshw. Biol.* 49:65–76.

KIMBALL, B. A., and NOLTE, D. L. 2005. Herbivore experience with plant defense compounds influences acquisition of new flavor aversions. *Appl. Anim. Behav. Sci.* 91:17–34.

KIMBALL, B. A., NOLTE, D. L., and PERRY, K. B. 2005. Hydrolyzed casein reduces browsing of trees and shrubs by white-tailed deer. *HortScience* 40:1810–1814.

KIMBALL, B. A., PROVENZA, F. D., and BURRITT, E. A. 2002. Importance of alternative foods on the persistence of flavor aversions: Implications for applied flavor avoidance learning. *Appl. Anim. Behav. Sci.* 76:249–258.

KLEINKOPF, G. E., and WALLACE, A. 1974. Physiological basis for salt tolerance in *Tamarix ramosissima*. *Plant Sci. Lett.* 3:157–163.

LESICA, P., and MILES, S. 2004. Beavers indirectly enhance the growth of russian olive and tamarisk along eastern Montana rivers. *West. N. Am. Nat.* 64:93–100.

MANNING, S. J., CASHORE, B. L., and SZEWCZAK, J. M. 1996. Pocket gophers damage saltcedar (*Tamarix ramosissima*) roots. *Great Basin Nat.* 56:183–185.

MARTELL, K. A., FOOTE, A. L., and CUMMING, S. G. 2006. Riparian disturbance due to beavers (*Castor canadensis*) in Alberta's boreal mixedwood forests: Implications for forest management. *Ecoscience* 13:164–171.

MULLER-SCHWARZE, D., BRASHEAR, H., KINNEL, R., HINTZ, K. A., LIOUBOMIROV, A., and SKIBO, C. 2001. Food processing by animals: Do beavers leach tree bark to improve palatability? *J. Chem. Ecol.* 27:1011–1028.

MULLER-SCHWARZE, D., SCHULTE, B. A., SUN, L. X., MULLER-SCHWARZE, A., and MULLER-SCHWARZE, C. 1994. Red maple (*Acer rubrum*) inhibits feeding by beaver (*Castor canadensis*). *J. Chem. Ecol.* 20:2021–2034.

NOLTE, D. L. 1999. Behavioral approaches for limiting depredation by wild ungulates, pp. 60–69, in K. L. Launchbaugh, D. Sanders, and J. C. Mosely (eds.). Grazing Behavior of Livestock and WildlifeUniversity of Idaho, Moscow, Idaho.

NOLTE, D. L., LUTMAN, M. W., BERGMAN, D. L., ARJO, W. M., and PERRY, K. R. 2003. Feasibility of non-lethal approaches to protect riparian plants from foraging beavers in North America, pp. 75–79, in G. R. Singleton, L. A. Hinds, C. J. Krebs, and D. M. Spratt (eds.). Rats, Mice, and People: Rodent Biology and ManagementAustralian Centre for International Agriculture Research, Canberra, Australia.

PROVENZA, F. D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. *J. Range Manag.* 48:2–17.

RICHARDS, R. and WHITESIDES, R. E. 2006. Tamarisk control by grazing with goats compared to herbicides. Abstract, *Tamarix* Research Conference: Current Status and Future Directions, Fort Collins, CO, 3–4 October.

SHARMA, S. K., and PARMAR, V. S. 1998. Novel constituents of *Tamarix* species. *J. Sci. Ind. Res.* 57:873–890.

STRICKER, E. M., and VERBALIS, J. G. 1991. Caloric and noncaloric controls of food-intake. *Brain Res. Bull.* 27:299–303.

STURTEVANT, B. R. 1998. A model of wetland vegetation dynamics in simulated beaver impoundments. *Ecol. Model.* 112:195–225.

TITUS, C. H., PROVENZA, F. D., PEREVOLOTSKY, A., and SILANIKOVE, N. 2000. Preferences for foods varying in macronutrients and tannins by lambs supplemented with polyethylene glycol. *J. Anim. Sci.* 78:1443–1449.

TITUS, C. H., PROVENZA, F. D., PEREVOLOTSKY, A., SILANIKOVE, N., and ROGOSIC, J. 2001. Supplemental polyethylene glycol influences preferences of goats browsing blackbrush. *J. Range Manag.* 54:161–165.

TORDOFF, M. G., and BACHMANOV, A. A. 2003. Mouse taste preference tests: Why only two bottles? *Chem. Senses* 28:315–324.

VILLALBA, J. J., and PROVENZA, F. D. 1996. Preference for flavored wheat straw by lambs conditioned with intraruminal administrations of sodium propionate. *J. Anim. Sci.* 74:2362–2368.

WIGGINS, N. L., MARSH, K. J., WALLIS, I. R., FOLEY, W. J., and MCARTHUR, C. 2006. Sideroxylonal in *Eucalyptus* foliage influences foraging behaviour of an arboreal folivore. *Oecologia* 147:272–279.

WILLINK, R. 2005. A confidence interval and test for the mean of an asymmetric distribution. *Commun. Stat. Theory Methods* 34:753–766.

led to the discovery of sex attractants for more than 90 species in highly evolved families (Ando 2008; El-Sayed 2008).

In Type II pheromone components, further structural modification of the dietary fatty acids is expected because of the species diversity in the families such as the Arctiidae and Geometridae. The 6,9-dienes, 3,6,9-trienes, and their epoxy derivatives might be insufficient to produce blends for the many species in these families. In fact, the first pheromone identified from a geometrid species was a 1,3,6,9-tetraene (Bestmann et al. 1982; Roelofs et al. 1982). In addition, hydrocarbons unsaturated at the 11-position and their epoxy derivatives have been characterized as pheromone components in some species (Ando et al. 2004). Since the pheromone content in a gland is quite low, it is not easy to determine the structure of these compounds even when modern analytical instruments are used. In pheromone studies, synthetic pheromone candidates generally play an important role; for example, they are used as the authentic standard in gas chromatography-mass spectrometry (GC-MS) analyses. Identification can be accomplished by comparing the retention time (Rt) values as well as the mass spectra of natural and synthetic compounds. If the synthetic standard is unavailable and a direct comparison is impossible, the published diagnostic ions would contribute to the analysis of a natural pheromone.

We have studied Type II pheromones by utilizing synthetic 6,9-dienes and 3,6,9-trienes (Ando et al. 2004). However, because the chemical data of other polyunsaturated hydrocarbons are still limited, in this study we synthesized the compounds with a 6,9,11-, 3,6,9,11-, or 1,3,6,9-polyene system in a C_{17}–C_{21} chain and report on their spectroscopic data. For the former two polyenes, we targeted geometrical isomers with an (*E*)-double bond at the 11-positon, since preparation was easily accomplished by a Wittig reaction utilizing (*E*)-2-alkenals. Furthermore, we carried out random screening tests of synthetic polyenes in the field to find new sex attractants for the Lepidoptera.

Methods and Materials

Analytical Instruments ^{1}H and ^{13}C NMR spectra were recorded by a Jeol Alpha 500 Fourier transform spectrometer (JEOL Ltd., Tokyo, Japan) at 500.2 and 125.7 MHz, respectively, for $CDCl_3$ solutions containing tetramethylsilane (TMS) as an internal standard. ^{1}H–^{1}H COSY, HSQC, and HMBC spectra were also measured with the same spectrometer with the usual pulse sequences and parameters. GC-MS was conducted in the EI mode with an HP 5973 mass spectrometer (Hewlett-Packard, Wilmington, DE, USA) equipped with a DB-23 capillary column (0.25 mm ID×30 m, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). The column temperature program was 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C. A split/splitless injector was used in the splitless mode for 1 min and kept at 220°C. The ionization voltage was 70 eV, and the flow rate of the carrier gas (He) was 1.0 ml/min. IR spectra were recorded as a thin film (neat liquid) with a Jasco FT/IR-350 (JASCO Corporation).

Chemicals The chemical structures of polyunsaturated hydrocarbons are abbreviated as follows: Z = (*Z*)-double bond, *E* = (*E*)-double bond, number before the hyphen = position of the double bond, number after the hyphen = carbon number of the straight chain, and H = compound without a terminal functional group. The trienes and tetraenes with a C_{17}–C_{21} chain were prepared by the following procedures (Fig. 1).

*1-Iodo-(Z)-3-nonene (***2a***)* Solutions of triphenylphosphine (4.5 g, 17 mmol) and imidazole (1.2 g, 17 mmol) with dry

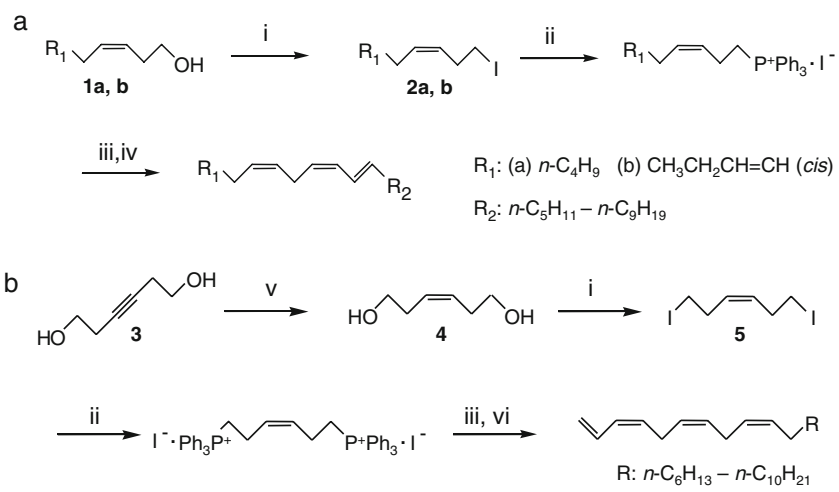

Fig. 1 Synthesis of polyunsaturated hydrocarbons with a C_{17}–C_{21} straight chain. (**a**) (*Z,Z,E*)-6,9,11-trienes and (*Z,Z,Z,E*)-3,6,9,11-tetraenes, and (**b**) (*Z,Z,Z*)-1,3,6,9-tetraenes. *i*, I_2-PPh_3/imidazole; *ii*, PPh_3/benzene; *iii*, $NaN(SiMe_3)_3$/THF; *iv*, R_2CH=CHCHO (*E*); *v*, H_2/Pd-$BaSO_4$/quinoline; and *vi*, acrolein and RCH_2CHO

ether (45 ml) and CH_3CN (15 ml), I_2 (4.4 g, 17 mmol) were mixed by stirring at 0°C under N_2 gas, and the mixture was warmed to room temperature. After 15 min of stirring, the solution was cooled again to 0°C and added dropwise to (*Z*)-3-nonen-1-ol (**1a**, 2.0 g, 14 mmol) dissolved in dry ether (3 ml). After 2 hr of stirring at room temperature, the precipitates were filtered. The eluent was poured into water, and crude products were extracted with hexane, washed with an aqueous solution of $Na_2S_2O_3$, and dried with Na_2SO_4. After evaporation of the extract, the residue was chromatographed on a silica gel column to give **2a** (3.2 g, 12 mmol) in 86% yield. ^{1}H NMR (δ, ppm): 0.89 (3H, t, *J*= 7 Hz), ~1.3 (6H, m), 2.02 (2H, td, *J*=7, 7 Hz), 2.63 (2H, td, *J*=7, 7 Hz), 3.13 (2H, t, *J*=7 Hz), 5.31 (1H, dtt, *J*=11, 7, 1.5 Hz), 5.53 (1H, dtt, *J*=11, 7, 1.5 Hz). ^{13}C NMR (*δ*, ppm): 5.5, 14.1, 22.5, 27.4, 29.2, 31.49, 31.53, 127.7, 132.7. IR (λ_{max} cm^{-1}): 3008, 2955, 2925, 2855, 1464, 1241, 1168, 725.

*1-Iodo-(3Z,6Z)-3,6-nonadiene (***2b***)* By the same procedure for **2a**, dienyl iodide (**2b**) was synthesized from (*Z,Z*)-3,6-nonadien-1-ol (**1b**) in 88% yield. ^{1}H NMR (*δ*, ppm): 0.98 (3H, t, *J*=7.5 Hz), 2.07 (2H, td, *J*=7.5, 7.5 Hz), 2.66 (2H, td, *J*=7.5, 7.5 Hz), 2.78 (2H, dd, *J*=7.5, 7.5 Hz), 3.14 (2H, t, *J*=7.5 Hz), 5.30 (1H, dtt, *J*=11, 7.5, 1.5 Hz), 5.35 (1H, dtt, *J*=11, 7.5, 1.5 Hz), 5.40 (1H, dtt, *J*=11, 7.5, 1.5 Hz), 5.52 (1H, dtt, *J*=11, 7.5, 1.5 Hz). ^{13}C NMR (*δ*, ppm): 5.2, 14.2, 20.6, 25.7, 31.4, 126.3, 127.8, 130.6, 132.1. IR (λ_{max} cm^{-1}): 3053, 3010, 2962, 2929, 1655, 1433, 1240, 1169, 742, 696, 501.

(E)-2-Alkenals (*E*)-2-Octenal and (*E*)-2-nonenal were purchased from Sigma-Aldrich Co. (Milwaukee, WI, USA). (*E*)-2-Decenal, (*E*)-2-undecenal, and (*E*)-2-dodecenal were synthesized from octanal, nonanal, and decanal, respectively, by an established method including the following three steps (Nishida et al. 2003); a coupling reaction with methoxycarbonylmethylenetriphenylphosphorane, reduction with $LiAl(OC_2H_5)_2H_2$, and oxidation with pyridinium chlorochromate. The following nuclear magnetic resonance (NMR) data were commonly obtained for the (*E*)-2-alkenals. ^{1}H NMR (*δ*, ppm): 9.50 (1H, d, *J*=8 Hz, C-1), 6.12 (1H, dd, *J*=15.5, 8 Hz, C-2), 6.86 (1H, dt, *J*=15.5, 7 Hz, C-3), 2.33 (2H, dt, *J*=7, 7 Hz, C-4). ^{13}C NMR (*δ*, ppm): 194.2 (C-1), 133.0 (C-2), 159.0 (C-3), 32.7 (C-4).

(6Z,9Z,11E)-6,9,11-Trienes and (3Z,6Z,9Z,11E)-3,6,9,11-Tetraenes A mixture of **2a** (250 mg, 1.0 mmol), triphenylphosphine (260 mg, 1.0 mmol), and dry benzene (10 ml) was heated under refluxing for 12 hr. After cooling, phosphonium salt was washed with benzene. The salt dissolved in dry tetrahydrofuran (THF, 5 ml) and hexamethylphosphoramide (HMPA, 0.2 ml) was stirred at −35°C under Ar gas, and then $NaN(SiMe_3)_2$ (1 M in THF, 1.0 ml) was added dropwise to the solution to make an ylide. After stirring at room temperature for 30 min, the ylide solution was cooled to −35°C again, and (*E*)-2-octenal (150 mg, 1.2 mmol) in THF (2.0 ml) was added dropwise to it to couple with the ylide. The mixture was further stirred at room temperature for 1 hr and poured into water. Crude products were extracted with hexane, washed successively with 1 N HCl and a saturated aqueous solution of $NaHCO_3$, and then chromatographed on a silica gel column impregnated with $AgNO_3$ with hexane and mixtures of hexane and benzene as eluents. Z6,Z9,E11-17:H (180 mg, 0.77 mmol, >95% purity) was obtained in 76% yield. Other 6,9,11-trienes (>95% purity) with a C_{18}–C_{21} chain were prepared with (*E*)-2-alkenal with a C_9–C_{12} chain in the same manner and more than 60% yield. The 3,6,9,11-tetraenes (>95% purity) were synthesized by the same coupling reaction between the ylide derived from dienyl iodide (**2b**) and (*E*)-2-alkenal with an appropriate carbon chain in the similar yield.

*(Z)-3-Hexene-1,6-diol (***4***)* Pb-$BaSO_4$ (200 mg) and quinoline (10 mg) were added to 3-hexyne-1,6-diol (**3**, 2.5 g, 22 mmol) dissolved in methanol (20 ml). The mixture was stirred at room temperature under positive pressure of H_2 gas for 1 hr. The reaction mixture was filtered, and the eluent was concentrated *in vacuo* to give the olefinic compound (**4**, 2.3 g, 20 mmol) in 91% yield. ^{1}H NMR (*δ*, ppm): 2.31 (4H, td, *J*=7, 4.5 Hz), 3.56 (4H, t, *J*=7 Hz), 5.49 (2H, t, *J*=4.5 Hz). ^{13}C NMR (*δ*, ppm): 31.6, 62.5, 129.8. IR (λ_{max} cm^{-1}): 3338, 3012, 2952, 2879, 1707, 1425, 1049, 723.

*1,6-Diiodo-(Z)-3-hexene (***5***)* The diol (**4**, 2.3 g, 20 mmol) was transferred to diiodide (**5**) by the same procedure for **2a** with two equivalent amounts of the reagents and solvents. The crude products were chromatographed on a silica gel column to give **5** (5.4 g, 16 mmol) in 80% yield. ^{1}H NMR (*δ*, ppm): 2.64 (4H, td, *J*=7, 4.5 Hz), 3.17 (4H, t, *J*=7 Hz), 5.50 (2H, t, *J*=4.5 Hz). ^{13}C NMR (*δ*, ppm): 4.9, 31.5, 130.4. IR (λ_{max} cm^{-1}): 3008, 2954, 1421, 1240, 1169, 717.

(3Z,6Z,9Z)-1,3,6,9-Tetraenes Phosphonium salt of diiodide (**5**) was prepared in the same manner as the salt of **2a**. The salt of **5** (1.3 g, 1.5 mmol) in dry THF (10 ml) and HMPA (1.0 ml) was mixed with $NaN(SiMe_3)_2$ (1 M in THF, 3.0 ml) at −80°C under Ar gas to make bis(ylide), which was further treated with a mixture of acrolein (84 mg, 1.5 mmol) and octanal (190 mg, 1.5 mmol) dissolved in THF (2.0 ml). After stirring at room temperature for 3 hr, the crude products were extracted with hexane, cleaned by the usual workup, and chromatographed on a silica gel column impregnated with $AgNO_3$ using hexane and mixtures of

hexane and benzene as eluents. 1,Z3,Z6,Z9-17:H (74 mg, 0.32 mmol, >95% purity) was obtained in 21% yield. Using the phosphonium salt of **5**, other 1,3,6,9-tetraenes with a C_{18}–C_{21} chain (>95% purity) were prepared by treatment with a mixture of acrolein and an aldehyde with a C_9–C_{12} chain in about 20% yield.

Field Tests In 2004 and 2005, synthetic polyenes were tested for behavioral activity at a mixed forest area in the suburbs of Tokyo (Rolling Land Laboratory, Tokyo University of Agriculture and Technology, Hachioji-shi, Tokyo) and on the Iriomote Islands. Rubber septa (white rubber, o.d. 8 mm, Sigma-Aldrich, St. Louis, MO, USA) were used as dispensers, and 1 mg of each compound dissolved in hexane (100 μl) was applied to them. Each lure was placed at the center of a sticky board trap (30×27 cm bottom plate with a roof, Takeda Chem. Co., Tokyo, Japan), which was set separately by at least 10 m at about 1.5 m above the ground. Two traps were used for each synthetic polyene, and two other traps included septa treated with 100 μl hexane as controls. Field evaluation of each lure was carried out for at least 12 mo. The lures were renewed every 3 mo, and the number of captured males was counted every 2 wk.

Results

Synthesis (*Z,Z,E*)-6,9,11-Trienes and (*Z,Z,Z,E*)-3,6,9,11-tetraenes were synthesized starting from (*Z*)-3-nonen-1-ol (**1a**) and (*Z,Z*)-3,6-nonadien-1-ol (**1b**), respectively, (Fig. 1a). After conversion of these alcohols into iodides (**2a** and **b**), their phosphonium salts were prepared. The ylide derived from each salt was treated with (*E*)-2-alkenal with a C_8–C_{12} chain to yield the objective C_{17}–C_{21} polyunsaturated hydrocarbons. Since HMPA was used as a co-solvent for the Wittig reaction, the production of the undesired (*Z,E,E*)- or (*Z,Z,E,E*)-isomers was restrained under 10%. Finally, column chromatography with silica gel impregnated with $AgNO_3$ gave the pure polyenes. (*Z,Z,Z*)-1,3,6,9-Tetraenes were synthesized by modifying the method reported by Pohnert and Boland (2000) (Fig. 1b). Restricted hydrogenation of 3-hexyn-1,6-diol (**3**) (Eya et al. 1990) gave (*Z*)-3-hexen-1,6-diol (**4**), which was halogenated to 1,6-diiodo-(*Z*)-3-hexene (**5**). Bis(ylide) was prepared from **5** after preparation of the phosphonium salt and coupled with acrolein and an alkanal with a C_8–C_{12} chain to yield the objective tetraenes with a C_{17}–C_{21} chain. In addition to targeting the (*Z*)-tetraenes, this one-pot double-Wittig reaction produced undesired geometrical isomers with an *E* configuration and symmetric products that included two molecules of acrolein or alkanal. The pure tetraenes were obtained after the chromatography of silica gel impregnated with $AgNO_3$.

NMR Analysis The structures of synthetic polyunsaturated hydrocarbons were confirmed by NMR analysis. The ^1H and ^{13}C signal assignments of the polyenes with a C_{19} chain were achieved by two-dimensional experiments (Table 1). The configurations at the conjugated dienyl part of each compound were revealed by coupling constants between two vicinal olefinic protons, i.e., $J_{9,10}$ (11 Hz) and $J_{11,12}$ (15–15.5 Hz) of Z6,Z9,E11-19:H and Z3,Z6,Z9,E11-19:H, and $J_{3,4}$ (11 Hz) of 1,Z3Z6,Z9-19:H. The *J* values of the (*E*)-double bonds were larger than those of the (*Z*)-double bonds. The (*Z*)-double bond at the 6-position of Z6,Z9,E11-19:H and Z3,Z6,Z9,E11-19:H was also confirmed by $J_{6,7}$ (10.5–11 Hz). The configurations of the other double bonds at the 3-position of Z3,Z6,Z9,E11-19:H and the 6- and 9-positions of 1,Z3Z6,Z9-19:H were not determined by the ^1H NMR analysis because the signals of these olefinic protons overlapped with each other; however, the chemical shifts of their allylic carbons indicated their Z configurations. The allylic carbons of a (*Z*)-double bond resonate in a higher field (about 5 ppm) than those of an (*E*)-double bond (Ando et al. 1983), and the carbon signal at the 2-position of (*Z,Z,Z*)-3,6,9-trienes appeared at 20.6 ppm, whereas those at the doubly allylic 5- and 8-positions appeared at 25.6 ppm (Ando et al. 1993). The chemical shifts of the allylic carbons (Table 1) indicate that all of the unconjugated double bonds have Z configurations. Furthermore, the dienyl parts in the ^{13}C spectra of the (*Z,Z,E*)-6,9,11-trienes and (*Z,Z,Z,E*)-3,6,9,11-tetraenes are similar to a (*Z,E*)-isomer of conjugated dienes and those of the (*Z,Z,Z*)-1,3,6,9-tetraenes are similar to a (*Z*)-isomer of terminal conjugated dienes, reflecting the characteristic common structures (Ando et al. 1983).

GC-MS Analysis Analysis of each synthetic polyene (GC-MS equipped with a DB-23 column) yielded retention times (Rt) and Kovat's retention indices (RI) of the polyenes with a C_{17}, C_{19}, or C_{21} chain (Table 2). The effluent from the polar column is regularly delayed as the number of double bonds increases. The RI values of the (*Z,Z*)-6,9-dienes increase by about 55 with the introduction of one unconjugated (*Z*)-double bond and by about 130 with that of one conjugated (*E*)-double bond. In the case of (*Z,Z,Z*)-3,6,9-trienes, an increment of 120–130 was observed by desaturation to the tetraenes. The introduction of one conjugated double bond at the 11-position affected the effluent more strongly than that of one at the 1-position. The intensity of M^+ of the 6,9,11-trienes was remarkably larger than that of the 3,6,9,11- and 1,3,6,9-tetraenes (Table 3). A series of spectra measured with the polyenes with a different carbon chain was analyzed in reference to published spectral data of the 6,9-dienes and 3,6,9-trienes (Ando et al. 1993; Millar 2000), and some diagnostic fragment ions were proposed for each polyene (Fig. 2).

Table 1 ^{1}H and ^{13}C NMR assignments for C_{19} polyenes

Position	Chemical shift (δ, ppm)					
	^{1}H NMR			^{13}C NMR		
	Z6,Z9,E11-Triene[a]	Z3,Z6,Z9,E11-Tetraene[b]	1,Z3,Z6,Z9-Tetraene[c]	Z6,Z9,E11-Triene[d]	Z3,Z6,Z9,E11-Tetraene[d]	1,Z3,Z6,Z9-Tetraene[d]
1	0.89	0.98	5.11, 5.20	14.1	14.3	117.4
2	~1.28	2.08	6.67	22.5	20.6	132.0
3	~1.28	~5.39	6.02	31.7	131.8	129.3
4	~1.3	~5.39	~5.39	~29	126.8	130.4
5	2.07	2.83	2.97	27.2	25.5	26.1
6	5.41[e]	5.32[f]	~5.39	130.3[g]	128.4[h]	127.3[i]
7	5.35[e]	~5.39[f]	~5.39	127.3[g]	127.6[h]	129.0[i]
8	2.91	2.94	2.81	26.0	26.0	25.7
9	5.26	5.27	~5.39	127.6	127.2	127.4
10	5.96	5.97	~5.39	128.5	128.7	130.6
11	6.32	6.33	2.05	125.2	125.1	27.3
12	5.69	5.70	~1.27	135.1	135.3	~29.5
13	2.09	2.10	~1.27	32.9	32.9	~29.5
19	0.89	0.89	0.88	14.1	14.1	14.1

[a] $J_{1,2}$=7.5 Hz, $J_{5,6}$=7 Hz, $J_{6,7}$=11 Hz, $J_{7,8}$=7 Hz, $J_{8,9}$=7 Hz, $J_{9,10}$=11 Hz, $J_{10,11}$=11 Hz, $J_{11,12}$=15 Hz, $J_{12,13}$=7 Hz.
[b] $J_{1,2}$=7.5 Hz, $J_{2,3}$=7 Hz, $J_{4,5}$=6.5 Hz, $J_{5,6}$=6.5 Hz, $J_{6,7}$=10.5 Hz, $J_{7,8}$=6.5 Hz, $J_{8,9}$=6.5 Hz, $J_{9,10}$=11 Hz, $J_{10,11}$=11 Hz, $J_{11,12}$=15.5 Hz, $J_{12,13}$=7 Hz.
[c] $J_{1,2}$=17, 10 Hz, $J_{2,3}$=11 Hz, $J_{3,4}$=11 Hz, $J_{4,5}$=6.5 Hz, $J_{5,6}$=6.5 Hz, $J_{7,8}$=6.5 Hz, $J_{8,9}$=6.5 Hz, $J_{10,11}$=7 Hz.
[d] C(17) 31.9 ppm, C(18) 22.7 ppm.
[e–i] Chemical shift values may be reversed.

While the polyenes synthesized in this study universally showed a base peak at *m/z* 79, the structure of each compound was estimated by different diagnostic ions at the following values: *m/z* 110, 163, and M-85 of the 6,9,11-trienes, *m/z* 108 and M-82 of the 3,6,9,11-tetraenes, and *m/z* 91, 106, and M-54 of the 1,3,6,9-tetraenes. These ions would help to identify natural components in a pheromone extract.

Attraction of Lepidopteran Males In field screening tests of the synthetic polyenes, the compounds were presented as single component lures. Four species in the family of Geometridae were attracted in a forest in Tokyo: *Culpinia diffusa* Walker by Z6,Z9,E11-17:H, *Wilemania nitobei* Nitobe by Z3,Z6,Z9,E11-19:H, *Inurois fletcheri* Inoue by Z3,Z6,Z9,E11-20:H, and *Operophtera rectipostmediana* Inoue by 1,Z3,Z6,Z9-19:H (Table 4). In the Iriomote

Table 2 Retention times (Rt) and Kovát's retention indices (RI) of synthetic polyenes from GC-MS analysis with a DB-23 column (0.25 mm× 30 m)[a]

Unsaturation	Chain length					
	C_{17}		C_{19}		C_{21}	
	Rt, min	RI (Δ)[b]	Rt, min	RI (Δ)[b]	Rt, min	RI (Δ)[b]
Z6,Z9-Diene [c]	11.73	1730	13.51	1922	15.53	2114
Z3,Z6,Z9-Triene [c]	12.17	1786 (56)	14.03	1975 (53)	16.20	2169 (55)
Z6,Z9,E11-Triene	12.93	1862 (132)	14.84	2052 (130)	17.08	2240 (126)
Z3,Z6,Z9,E11-Tetraene	13.43	1914 (128)	15.45	2107 (132)	17.87	2301 (132)
1,Z3,Z6,Z9-Tetraene	13.34	1905 (119)	15.31	2095 (120)	17.68	2286 (117)

[a] The column temperature program was 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C.
[b] Δ means the difference of RI values between a triene and the corresponding Z6,Z9-diene or between a tetraene and the corresponding Z3,Z6,Z9-triene. RIs were calculated relative to the respective C_{17} to C_{23} *n*-alkanes.
[c] Ando et al. (1993).

Table 3 Relative intensities of M^+ and diagnostic fragment ions in EI mass spectra of synthetic polyenes

Compound	Relative intensity (%) of ions at indicated *m/z*												
	m/z	67	79	81	91	106	108	110	163	M-85	M-82	M-56	M-54	M
6,9-Diene[a]														
Z6,Z9-19:H		100	35	90	10	0	1	41	0	1	0	0	0	21
3,6,9-Triene[a]														
Z3,Z6,Z9-19:H		51	100	29	21	3	55	3	2	1	1	16	3	2
6,9,11-Triene[b]														
Z6,Z9,E11-17:H		68	100	52	47	3	5	13	20	16	0	5	4	12
Z6,Z9,E11-19:H		69	100	54	47	5	8	20	19	10	0	1	5	13
Z6,Z9,E11-21:H		75	100	57	50	6	7	22	19	6	0	0	4	11
3,6,9,11-Tetraene[b]														
Z3,Z6,Z9,E11-17:H		21	100	8	32	4	56	0	0	2	7	1	0	1
Z3,Z6,Z9,E11-19:H		21	100	8	31	4	69	1	0	1	6	1	0	1
Z3,Z6,Z9,E11-21:H		23	100	10	29	6	79	1	0	0	4	1	0	1
1,3,6,9-Tetraene[b]														
1,Z3,Z6,Z9-17:H		40	100	19	93	22	2	2	0	0	0	0	13	1
1,Z3,Z6,Z9-19:H		42	100	24	93	28	3	2	1	0	0	0	15	2
1,Z3,Z6,Z9-21:H		40	100	25	94	38	4	2	0	0	0	0	15	3

[a] Ando et al. (1993).
[b] Synthesized in this work.

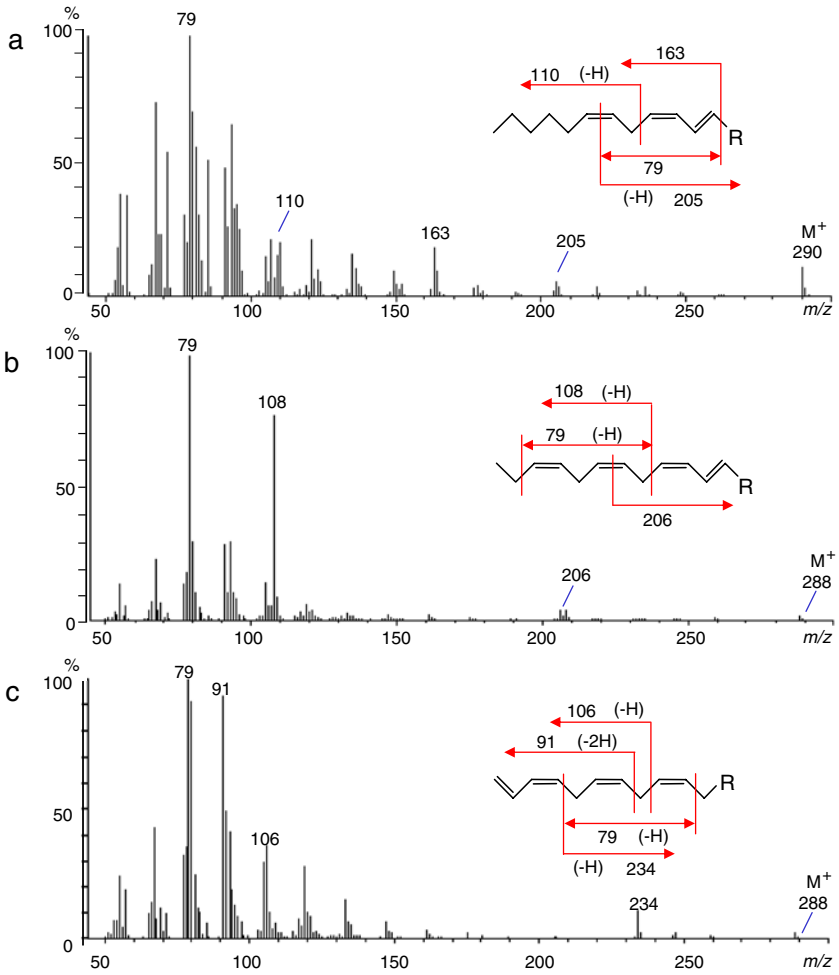

Fig. 2 Mass spectra and fragmentations proposed for polyunsaturated hydrocarbons with a C_{21} straight chain. (**a**) Z6,Z9, E11-21:H, (**b**) Z3,Z6,Z9,E11-21: H, and (**c**) 1,Z3,Z6,Z9-21:H

Islands, one species in the family of Noctuidae was attracted by 1,Z3,Z6,Z9-19:H, but the taxon was not determined to species. Furthermore, in the case of 1,Z3, Z6,Z9-19:H, its binary mixture with Z3,Z6,Z9-19:H was examined. The response of males of the two species to the tetraene was decreased by including the triene in the lure (data not shown).

Discussion

In our previous random screening tests, the specific attraction of male moths was observed for three species by 6,9-dienes with a C_{17}–C_{21} chain and seven species by 3,6,9-trienes with a C_{18}–C_{21} chain (Ando et al. 1993, 1995). In addition, the field evaluation of 6,9,11-trienes and 3,6,9,11- and 1,3,6,9-tetraenes with a C_{17}–C_{21} chain, which were systematically synthesized by two routes in this study (Fig. 1), revealed the attraction of five other lepidopteran species (Table 4). Four of them belong to the family of Geometridae. To date, female-produced sex pheromones of 43 species and male attractants of 76 species have been reported in the Geometridae (Ando 2008). In this family, however, the chemical communication of the insects in the genera *Culpinia* and *Wilemania* has not been reported (Ando 2008; El-Sayed 2008). The male attractants of *C. diffusa* and *W. nitobei* are the first ones in these genera, indicating the significance of devising a screening test for the study of lepidopteran sex pheromones. Moreover, this study evaluated 6,9,11-trienes as a new pheromone candidate. While *trans*-11,12-epoxy-(*Z,Z*)-6,9-henicosadiene (posticlure), which is expected to be biosynthesized *via* Z6,Z9,E11-21:H, has been identified from *Orgyia postica* (Lymantriidae) (Wakamura et al. 2001, 2005), no 6,9,11-trienes have been identified as a pheromone component. The field attraction of the *C. diffusa* males by Z6,Z9,E11-17:H suggests that the 6,9,11-trienes are utilized as a cue for the mating communication of some species in highly evolved lepidopteran families.

The *W. nitobei* males were attracted by Z3,Z6,Z9,E11-19:H, which has been identified from *Alsophila pometaria* (Geometridae: Oenochrominae) (Wong et al. 1984). Among the 3,6,9,11-tetraenes, the C_{20} compound attracted another geometrid species, i.e., *Inuroi fletcheri* males were attracted by Z3,Z6,Z9,E11-20:H, which has not been identified from any insects. The genus *Inuroi* is one of the groups of winter moths, whose females are wingless or have degraded wings. This genus includes nine species in Japan, and our previous study determined the male attraction of two other *Inuroi* species, *I. fumosa* and *I. membranaria*, by *cis*-3,4-epoxy-(*Z,Z*)-6,9-henicosadiene (Ando et al. 1993).

The 1,3,6,9-tetraenes have been more widely studied. 1, Z3,Z6,Z9-19:H is a pheromone component of *Operophtera* spp. (Geometridae: Larentiinae), *O. brumata* (Bestmann et al. 1982; Roelofs et al. 1982), *O. bruceata* (Underhill et al. 1987), and *O. fagata* (Szöcs et al. 2004), and 1,Z3,Z6,Z9-21:H is a component of *Epirrita autumnata* (Geometridae: Larentiinae) (Zhu et al. 1995) and three species in the Arctiidae, *Utetheisa ornatrix* (Jain et al. 1983), *Arctia villica* (Einhorn et al. 1984), and *Pareuchaetes pseudoinsulata* (Frérot et al. 1993). The genus *Operophtera* is another group of winter moths. The above three species, whose sex pheromones have been identified, do not inhabit Japan. *Operophtera brumata* had been recognized as a species inhabiting Japan, but the taxon identified as *O. brumata* in Japan is now defined to be *O. brunnea* or *O. variabilis*. These two species were not attracted in our field tests with 1,Z3,Z6,Z9-19:H, the sex pheromone of *O. brumata*. Rather, *O. rectipostmediana* males were captured by this tetraene in our study. Seven *Operophtera* species inhabit Japan, and our previous experiment revealed the male attraction of *O. relegata* by *cis*-9,10-epoxy-(*Z,Z*)-3,6-nonadecadiene (Qin et al. 1997).

Our field evaluation was carried out in a limited number of places. To clarify the diversity of lepidopteran Type II

Table 4 Sex attractants detected in field screening tests in a forest in Hachioji-shi (Tokyo) and Iriomote Island (Okinawa Prefecture) from 2004 to 2005

Family / Subfamily	Species	Attractant	Time of flight	Location and trap catch (*N*) [a]
Geometridae				
Geometrinae	*Culpinia diffusa* Walker	Z6,Z9,E11-17:H	June to Aug.	Tokyo (11)
Ennominae	*Wilemania nitobei* Nitobe	Z3,Z6,Z9,E11-19:H	Nov. to Dec.	Tokyo (14)
Oenochrominae	*Inurois fletcheri* Inoue	Z3,Z6,Z9,E11-20:H	Dec.	Tokyo (122)
Larentiinae	*Operophtera rectipostmediana* Inoue	1,Z3,Z6,Z9-19:H	Nov. to Dec.	Tokyo (141)
Noctuidae				
Ophiderinae	Gen. et sp.	1,Z3,Z6,Z9-19:H	Dec. to Feb.	Okinawa (72)

[a] Total number of male moths caught in two traps. No males of these species were captured in control traps.

pheromones further, it is necessary to conduct screening tests in many different areas and environments and observe the male attraction of many species. Moreover, this study dealt with only polyunsaturated hydrocarbons. Since their epoxy derivatives are also expected to be the pheromone components of some species in highly evolved families, such as Geometridae, we intend to systematically prepare them and examine their activity in the field. In addition to knowledge of the attractants, this study also accumulated chemical data of Type II pheromones and the candidates including a further modified structure (Tables 1, 2, and 3 and Fig. 2). The Rt values and mass spectra of the synthetic compounds are useful, particularly, for structural determination conducted with a trace amount of a natural pheromone. Utilizing these GC-MS data, the analysis of a pheromone gland extract can be accomplished without significant effort.

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References

ANDO, T. 2008. Internet database: http://www.tuat.ac.jp/~antetsu/LepiPheroList.htm.

ANDO, T., KUSA, K., UCHIYAMA, M., YOSHIDA, S., and TAKAHASHI, N. 1983. ^{13}C NMR analyses on conjugated dienic pheromones of Lepidoptera. *Agric. Biol. Chem.* 47:2849–2853.

ANDO, T., OHSAWA, H., UENO, T., KISHI, H., OKAMURA, Y., and HASHIMOTO, S. 1993. Hydrocarbons with a homoconjugated polyene system and their monoepoxy derivatives: Sex attractants of geometrid and noctuid moths distributed in Japan. *J. Chem. Ecol.* 19:787–798.

ANDO, T., KISHI, H., AKASHIO, N., QIN, X.-R., SAITO, N., ABE, H., and HASHIMOTO, S. 1995. Sex attractants of geometrid and noctuid moths: Chemical characterization and field test of monoepoxides of 6,9-dienes and related compounds. *J. Chem. Ecol.* 21:299–311.

ANDO, T., INOMATA, S., and YAMAMOTO, M. 2004. Lepidopteran sex pheromones, pp. 51–96, in S. Schulz (ed.). The Chemistry of Pheromones and Other Semiochemicals I. Topics in Current Chemistry, vol. 239. Springer, Berlin/Heidelberg.

BESTMANN, H. J., BROSCHE, T., KOSCHATZKY, K. H., MICHAELIS, K., PLATZ, H., ROTH, K., SÜß, J., VOSTROWSKY, O., and KNAUF, W. 1982. Pheromone-XLII. 1,3,6,9-Nonadecatetraen, das Sexualpheromon des Frostspanners *Operophtera brumata* (Geometridae). *Tetrahedron Lett.* 23:4007–4010.

EINHORN, J., BONIFACE, B., RENOU, M., and MILAT, M.-L. 1984. Study on the sex pheromone of *Arctia villica* L. (Lepidoptera, Arctiidae). *C. R. Acad. Sci., Ser. 3* 298:573–576.

EL-SAYED, A. M. 2008. Internet database: http://www.pherobase.com/.

EYA, B. K., OTSUKA, T., KUBO, I., and WOOD, D. L. 1990. Syntheses and NMR analyses of the eight geometrical isomers of 3,6,8-dodecatrien-1-ol, subterranean termite trial pheromone. *Tetrahedron* 46:2695–2706.

FRÉROT, B., MALOSSE, C., CHENON, R. D., DUCROT, P. H., and CAIN, A.-H. 1993. Identification of the sex pheromone components in *Pareuchaetes pseudoinsulata* Rego Barros (Lepidoptera, Arctiidae). *C. R. Acad. Sci., Ser. 2* 317:1045–1050.

JAIN, S. C., DUSSOURD, D. E., CONNER, W. E., EISNER, T., GUERRERO, A., and MEINWALD, J. 1983. Polyene pheromone components from an arctiid moth (*Utetheisa ornatrix*): Characterization and synthesis. *J. Org. Chem.* 48:2266–2270.

JURENKA, R. 2004. Insect pheromone biosynthesis, pp. 97–132, in S. Schulz (ed.). The Chemistry of Pheromones and Other Semiochemicals I. Topics in Current Chemistry, vol. 239. Springer, Berlin/Heidelberg.

MILLAR, J. G. 2000. Polyene hydrocarbons and epoxides: A second major class of lepidopteran sex attractant pheromones. *Annu. Rev. Entomol.* 45:575–604.

NISHIDA, T., VANG, L. V., YAMAZAWA, H., YOSHIDA, R., NAKA, H., TSUCHIDA, K., and ANDO, T. 2003. Synthesis and characterization of hexadecadienyl compounds with a conjugated diene system, sex pheromone of the persimmon fruit moth and related compounds. *Biosci. Biotechnol. Biochem.* 67:822–829.

POHNERT, G. and BOLAND, W. 2000. Highly efficient one-pot double-Wittig approach to unsymmetrical (1*Z*,4*Z*,7*Z*)-homoconjugated trienes. *Eur. J. Org. Chem.* 2000:1821–1826.

QIN, X.-R., ANDO, T., YAMAMOTO, M., YAMASHITA, M., KUSANO, K., and ABE, H. 1997. Resolution of pheromonal epoxydienes by chiral HPLC, stereochemistry of separated enantiomers, and their field evaluation. *J. Chem. Ecol.* 23:1403–1417.

ROELOFS, W. L., HILL, A. S., LINN, C. E., MEINWALD, J., JAIN, S. C., HERBERT, H. J., and SMITH, R. F. 1982. Sex pheromone of the winter moth, a geometrid with unusually low temperature precopulatory responses. *Science* 217:657–659.

SZÖCS, G., TÓTH, M., KÁRPÁTI, Z., ZHU, J., LÖFSTEDT, C., PLASS, E., and FRANCKE, W. 2004. Identification of polyenic hydrocarbons from the northern winter moth, *Operophtera fagata*, and development of a species specific lure for pheromone traps. *Chemoecology* 14:53–58.

UNDERHILL, E. W., MILLAR, J. G., RING, R. A., WONG, J. W., BARTON, D., and GIBLIN, M. 1987. Use of a sex attractant and an inhibitor for monitoring winter moth and bruce spanworm populations. *J. Chem. Ecol.* 13:1319–1330.

WAKAMURA, S., ARAKAKI, N., YAMAMOTO, M., HIRADATE, S., YASUI, H., YASUDA, T., and ANDO, T. 2001. Posticlure: A novel *trans*-epoxide as a sex pheromone component of the tussock moth, *Orgyia postica* (Walker). *Tetrahedron Lett.* 42: 687–689.

WAKAMURA, S., ARAKAKI, N., YAMAMOTO, M., HIRADATE, S., YASUI, H., KINJO, K., YASUDA, T., YAMAZAWA, H., and ANDO, T. 2005. Sex pheromone and related compounds in the Ishigaki and Okinawa strains of the tussock moth *Orgyia postica* (Walker) (Lepidoptera: Lymantriidae). *Biosci. Biotechnol. Biochem.* 69: 957–965.

WONG, J. W., PALANISWAMY, P., UNDERHILL, E. W., STECK, W. F., and CHISHOLM, M. D. 1984. Sex pheromone components of fall cankerworm moth, *Alsophila pometaria* synthesis and field trapping. *J. Chem. Ecol.* 10:1579–1596.

ZHU, J. W., LÖFSTEDT, C., PHILIPP, P., FRANCKE, W., TAMMARU, T., and HAUKIOJA, E. 1995. A sex pheromone component novel to the Geometridae identified from *Epirrita autumnata*. *Entomol. Exp. Appl.* 75:159–164.

Recently, the calling pheromone of *P. transgariepinus* was identified as 1-tridecene (Geiselhardt et al. 2008). This compound is well-known from pygidial defensive secretions of numerous tenebrionids (Tschinkel 1975a) and is also part of this secretion in *P. transgariepinus*. Nevertheless, the pheromone is not produced in the pygidial glands but is secreted from the aedeagal glands. These glands are part of the internal male reproductive system (Brits 1982) and are found only in *Parastizopus* and the sister genus *Ennychiatus* (Geiselhardt, unpublished). In *P. armaticeps*, these glands are 2.0 mm in length and 0.4 mm in diameter, and are connected with the ectodermal sac around the aedeagus by a muscle-controlled opening. Brits (1982) mentioned that the secretion of the glands flows over the aedeagus during evagination and postulated a possible function as a pheromone.

In the present study, we collected the volatiles emitted from the exposed aedeagus of *P. armaticeps* by using solid phase microextraction (SPME) and compared the composition with that of the aedeagal gland secretion. To obtain information about the biologically active compounds and their sex-specificty, we performed chemo-orientation experiments on a servosphere.

Methods and Materials

Beetles Insects were wild-caught *P. armaticeps* from Twee Rivieren, Kalahari Gemsbok National Park, South Africa, and their F1 offspring. They were separated according to sex and kept at 30°C and 40% relative humidity under a 14:10 L/D regime in terraria containing Kalahari sand, hiding places, and a water source. The beetles were fed with oat flakes or dried twigs of *Lebeckia linearifolia* (Fabaceae), their natural food plant.

Collection of Volatiles with SPME Groups of three males were kept in small open terraria (30×20×20 cm) with 1 cm moist Kalahari sand on the floor under the environmental conditions described above. To stimulate the beetles, they were sprayed with water simulating rainfall. When a male did a 'headstand' and exposed its aedeagus, the volatiles emitted were sampled by SPME, holding a polydimethylsiloxane fiber (100 μm, Supelco) for at most 5 s at 1–2 mm distance from the aedeagus.

Composition of the Aedeagal Gland Secretion The male genitalia were dissected in water (Brits 1982), and the aedeagal glands were removed and extracted in 100 μl of *n*-pentane. For quantification, 10 μl of 1,4-benzoquinone (1 mg/ml in *n*-pentane) were added to the samples as an internal standard.

Chemical Analysis Samples were analyzed on a coupled gas chromatography–mass spectrometer system (HP 6890 series GC–HP 5973 MSD) equipped with a split/splitless injector (300°C) and an autosampler (injection volume, 1 μl). Desorption time of SPME fibers was 2 min. A fused silica column (DB-1, 30 m×0.25 mm ID, 0.25 μm, J & W Scientific, Folsom, CA, USA) was used with a helium carrier gas flow of 1 ml/min. The oven temperature was programmed 2 min at 35°C, 20°C/min to 100°C, then 6°C/min to 300°C. Mass spectra were obtained by electron impact ionization at 70 eV.

All volatiles were identified by comparison of mass spectra and retention times with those of authentic standards. Fragmentation patterns of fatty acid esters were compared with those in the Wiley mass spectra library (John Wiley & Sons, Ltd.), and double-bond positions were determined after derivatization with dimethyl disulfide (Scribe et al. 1988). The identification of the hydrocarbons was based on their fragmentation patterns (Nelson and Sukkestad 1970; Nelson et al. 1972; Pomonis et al. 1980) and corroborated by their retention indices (Carlson et al. 1998).

Chemo-Orientation Experiments were performed with the servosphere TrackSphere LC300 (Syntech, Hilversum, The Netherlands). Walking movements of the beetles were recorded as *x*, *y* coordinates at a rate of ten data points per second and displayed in real time by the TrackSphere 2.2 software. Bioassays were conducted in the dark at 25–30°C during the first 3 h of the scotophase. The air stream (0.3 m/s) passed through a glass tube (ID, 5 mm), with its opening located 25 cm from the top of the sphere. Beetles were allowed to acclimate for a few minutes on the servosphere before the trials started. A test consisted of two 3-min periods, i.e., before an odor was applied and during odor presentation. The test criterion for a responding female was a posture in which the beetle elevated the front part of its body and raised the antennae to a vertical position. If the ternary test mixture triggered this reaction in a female, the beetle was subsequently tested with all odor combinations. Males also were tested, if they showed calling behavior. Each series was started and finished with the ternary mixture.

Synthetic Compounds Tested on the Servosphere The concentrations of 3-methylphenol (>99%, Fluka), ethyl-1,4-benzoquinone (>99%, synthesized according to Peschke and Metzler 1982), and 3-ethylphenol (>99.4%, Riedel de Haen) were 52, 46, and 2 μg/ml, respectively, regardless of their use as single components or in binary and ternary mixtures. All components were dissolved in *n*-pentane, which had no behavioral effect on the beetles. Ten microliters of the test mixtures or single components were applied to a piece of filter paper (0.5 cm^2), which was

inserted in the air-stream glass tube. The amounts applied corresponded approximately to the amounts extracted from one aedeagal gland.

Statistics Before the data from the servosphere bioassays were analyzed, the basic track recording intervals of 0.1 s were merged to provide intervals of 1 s to smooth the data. Stops were assumed when the walking speed fell below 5 mm/s because the sphere made small random movements even when the beetles were not moving. All displacements slower than this were discarded. From these modified track recordings, we calculated mean walking speed (without stops), stop number, and stop duration. For each period, we then calculated the mean vector m (length r_{pop}; direction Φ) and the path straightness (r). Values of r range from 0 (ends at the origin) to +1 (straight). Differences in walking speed, stop number, stop duration, and path straightness between the periods before and during odor presentation were compared by using the two-tailed Wilcoxon test for matched pairs. Walking parameters of males and females under clean air conditions were compared with the Mann–Whitney U test. Directional data were subject to the modified Rayleigh V test (Batschelet 1981).

Results

Chemical Composition of the Pheromone and the Aedeagal Glands Extract The volatiles collected with SPME from the air close to the aedeagus of a calling male *P. armaticeps* were identified as 3-methylphenol, ethyl-1,4-benzoquinone, and 3-ethylphenol (N=14; Fig. 1). The proportions of 3-methylphenol and ethyl-1,4-benzoquinone were nearly equal (51.8% and 46.4%, respectively), whereas 3-ethylphenol was only a minor component (1.8%).

In extracts of the aedeagal glands, all three volatile components were present but only as minor components [Table 1 and Fig. 2, 3-ethylphenol (0.03%) is not listed]. The main fraction consisted of several fatty acid esters (23.6%, dominated by ethyl 11-eicosenoate 10.8%) and of *n*-alkanes, and mono- and dimethylalkanes, mainly with carbon backbones from 29 to 33 (69.1%). *n*-Alkanes were minor components (1.9%), whereas mono- and dimethylalkanes amounted to 29.0% and 33.8%, respectively. Although esters and hydrocarbons made up more than 95% of the gland extracts, they were never detected in the airspace around the aedeagus during calling. The total amount (mean ± SD) of volatiles extracted from the aedeagal gland reservoirs was 0.92±0.83 μg per beetle (N=10).

Behavior in the Clean Air Stream Neither males nor females of *P. armaticeps* showed a preferred direction with respect to the direction of the air stream (males' mean vector: N=15, r_{pop}=0.14, Φ=+57°, P>0.05; females' mean vector$_{initial\ trial}$: N=11, r_{pop}=0.08, Φ=+30°, P>0.05; modified Rayleigh V test; Fig. 3, Table 2). Furthermore, males and females did not differ in their walking performance parameters such as speed (median, 22 mm/s vs. 24 mm/s; Mann–Whitney U test, U=79, z=0.18, P>0.05), stop number (median 2 vs. 7; U=41, z=1.93, P>0.05), stop duration (median 1% vs. 17% of the trial duration; U=49, z=−1.74, P>0.05) or path straightness (median 0.24 vs. 0.48; U=57, z=−1.32, P>0.05).

Response to Pheromone Mixtures and Single Components Male *P. armaticeps* did not respond to the complete pheromone blend (mean vector: r_{pop}=0.21, Φ=−10°, P>0.05, modified Rayleigh V test; Fig. 3a and Table 2), whereas females were strongly attracted to this ternary mixture (mean vector$_{initial\ trial}$: r_{pop}=0.88, Φ=−4°, P<0.001; mean vector$_{final\ trial}$: r_{pop}=0.85, Φ=−7°; P<0.001; Fig. 3b,c and Table 2). Although females were attracted to the ternary mixture in the initial and final trial, they were not attracted in any of the trials with binary mixtures or single components alone (Table 2).

With respect to walking performance, males did not differ in any of the measured parameters compared to the clean air condition (Table 2). When stimulated with the ternary mixture, females stopped more often (median, 7_{air} vs. $16_{initial\ trial}$ and 6_{air} vs. $10_{final\ trial}$; P<0.05 in both cases, Wilcoxon test for matched pairs) and longer than in clean air (median, $17\%_{air}$ vs. $35\%_{initial\ trial}$ and $7\%_{air}$ vs. $32\%_{final\ trial}$; P<0.05 in both cases). When ethyl-1,4-benzoquinone was part of the stimulus blend, a similar response was observed as well (Table 2). Among treatments, the number and

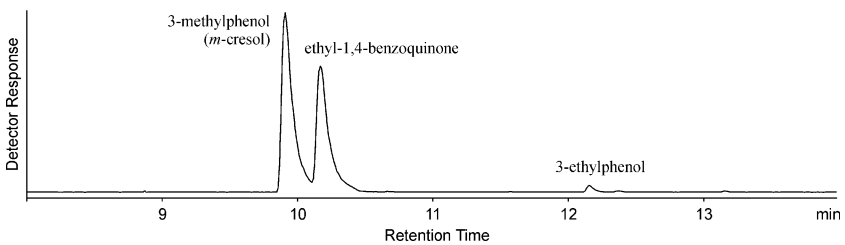

Fig. 1 Gas chromatogram of volatiles collected with SPME from an exposed aedeagus of a calling *Parastizopus armaticeps* male

Table 1 Chemical composition of the main components (>1%) of aedeagal gland extracts of male *Parastizopus armaticeps* (*N*=13)

Peak no.	RI	Component	Composition (%)	
			Median	Range
1	1043	3-Methylphenol	1.1	0.3–4.8
2	1053	Ethyl-1,4-benzoquinone	1.1	0.0–2.8
3	2353	Ethyl 11-eicosenoate	10.8	1.1–24.4
4	2381	Isopropyl 11-eicosenoate	2.4	1.2–3.6
5	2446	Propyl 11-eicosenoate	1.2	0.3–2.6
6	2553	Ethyl 13-docosenoate	1.9	0.2–3.1
7	2745	7-Methylheptacosane	1.5	0.8–2.1
8	2776	3-Methylheptacosane	1.0	0.6–1.4
9	2900	*n*-Nonacosane	1.2	0.3–2.5
10	2930	13-/11-/9-Methylnonacosane	6.4	4.6–9.0
11	2940	7-Methylnonacosane	1.1	0.7–2.5
12	2953	5-Methylnonacosane	4.7	3.0–7.7
13	2968	9,19-Dimethylnonacosane	1.6	1.0–2.5
14	2978	3-Methylnonacosane	2.5	1.2–4.5
15	2984	5,15-Dimethylnonacosane	2.8	2.0–5.0
16	3010	3,11-/3,9-/3,7-Dimethylnonacosane	1.3	0.9–1.6
17	3132	15-/13-/11-/9-Methylhentriacontane	5.7	3.9–8.9
18	3168	9,19-Dimethylhentriacontane	6.3	5.3–9.2
19	3183	5,15-Dimethylhentriacontane	2.6	1.9–4.3
20	3332	17-/15-/13-/11-Methyltritriacontane	2.5	1.2–3.2
21	3364	11,21-Dimethyltritriacontane	11.2	8.3–15.9
22	3383	5,15-Dimethyltritriacontane	1.2	0.5–2.6

duration of stops differed, as did the behavior during the stops. Females often lifted the front part of their bodies, raised the antennae to a vertical position, and turned slowly on their own axis, a behavior that was not seen in clean air. In the initial trial as well as in the trial with ethyl-1,4-benzoquinone, the walking speed of females was significantly lower than in the controls (median, 24 mm/s_{air} vs. 18 mm/$s_{initial\ trial}$ and 32 mm/s_{air} vs. 27 mm/s_{EtBQ}; $P<0.01$ and $P<0.05$, respectively). This seemed to be a result of increased and decreased locomotion associated with more frequent stops rather than of average walking speed itself. None of the chemical stimuli tested affected the path straightness of the beetles' walks in any way (Table 2).

Discussion

Our study demonstrated that the male sex pheromone of *P. armaticeps* consists of 3-methylphenol, ethyl-1,4-benzoquinone, and 3-ethylphenol. In a previous study, Geiselhardt et al. (2008) showed that males of the sister species *Parastizopus transgariepinus* emit 1-tridecene during calling. Although the chemical compositions of the pheromones are entirely different for the two species, the constituent compounds are well-known from defensive secretions of many arthropods (Blum 1981). Most tenebrionids, including *Parastizopus*, possess pygidial defensive glands, which secrete mixtures of 1,4-benzoquinones and 1-alkenes (Brown

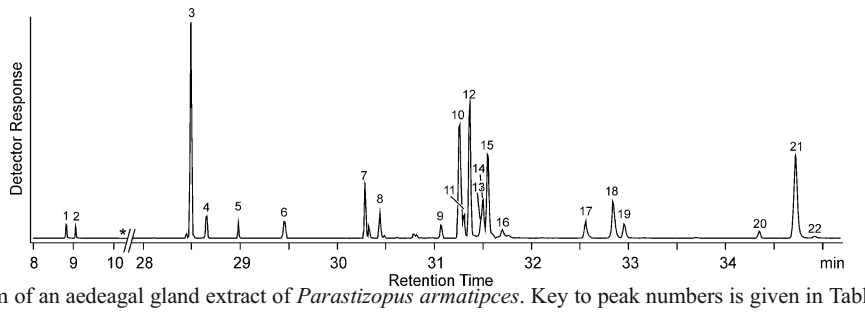

Fig. 2 Gas chromatogram of an aedeagal gland extract of *Parastizopus armatipces*. Key to peak numbers is given in Table 1. *3-ethylphenol (not listed in Table 1)

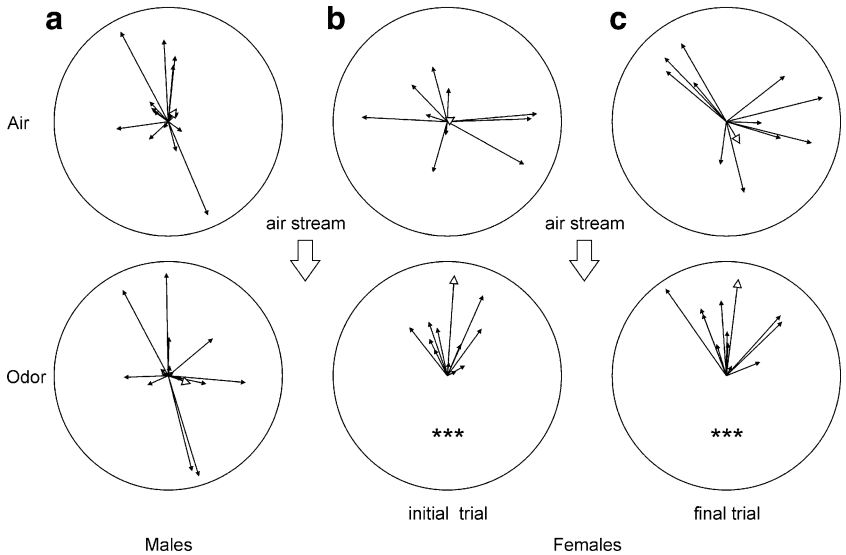

Fig. 3 Polar plots of the distribution of the mean direction of tracks of **a** male (N=15) and **b**, **c** female (N=11) *Parastizopus armaticeps* on a servosphere during consecutive 3-min periods in an air stream alone and in an air stream with the ternary pheromone mixture (1 μg). *Arrow lengths* are proportional to path straightness (Batschelet 1981). *White arrows* correspond to the mean vectors. The *asterisks* indicate significant preference for the wind direction (modified Rayleigh V test, ***P<0.001)

et al. 1992; Tschinkel 1975a, b), but *Parastizopus* additionally produces appreciable amounts of monoterpene hydrocarbons in the defensive glands (Geiselhardt et al. 2006). The defensive secretions of *P. armaticeps* and *P. transgariepinus* are very similar, comprising the same set of compounds in similar ratios (Geiselhardt et al. 2006, 2008).

Although some pheromone compounds are found in the pygidial glands, such as 1-tridecene in *P. transgariepinus*

Table 2 Track parameters (median, range) of male and female *Parastizopus armaticeps* walking on a servosphere while exposed to an air stream with clean air or different mixtures of 3-methylphenol (MePh), ethyl-1,4-benzoquinone (EtBQ), and 3-ethylphenol (EtPH)

Applied odor	N	Mean vector			Walking speed (mm/s)			Number of stops			Stop duration (% of trial)			Path straightness		
		r_{pop}	Φ	P^1	$\tilde{x}$	Range	P^2	$\tilde{x}$	Range	P^2	$\tilde{x}$	Range	P^2	$\tilde{x}$	Range	P^2
Males																
Air	15	0.14	+57°	NS	22	10–51	NS	2	0–12	NS	1	0–58	NS	0.24	0.04–0.89	NS
MePh + EtBQ + EtPh	15	0.21	−19°	NS	26	14–61		3	2–14		5	0–27		0.34	0.02–0.92	
Females																
Air	11	0.08	+30°	NS	24	13–31	**	7	2–17	*	17	2–41	*	0.48	0.12–0.79	NS
MePH+EtBQ+EtPh (initial trial)	11	0.88	−4°	***	18	10–24		16	9–30		35	19–76		0.36	0.11–0.77	
Air	11	0.03	+57°	NS	32	20–56	NS	3	0–15	NS	8	0–24	NS	0.79	0.07–0.92	NS
MePH+EtBQ	11	0.06	+18°	NS	29	17–51		6	1–15		11	1–40		0.41	0.05–0.93	
Air	11	0.31	−27°	NS	25	19–49	NS	5	0–10	NS	8	1–42	NS	0.33	0.04–0.96	NS
MePH+EtPh	11	0.42	−64°	NS	27	15–52		5	1–18		9	2–36		0.49	0.05–0.92	
Air	11	0.16	−73°	NS	27	14–49	NS	3	0–14	NS	7	1–22	*	0.43	0.13–0.93	NS
EtBQ+EtPH	11	0.14	−77°	NS	27	17–51		5	2–14		18	1–52		0.34	0.19–0.92	
Air	11	0.28	−16°	NS	21	6–48	NS	5	0–15	NS	8	1–97	NS	0.25	0.04–0.92	NS
MePH	11	0.35	−97°	NS	25	7–53		6	2–10		11	2–99		0.54	0.06–0.94	
Air	11	−0.27	−120°	NS	32	20–55	*	2	0–13	*	6	0–20	**	0.72	0.03–0.95	NS
EtBQ	11	0.32	−20°	NS	27	8–50		6	1–17		26	8–89		0.48	0.14–0.93	
Air	11	0.05	−56°	NS	28	10–49	NS	4	0–10	NS	13	4–79	NS	0.53	0.05–0.96	NS
EtPH	11	0.19	−40°	NS	28	18–49		6	2–12		10	4–53		0.66	0.22–0.96	
Air	11	0.23	−59°	NS	27	8–34	NS	6	0–22	*	7	0–79	**	0.65	0.31–0.83	NS
MePh+EtBQ+EtPh (final trail)	11	0.85	−7°	***	19	9–34		10	3–23		32	4–80		0.58	0.10–0.93	

[1] modfied Rayleigh V-test
[2] two-tailed Wilcoxon test for matched pairs
*P<0.05; **P<0.01; ***P<0.001

(Geiselhardt et al. 2008) and ethyl-1,4-benzoquinone in *P. armaticeps*, respectively, the pheromone is not produced in these glands but in the aedeagal glands that are connected to the aedeagal pouch. The occurrence of the same compounds in different glands has been reported for the flour beetle *Tribolium castaneum*, whose pygidial and prothoracic defensive gland secretions have identical compositions of 1,4-benzoquinones (Loconti and Roth 1953). In the tenebrionid *Zophobas rugipes*, the pygidial glands secrete 1,4-benzoquinones, whereas the prothoracic glands produce phenols (Tschinkel 1969). The author suggested that the biosynthesis of 1,4-benzoquinones proceeds through phenolic precursors. Although the pygidial glands of *Parastizopus* did not secrete phenols, 3-methyl- and 3-ethylphenol are part of the defensive secretions of some closely related Stizopina species (Geiselhardt, unpublished).

The evolution of allomones to function also as sex pheromones has been reported from several insects, including ants (Walter 1993) and beetles (Keville and Kannowski 1975; Peschke 1983). This seems to be a widespread phenomenon especially in melolonthine scarab beetles (Leal 1997), including examples of the incorporation of 1,4-benzoquinones or phenols in the female mate attraction system, such as 1,4-benzoquinone in the cockchafer *Melolontha hippocastani* (Ruther et al. 2001), methyl-1,4-benzoquinone in *M. melolontha* (Reinecke et al. 2002), and phenol in both species (Ruther et al. 2002). In these examples, one gland produces a bifunctional secretion that serves as pheromone and allomone, whereas *Parastizopus* allocates these functions to different glands. As the composition of the pygidial defensive secretions is uniform in the genus *Parastizopus* and the subtribe Stizopina in general (Geiselhardt et al. 2006, 2008), the shift of only a subset of compounds from the pygidial glands to the aedeagal glands opens up the possibility to formulate a species-specific sex pheromone. However, this scenario is still speculative, and further studies are needed to show whether the newly evolved aedeagal glands have adopted the biosynthetic pathways from the phylogenetically older pygidial glands or whether both glands have evolved independently.

The orientation bioassays demonstrated that the emitted pheromone is primarily a sex pheromone rather than an aggregation pheromone, although Rasa et al. (1998) observed more than one male per courtship group and inferred that the pheromone attracts both sexes. However, males in our study showed neither a preference for the upwind direction nor any other observable change in their behavior when exposed to the ternary blend. In contrast, females showed a strong reaction when they perceived the pheromone. They stopped walking and adopted an antennae-raised posture, resembling that of other walking insects that respond to olfactory cues, such as the dermestid beetle *Trogoderma variabile* (Tobin and Bell 1986) or the triatomine bug *Rhodnius prolixus* (Otálora-Luna et al. 2004). During the stops, the females turned slowly on their own axis and sampled the air stream before staring to walk upwind. Therefore, the stops may be important for course control during orientation, as proposed for triatomine bugs that orientate to olfactory cues (Otálora-Luna et al. 2004) and cockroaches (Willis and Avondet 2005). Furthermore, this sampling behavior, in addition to a high stop frequency and duration, may be an adaptation to the male pheromone-emitting behavior, with short calling and long inter-calling periods. Thus, females may perceive the pheromone only for a few seconds before they lose the chemostimulus for about 1 min. The best way for a female to locate a calling male may be a stop-and-go strategy or saltatory search (O'Brien et al. 1990), walking upwind in contact with the pheromone and stopping at loss of the stimulus, waiting for the next pheromone pulse.

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References

BATSCHELET, E. 1981. Circular Statistics in Biology. Academic, London.

BLUM, M. S. 1981. Chemical Defenses of Arthropods. Academic, New York.

BRITS, J. A. 1982. The anatomy, histology and physiology of the internal adult male reproductive system of *Parastizopus armaticeps* Péringuey (Coleoptera: Tenebrionidae). *J. Entomol. Soc. South. Afr.* 45:239–260.

BROWN, W. V., DOYEN, J. T., MOORE, B. P., and LAWRENCE, J. F. 1992. Chemical composition and taxonomic significance of defensive secretions of some Australian Tenebrionidae (Coleoptera). *J. Aust. Entomol. Soc.* 31:79–89.

CARLSON, D. A., BERNIER, U. R., and SUTTON, B. D. 1998. Elution patterns from capillary GC for methyl-branched alkanes. *J. Chem. Ecol* 24:1845–1865.

GEISELHARDT, S., OCKENFELS, P., and PESCHKE, K. 2008. 1-Tridecene—male-produced sex pheromone of the tenebrionid beetle *Parastizopus transgariepinus*. *Naturwissenschaften* 95:247–251.

GEISELHARDT, S., SZEPAT, T., RASA, O. A. E., and PESCHKE, K. 2006. Defensive secretion components of the host *Parastizopus armaticeps* as kairomones for the cleptoparasite *Eremostibes opacus*. *J. Chem. Ecol.* 32:767–778.

KEVILLE, R., and KANNOWSKI, P. B. 1975. Sexual excitation by pheromones of the confused flour beetle. *J. Insect Physiol.* 21:81–84.

KOCH, C. 1963. The Tenebrionidae of Southern Africa XXIX. Luebbertia plana gen. et spec. Nov., with a dichotomic analysis of Stizopina (Opatrini). Scientific paper Namib Des. Res. Station 18.

LEAL, W. S. 1997. Evolution of sex pheromone communication in plant-feeding scarab beetles, pp, pp. 505–513, in R. T. Cardé, and A. K. Minks (eds.). *Insect Pheromone Research: New Directions* Chapman and Hall, New York.

LOCONTI, J. D., and ROTH, L. M. 1953. Composition of the odorous secretion of *Tribolium castaneum*. *Ann. Entomol. Soc. Am* 46:281–289.

NELSON, D. R., and SUKKESTAD, D. R. 1970. Normal and branched aliphatic hydrocarbons from eggs of the tobacco hornworm. *Biochemistry* 9:4601–4611.

NELSON, D. R., SUKKESTAD, D. R., and ZAYLSKIE, R. G. 1972. Mass spectra of methyl-branched hydrocarbons from eggs of the tobacco hornworm. *J. Lipid Res* 13:413–421.

O'BRIEN, W. J., BROWMAN, H. I., and EVANS, B. I. 1990. Search strategies of foraging animals. *Am. Sci.* 78:152–160.

OTÁLORA-LUNA, F., PERRET, J.-L., and GUERIN, P. M. 2004. Appetence behaviours of the triatomine bug Rhodnius prolixus on a servosphere in response to the host metabolites carbon dioxide and ammonia. *J. Comp. Physiol. A* 190:847–854.

PESCHKE, K. 1983. Defensive and pheromonal secretion of the tergal gland of *Aleochara curtula* II. Release and inhibition of male copulatory behavior. *J. Chem. Ecol* 9:13–31.

PESCHKE, K., and METZLER, M. 1982. Defensive and pheromonal secretion of the tergal gland of *Aleochara curtula.* II. The chemical composition. *J. Chem. Ecol*. 8:773–778.

POMONIS, J. G., NELSON, D. R., and FATLAND, C. L. 1980. Insect hydrocarbons. 2. Mass spectra of dimethylalkanes and the effect of the number of methylene units between groups on fragmentation. *J. Chem. Ecol* 6:965–972.

RASA, O. A. E. 1990. Evidence for subsociality and division of labor in a desert tenebrionid beetle *Parastizopus armaticeps* Peringuey. *Naturwissenschaften* 77:591–592.

RASA, O. A. E., BISCH, S., and TEICHNER, T. 1998. Female mate choice in a subsocial beetle: male phenotype correlates with helping potential and offspring survival. *Anim. Behav.* 56:1213–1220.

REINECKE, A., RUTHER, J., and HILKER, M. 2002. The scent of food and defence: green leaf volatiles and toluquinone as sex attractant mediate mate finding in the European cockchafer *Melolontha melolontha*. *Ecol. Lett.* 5:257–263.

RUTHER, J., REINECKE, A., TOLASCH, T., and HILKER, M. 2001. Make love not war: a common arthropod defence compound as sex pheromone in the forest cockchafer *Melolontha hippocastani*. *Oecologia* 128:44–47.

RUTHER, J., REINECKE, A., TOLASCH, T., and HILKER, M. 2002. Phenol—another cockchafer attractant shared by *Melolontha hippocastani* Fabr. and *M. melolontha* L. Z. *Naturforsch* 57:910–913.

SCRIBE, P., GUEZENNEC, J., DAGAUT, J., PEPE, C., and SALIOT, A. 1988. Identification of the position and the stereochemistry of the double bond in monounsaturated fatty acid methyl esters by gas chromatography/mass spectrometry of dimethyl disulfide derivatives. *Anal. Chem* 60:928–931.

TOBIN, T. R., and BELL, W. J. 1986. Chemo-orientation of male Trogoderma variabile (Coleoptera, Dermestidae) in a simulated corridor of female sex pheromone. *J. Comp. Physiol A* 158:729–739.

TSCHINKEL, W. R. 1969. Phenols and quinones from the defensive secretions of the tenebrionid beetle, *Zophobas rugipes*. *J. Insect Physiol.* 15:191–200.

TSCHINKEL, W. R. 1975a. A comparative study of the chemical defensive system of tenebrionid beetles—chemistry of the secretions. *J. Comp. Physiol* 21:753–783.

TSCHINKEL, W. R. 1975b. A comparative study of the chemical defensive system of tenebrionid beetles—III. Morphology of the glands. *J. Morphol.* 145:355–370.

WALTER, F. 1993. Identification of the sex pheromone of an ant, *Formica lugubris* (Hymenoptera, Formicidae). *Naturwissenschaften* 80:30–34.

WILLIS, M. A., and AVONDET, J. L. 2005. Odor-modulated orientation in walking male cockroaches *Periplaneta americana*, and the effect of odor plumes of different structure. *J. Exp. Biol.* 208:721–735.

Table 1 The mean correlation values (r^2) between the relative amounts of major alkanes and alkenes in the cuticular hydrocarbon profile of *Formica exsecta* at species and colony levels

	F. exsecta	
	Species, N=97 (20)	Colony[a], N=4–5 (20)
C_{23} vs. C_{25}	0.46	0.73±0.24
C_{25} vs. C_{27}	0.81	0.88±0.18
C_{27} vs. C_{29}	0.58	0.86±0.17
$C_{23:1}$ vs. $C_{25:1}$	0.71	0.97±0.04
$C_{25:1}$ vs. $C_{27:1}$	0.47	0.98±0.04
$C_{27:1}$ vs. $C_{29:1}$	0.71	0.95±0.07

[a] The number of individuals analyzed (N) and number of colonies sampled (in parentheses) are given; error is represented by SD.

to the precise context, differences in the overall CHC profiles among individuals, castes, or colonies inevitably occur. Although the underlying causes of these differences are not known, researchers have attempted to discriminate signals by using sensitive multivariate statistical methods (principal component and discriminate analysis). These methods provide limited insight into what biological processes underlie the often very small differences detected, as they combine rather than separate out the various signals (Howard and Blomquist 2005). It is normally assumed that each peak or compound in a profile is an independent variable potentially containing information. However, what has long been known (Lockey 1988) but often overlooked is that the vast majority of CHCs (alkanes, alkenes, and monomethylalkanes) within any profile are structurally related, belonging to a homologous series in which the chain length increases by two carbons (e.g., C_{25}, C_{27}, and C_{29}), but the position of double bonds or methyl groups remains constant (e.g., 3-MeC_{25}, 3-MeC_{27}, and 3-MeC_{29}). In some insects, CHCs are produced with only one chain length or in highly limited homologous series; often, such CHCs are dominated by dimethylalkanes. Odd chain-length homologous series dominate the CHCs of most insects (Lockey 1988), and even-chain compounds, if present, are always in smaller proportions. However, if the ratios (relative amounts) of the compounds within a homologous series are constant [i.e., highly correlated (r^2>0.9)] within a colony, population, or species, then that series can be treated as a single variable, greatly reducing the apparent complexity of the profile for multivariate analysis.

In order to decode the information contained within the CHC profile, we first investigated the CHC profiles of 13 species of *Formica* ants from geographically and ecologically different populations (Martin et al. 2008b). This allowed us to distinguish between species-specific cues, that must be common to all members of that species, and any potential colony-specific cues, that must vary between colonies but be consistent among colony members (nest mates). This work revealed two species, *F. fusca* and *F. exsecta*, that had characteristics in their CHC profiles and behavioral traits that made them particularly suitable for further study. These characteristics were that (1) both species possess a strong colony identity as demonstrated by inter-colonial aggression (Wallis 1962; Czechowski 1990); (2) *F. fusca* has the most diverse CHC profile, with respect to dimethylalkanes, of any known *Formica* species (Martin et al. 2008b), and profiles of these compounds appeared to vary between colonies; and (3) *F. exsecta* has a simple CHC profile but retains a strong colony identity.

By studying the relationships of compounds within each homologous series and how these series form the CHC profile, from a large number of *F. fusca* and *F. exsecta* colonies, we provide evidence that only part of the CHC profile contains colony-specific information. Furthermore, this information is encoded in two quite different ways despite the two species belonging to the same genus.

Methods and Materials

Ant Samples

Groups of up to ten adult workers were randomly sampled from 30 *F. fusca* and 20 *F. exsecta* colonies at different

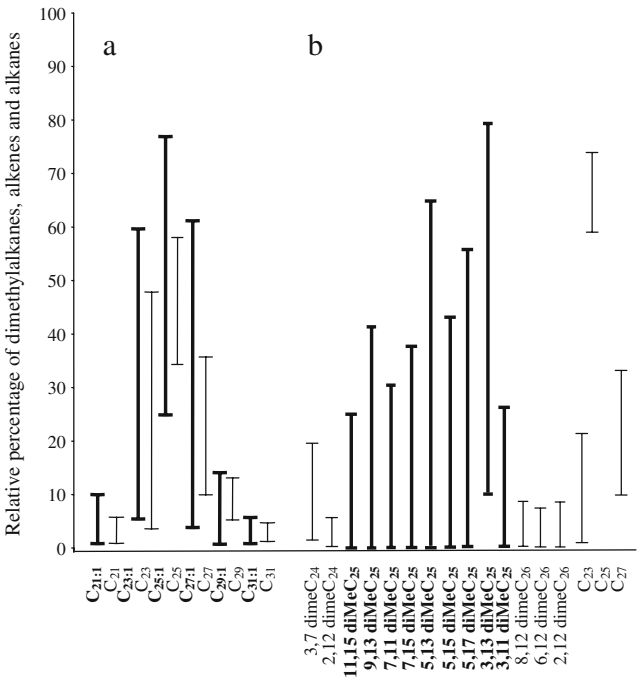

Fig. 1 Variation in relative percentages of the major compounds (alkanes, alkenes, and dimethylalkanes) found in *Formica exsecta* (*a*) and *F. fusca* (*b*) at the species level. The variation for each compound was calculated within each chemical group; e.g., when only the alkanes were considered, C_{25} represents 60–75% of the alkanes in *F. fusca*. The compounds selected for further statistical analysis are shown in *bold*

locations in Finland during 2005 and 2006. All ants were sampled from ten locations within 30km of the Tvärminne Zoological Station in Hanko, south-western Finland. At all collection sites, both species occurred sympatrically in forest clearings created by logging. In total, 111 *F. fusca* and 97 *F. exsecta* workers were sampled. Ants were killed by freezing and stored individually in glass vials at −5°C until extraction. *F. exsecta* colonies in this region are monogynous (Sundström et al. 1996), and only *F. fusca* colonies in which a single queen was found were used in this study, although workers may have been produced by more than one queen (Sundström et al. 2005).

Chemical Analysis

Individual ants were placed in glass vials, 50 µl of high-performance liquid chromatography-grade hexane were added and extracted for 10 min. After this, ants were removed, the hexane allowed to evaporate, and the vials sealed and stored at 5°C. Just before analysis, 30 µl of hexane were added to the vials. Samples were analyzed on an HP 6890 gas chromatograph (GC) connected to an HP5973 MSD (quadrupole) mass spectrometer (MS; −70 eV, electron impact ionization). The GC was equipped with an HP-5MS column (length, 30 m; ID, 0.25 mm; film thickness, 0.25 µm), and the oven temperature was programmed from 70°C to 200°C at 40°C min^{-1} and then from 200°C to 320°C at 25°C min^{-1}. Samples were injected in splitless mode, with helium as carrier gas, at a constant flow rate of 1.0 ml min^{-1}. CHCs were characterized by using standard MS databases, diagnostic ions, and Kovats indices. In *F. exsecta*, the double-bond positions of the alkenes were determined by dimethyl disulfide (DMDS) derivatization (Carlson et al. 1989) of four pooled extracts of ten adults that contained two ants from each colony chosen at random from the 20 colonies. In the few cases in which isomers of methyl-branched hydrocarbons overlapped, a characteristic ion of a compound was used to

Table 2 The average percentages (of the entire cuticular hydrocarbon profile) of six (*Z*)-9-alkenes in 20 *Formica exsecta* colonies, based on $n-1$ samples

Colony code	G	H	B	J	D	I	C	F	A	E	8	60	22	56	71	35	64	40	53	69
N	5	5	5	10	4	5	4	5	10	5	3	4	4	4	4	4	4	4	4	4
$C_{21:1}$	5	2	3						1											
$C_{23:1}$	36	29	24	22	12	12	4	6	4	4	34	32	26	20	6	7	6	6	5	4
$C_{25:1}$	17	25	28	22	31	33	13	18	14	18	33	33	28	33	20	25	24	23	24	23
$C_{27:1}$	3	7	7	11	19	16	16	37	30	37	9	11	17	18	25	31	34	39	38	40
$C_{29:1}$	1	1	2	4	1	1	3	3	6	6	2	2	1	4	2	3	3	4	3	4
$C_{31:1}$				3			1	1	2	1	1			1						
Blind samples																				
G	●																			
H		●																		
B			●																	
J				●																
D					●															
I						●														
C							●													
F								●												
A									●											
E										●										
8											●									
60												●								
22													●							
56														●						
71															●					
35															●	●				
64																	●			
40																		●		
53																			●	
69																				●

The black dots indicate the blind samples that matched a particular colony based on the rule that the proportion of each 9-alkene was within ±5% of the proportion in the colony.

delineate the area of the total ion chromatogram for integration. This method introduced some margin of error, but this was expected to be equal across all samples analyzed. In cases in which isomers overlapped completely (e.g., 3,11-diMeC$_{25}$ and 3,13-diMeC$_{25}$), peak area was divided according to the ratio in which the diagnostic ions were present.

Method Used to Search for Colony-Specific Signals

All CHC profiles were of two types. Those with limited diversity, comprising hydrocarbons of only one chain length or a short homologous series of hydrocarbons, which were usually dimethylalkanes, and those comprising long homologous series (alkanes, alkenes, and monomethylalkanes) of hydrocarbons. We assumed that the amount of information contained within a homologous series depended on whether or not the ratios (relative amounts) of the individual compounds were highly correlated (r^2>0.9) to each other. For a highly correlated homologous series, we assumed that the information encoded within the entire series was effectively the same as the information encoded in a single compound. We also assumed that a colony signal should be contained only within a homologous series that is highly correlated at the colony and not at the species level. A similar logic can also be applied to isolated compounds. That is, their occurrence and ratio of abundance to each other should be constant within a colony but vary among colonies. Therefore, correlation levels of major CHCs in each homologous series or among isolated compounds were determined at the species and colony level.

Once a group of compounds had been identified as containing potential colony signatures (referred to as signature CHCs), their ability to encode colony-specific information was tested by using two methods. First, one ant was

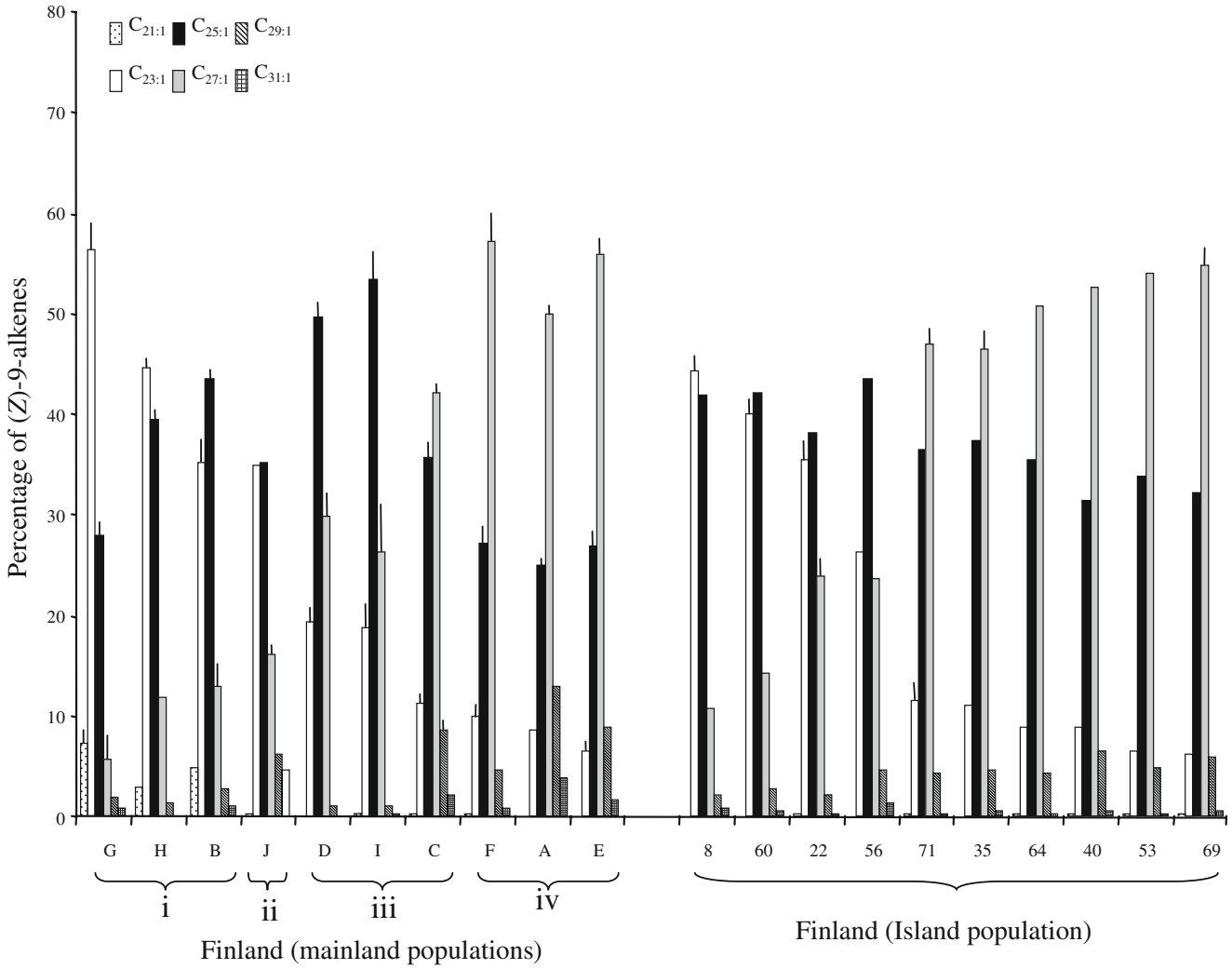

Fig. 2 Relative amounts of various 9-alkenes in *Formica exsecta* colonies sampled from four mainland locations (*i* Byvägen, *ii* Tvarminne, *iii* Gråkärr, and *iv* Harparskog) and a single island population in Finland. Standard deviation is shown where it is >1%

Table 3 The average percentages (of the entire cuticular hydrocarbon profile) of nine C_{25}-dimethylalkanes in 30 *Formica fusca* colonies, based on $n-1$ samples

Colony code	29	28	40	15	32	27	3	13	5	16	22	R13	56	104	97	100	93	103	106	91	99	76	61	64	S24	70	M5	M1	69	57
N	*3*	*10*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*10*	*3*	*3*	*3*	*3*	*4*	*3*	*3*	*3*	*3*	*5*	*3*	*3*	*3*	*3*	*5*	*3*	*5*	*5*	*3*	*3*
11,15-DiMeC$_{25}$								2	1	6	10	3	3				2		3					1	1			1		
9,13-DiMeC$_{25}$		1	6				5	2	1			1	2							14	6	1			1		2	1		12
7,11-DiMeC$_{25}$				2							6	1	2							6		2						3		
7,15-DiMeC$_{25}$					12	16				3			2								2	5								
5,13-DiMeC$_{25}$	23	15	6	12	2	3		6	5			5	12	11	16			8	11			6				14		8		5
5,15-DiMeC$_{25}$							4														17	7	21							
5,17-DiMeC$_{25}$						1	8			1						8			2	8				2		9			17	
3,11-DiMeC$_{25}$											12		1					7												
3,13-DiMeC$_{25}$	5	4	16	23	22	19	9	16	18	9	2	11	1	18	20	21	20		18	3	12	6	16	24	14	3	8	13	10	5
Blind samples																														
29	●																													
28		●																												
40			●																											
15				●																										
32					●																									
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103																		●												

Table 3 (continued)

Colony code	29	28	40	15	32	27	3	13	5	16	22	R13	56	104	97	100	93	103	106	91	99	76	61	64	S24	70	M5	M1	69	57
N	*3*	*10*	*3*	*3*	*3*	*3*	*3*	*3*	*3*	*10*	*3*	*3*	*3*	*3*	*4*	*3*	*3*	*3*	*3*	*5*	*3*	*3*	*3*	*3*	*5*	*3*	*5*	*5*	*3*	*3*
106																			●											
91																				●										
99																					●									
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61																							●							
64																								●						
S24																									●					
70																										●				
M5																											●			
M1																												●		
69																													●	
57																														●

The black dots indicate the blind samples that matched a particular colony based on the rule that the proportions of each C_{25}-dimethylalkane in the blind sample was within ±5% of the proportion in the colony.

randomly chosen from each colony, and the proportions of each signature CHC were calculated based on the total relative amount of CHCs in the profile. This was the blind sample. Then, the average proportion of each signature CHC for each colony was calculated from the remaining individuals of a colony. Each blind sample was assigned to the colony or colonies that possessed the same profile (±5%) with respect to the signature CHCs. A greater amount of inter-colony variation in signature CHCs was observed in *F. fusca* than in *F. exsecta*, and, therefore, there was greater ambiguity in assigning blind samples to a colony for this species. Second, we used hierarchical cluster analysis (Ward's method; SPPS v.14) to assign individual ants to a pre-defined number of clusters (colonies) by using the signature proportional data.

Subsequently, the proportions of the signature CHCs were recalculated by ignoring the rest of the profile so that the signature CHCs accounted for 100% of the profile. Although this did not change the overall result, it made the graphical comparisons clearer and may be more biologically relevant, as different groups of hydrocarbons (e.g., alkanes vs. alkenes) are perceived differently (Sachse et al. 1999; Chaline et al. 2005).

Results

Formica exsecta

The CHC profile of *F. exsecta* was dominated (>95%) by a group of (*Z*)-9-alkenes and a homologous series of alkanes. In *F. exsecta*, the alkenes were poorly correlated at the species level but were highly correlated (r^2>0.95) at the colony level (Table 1), thus making them prime candidates for encoding colony-specific signals. The variation of the six signature (*Z*)-9-alkenes across *F. exsecta* colonies was large (Fig. 1a), especially considering that the average intra-colony variation (SD) in the 20 colonies for all alkenes was small (2±1%). By using only the proportions of the six signature (*Z*)-9-alkenes (9-$C_{21:1}$ to 9-$C_{31:1}$), all 20 blind samples were correctly assigned to their colonies (Table 2), although two ants could also each be assigned to another colony that had a similar profile (Table 2). By using the hierarchical cluster analysis, we also correctly assigned 94 of the 97 individuals into the 20 clusters (colonies), with the three incorrectly assigned ants mixed between colonies D and I. Using only the alkanes, 52% (50 out of 97) of the individuals were assigned to their correct colonies. These data support the unique distributions of the (*Z*)-9-alkenes containing colony-/nest mate-specific information. Although colonies from the various populations may possess similar traits (e.g., the presence of $C_{21:1}$ in the Byvägen population), the amount of variation between colonies within each population was striking (Fig. 2).

Formica fusca

The CHC profile of 111 *F. fusca* workers was composed of homologous series of alkanes, monomethylalkanes, and, unusually, a relatively large number of positional isomers of C_{25}–dimethylalkanes. All the compounds within each homologous series of alkanes or monomethylalkanes were highly correlated at both the species ($r^2>0.9$) and colony level ($r^2>0.9$), indicating that these compounds probably do not contain colony-specific information. Of the 14 different dimethylalkanes detected in the Finnish population, nine exhibited large amounts of variation when compared to other CHCs (Fig. 2b). The nine positional isomers of dimethyl pentacosane contributed 15–37% of the total CHC profile. Each of the 30 colonies possessed two to seven of these nine compounds in a variety of combinations and proportions. By using only the proportions of these nine signature CHCs, all 30 blind samples were assigned to their correct colonies (Table 3). However, there was some ambiguity in assigning two of these ants due to similarity of profiles with other colonies (Table 3). The hierarchical cluster analysis supported the rule-based analysis by correctly assigning 107 of the 111 individuals into 30 clusters (colonies). The variation of these nine signature C_{25}–dimethylalkanes among the 30 *F. fusca* colonies was clearer when their proportions were analyzed independently from the rest of the CHCs in the profiles (Fig. 3). Despite the large variation (range=0–94%) of these signature CHCs among colonies (Fig. 3), the intra-colony variation was low within each of the 30 colonies, with the SD averaging only 3±2% across all signature compounds.

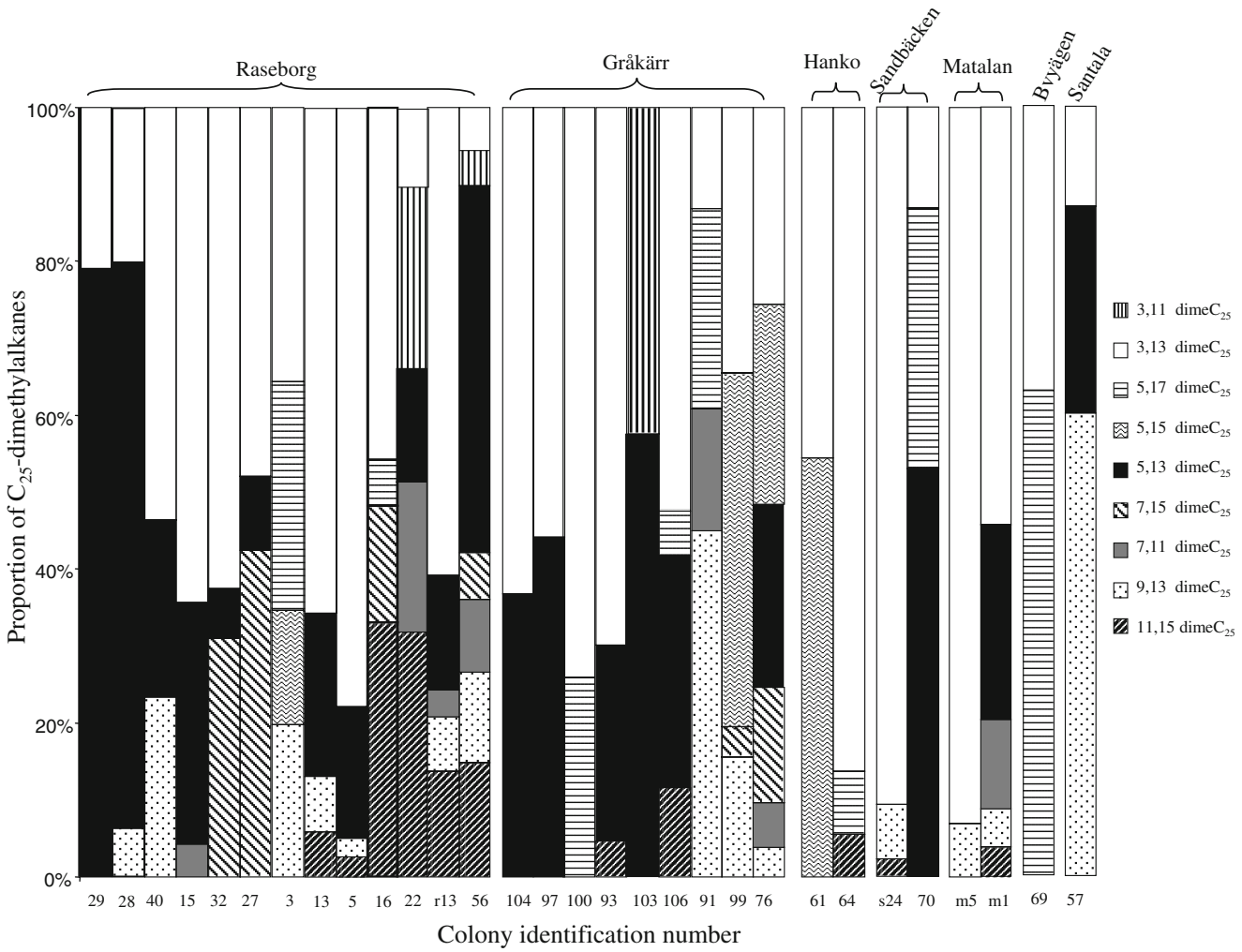

Fig. 3 The relative proportions of nine colony-specific C_{25}–dimethylalkanes from 30 Finish *Formica fusca* colonies collected at seven locations. The colonies are grouped within each population according to the number of C_{25}–dimethylalkanes present. The variation is not shown for the sake of clarity, but the SD averaged 3±2% for all compounds across all the colonies

Discussion

It has been suggested recently that the information encoded within CHC profiles, which social insects use to discriminate between nest mates and non-nest mates, resembles a blurred barcode (Boomsma and Franks 2006). However, we have shown in this study that colony-specific information can be encoded in the CHC profile in two distinct ways, both of which are clear and distinct once identified from the rest of the CHC profile.

Mechanism 1: Altering the Proportions of a Single Type of Hydrocarbon (*F. exsecta*)

Despite *F. exsecta* possessing one of the most basic CHC profiles among the *Formica* ants, it encodes colony-specific information in the proportions of individual hydrocarbons within a single homologous series (of 9-alkenes). Such encoding presumably requires precise control during each step of the chain-lengthening process or in the lipophorin transport system (Schal et al. 2001; Lucas et al. 2004). The encoding of information in a single homologous series of hydrocarbons limits the number of possible unique signatures across colonies. Despite this, *F. exsecta* populations are able to produce a wide variety of distinct colony-specific distributions of alkenes (Fig. 3). Recent experimental evidence (Martin et al. 2008a) with synthetic CHCs has shown that *F. exsecta* uses (*Z*)-9-alkenes to encode nest-mate recognition signals. Our interpretation is supported by data on *F. japonica* in Japan (Akino et al. 2004), which has a similar CHC profile to *F. exsecta*. When synthetic CHCs (9-alkenes and alkanes) were placed on a glass dummy in proportions that mimic a particular colony, *F. japonica* nest mates were not aggressive and non-nest mates were aggressive toward the dummy in the two colonies tested.

Mechanism 2: Synthesis of Colony-specific Compounds (*F. fusca*)

A review of the CHC literature shows that dimethylalkanes in a given species are synthesized principally in one-carbon chain length, as found in *F. fusca* or in limited homologous series (Howard and Blomquist 2005). If only the presence or absence of the nine C_{25}–dimethylalkanes found in *F. fusca* encoded a colony signature, then 512 (2^9) unique colony signatures could exist. Among the 30 colonies studied, 24 unique profiles were found based on combinations of presence or absence of specific compounds, thus illustrating the diverse production of CHC profiles within this species. However, the proportions of each C_{25}–dimethylalkane also varied between colonies, potentially increasing the number of unique colony profiles (e.g., up to 10^9 if a 10% discrimination window is used) dramatically. Behavioral work is needed to determine what differences in a given CHC profile are allowable before it is no longer recognized as that of a nest mate.

The role of methyl-branched hydrocarbons in nest-mate recognition has been implicated in *Polistes* wasps (Dani et al. 2001) and the ants *Camponotus vagus* (Clement et al. 1987), *Messor barbarus* (Provost et al. 1994), possibly *Cataglyphis* spp (Dahbi et al. 1996), and now *F. fusca* (this study). However, in all these studies, including this one, bioassays that use synthetic methyl-branched hydrocarbons have yet to be conducted. This work needs to be carried out to confirm that these compounds are indeed used in nest-mate recognition. From an evolutionary perspective, dimethylalkanes are ideally suited to function as communication molecules because there are numerous isomers for a given chain length, and a relatively minor chemical change, such as in position of a methyl group, can have a profound effect on the conformation of the molecule. This may explain why dimethylalkanes are by far the most diverse type of hydrocarbon found among the ants (Dahbi et al. 1996).

The two systems described here or variations of them may be expected to occur in many species of social insects that have strong colony identity. We believe that the addressing of the roles of chain length, detection thresholds, and biosynthesis in social insect CHCs will provide insight into the world of insect communication and the role of nest-mate recognition in the evolution of sociality.

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References

AKINO, T., YAMAMURA, K., WAKAMURA, S., and YAMAOKA, R. 2004. Direct behavioural evidence for hydrocarbons as nest mate recognition cues in *Formica japonica* (Hymenoptera: Formicidae). *Appl. Entomol. Zool.* 39:381–387.

BEYE, M., NEUMANN, P., CHAPUISAT, M., PAMILO, P., and MORITZ, R. F. A. 1998. Nest mate recognition and genetic relatedness of nests in the ant *Formica pratensis*. *Behav. Ecol. Sociobiol.* 43:67–72.

BEYE, M., NEUMANN, P., and MORITZ, R. F. A. 2004. Nest mate recognition and the genetic gestalt in the mound-building ant *Formica polyctena*. *Insectes. Soc.* 44:49–58.

BOOMSMA, J. J., and FRANKS, N. R. 2006. Social insects: from selfish genes to self organization and beyond. *Trends Ecol. Evol.* 21:303–308.

BREED, M. D. 1998. Recognition pheromones of the honey bee. *BioScience* 48:463–470.

CARLSON, D. A., ROAN, C-S., YOST, R. A., and HECTOR, J. 1989. Dimethyl disulfide derivatives of long chain alkenes, alkadienes, and alkatrienes for gas chromatography/mass spectrometry. *Anal. Chem.* 61:1564–1571.

CHALINE, N., SANDOZ, J. C., MARTIN, S. J., RATNIEKS, F. L. W., and JONES, G. R. 2005. Learning and discrimination of individual cuticular hydrocarbons by honey bees (*Apis mellifera*). *Chem. Senses* 30:327–333.

CLÉMENT, J. L., BONAVITA-COUGOURDAN, A., and LANGE, C. 1987. Nestmate recognition and cuticular hydrocarbons in *Camponotus vagus* Scop., p. 473, in J. Eder, and H. Rembold (eds.). Chemistry and Biology of Social Insects. Peperny, München.

CZECHOWSKI, W. 1990. Intraspecific conflict in *Formica exsecta* Nyl. (Hymenoptera, Formicidae). *Memorabilia Zool.* 44:71–81.

DAHBI, A., LENOIR, A., TINAUT, A., TAGHIZADEH, T., FRANCKE, W., and HEFETZ, A. 1996. Chemistry of the postpharyngeal gland secretion and its implication for the phylogeny of Iberian *Cataglyphis* species (Hymenoptera: Formicidae). *Chemoecology* 7:163–171.

DANI, F. R., JONES, G. R., DESTRI, S., SPENCER, S. H., and TURILAZZI, S. 2001. Deciphering the recognition signature within the cuticular chemical profiles of paper wasps. *Anim. Behav.* 62:165–171.

DENIS, D., BLATRIX, R., and FRESNEAU, D. 2007. How an ant manages to display individual and colonial signals by using the same channel. *J. Chem. Ecol.* 32:1647–1661.

FIELDE, A. M. 1901. Further study of an ant. *Proc. Acad. Nat. Sci. U.S.A* 53:425–449.

FIELDE, A. M. 1903. Artificial mixed nests of ants. *Biol. Bull.* 5: 320–325.

HOWARD, R. W., and BLOMQUIST, G. J. 2005. Ecological, behavioral, and biochemical aspects of insect hydrocarbons. *Annu. Rev. Entomol.* 50:371–393.

JACKSON, B. D., and MORGAN, E. D. 1993. Insect chemical communication: pheromones and exocrine glands of ants. *Chemoecology* 4:125–144.

LAHAV, S., SOROKER, V., HEFETZ, A., and VANDER MEER, R. K. 1999. Direct behavioral evidence for hydrocarbons as ant recognition discriminators. *Naturwissenschaften* 86:246–249.

LOCKEY, K. H. 1988. Lipids of the insect cuticle: origin, composition and function. *Comp. Biochem. Physiol.* 89B:595–645.

LUCAS, C., PHO, D. B., FRESNEAU, D., and JALLON, J. M. 2004. Hydrocarbon circulation and colonial signature in *Pachycondyla villosa*. *J. Insect Physiol*. 50:595–607.

MARTIN, S. J., VITIKAINEN, E., HELANTERÄ, H., and DRIJFHOUT, F. P. 2008a. Chemical basis of nest-mate discrimination in the ant *Formica exsecta*. *Proc. R. Soc., Lond. B* 275:1271–1278.

MARTIN, S. J., HELANTERÄ, H., and DRIJFHOUT, F. P. 2008b. Evolution of species-specific cuticular hydrocarbon patterns in formica Ants. *Biol. J. Linn. Soc.* (in press).

OZAKI, M., WADA-KATSUMATA, A., FUJIKAWA, K., IWASAKI, M., YOKOHARI, F., SATOJI, Y., NISIMURA, T., and YAMAOKA, R. 2005. Ant nestmate and non-nestmate discrimination by a chemosensory sensillium. *Science* 309:311–315.

PROVOST, E., RIVIÈRE, G., ROUX, M., BAGNÉRES, A. G., and CLÉMENT, J. L. 1994. Cuticular hydrocarbons whereby *Messor barbarus* ant workers putatively discriminate between monogynous and polygynous colonies. Are workers labeled by queens? *J. Chem. Ecol*. 20:2895–3003.

SACHSE, S., RAPPERT, A., and GALIZIA, C. G. 1999. The spatial representation of chemical structures in the antennal lobe of honeybees: steps towards the olfactory code. *Eur. J. Neurosci.* 11:3970–3982.

SCHAL, C., SEVALA, V., CAPURRO, M. de L., SNYDER, T. E., BLOMQUIST, G. J., and BAGNÈRES, A. G. 2001. Tissue distribution and lipophorin transport of hydrocarbons and sex pheromones in the house fly, *Musca domestica*. *J. Insect Sci*. 1:1–11.

SUNDSTRÖM, L., CHAPUISAT, M., and KELLER, L. 1996. Conditional manipulation of sex ratios by workers a test of kin selection theory. *Science* 274:993–995.

SUNDSTRÖM, L., SEPPÄ, P., and PAMILO, P. 2005. Genetic population structure and dispersal patterns in Formica ants—a review. *Ann. Zool. Fenn.* 42:163–177.

WALLIS, D. I. 1962. Aggressive behaviour in the ant *Formica fusca*. *Anim. Behav.* 10:267–274.

of species of several major groups of glandulate oribatids have been investigated, including representatives of Parhyposomata (Sakata and Norton 2001), a few species of Mixonomata and Desmonomata (Sakata et al. 1995, 2003; Raspotnig et al. 2001, 2005a, b; Shimano et al. 2002; Sakata and Norton 2003), and also examples of Brachypylina (Takada et al. 2005; Saporito et al. 2007). While Parhyposomata produce phenols and naphthols, the so-called astigmatid compounds (sensu Sakata and Norton 2001) evolved within ancient mixonomatans, and hence, are possibly found in all the above groups. These astigmatid compounds comprise a set of terpenes and aromatics, namely neral, geranial, neryl formate, 2-hydroxy-6-methyl-benzaldehyde, and γ-acaridial. The distribution of astigmatid compounds in oribatids is of special interest since these compounds not only provide chemical support for the evolutionary origin of astigmatid mites within ancient Oribatida (Norton 1998), but also define a presumably monophyletic subunit within glandulate Oribatida, the "astigmatid compounds-bearing" oribatids (Raspotnig 2006).

Several chemotaxonomic studies that examined these presumptive astigmatid compound-bearing groups have been undertaken and have yielded a fragmented picture of the distribution of astigmatid compounds. These compounds are difficult to detect in the more derived groups of Desmonomata (apart from Trhypochthoniidae) and generally in Brachypylina, suggesting that, in these groups, astigmatid compounds have been prone to evolutionary reduction and replacement. In some cases, desmonomatan and brachypyline species are chemically "dimorphic", with astigmatid compounds present only in juveniles and completely replaced in adults by novel compounds such as toxic alkaloids (Takada et al. 2005; Saporito et al. 2007). In contrast, the full (or nearly full) set of astigmatid compounds seems to characterize highly derived mixonomatans, low to moderately derived representatives of Desmonomata, and Astigmata. Specifically, these compounds have been detected in opisthonotal gland secretions of *Collohmannia gigantea* (Raspotnig et al. 2001), in all Trhypochthoniidae so far investigated (e.g., Sakata et al. 1995, 2003), and in astigmatid mites (e.g., Kuwahara 2004). However, there are large gaps in our knowledge of the distribution of astigmatid compounds: the most conspicuous gap being in the Euphthiracaroidea, a speciose group of box mites whose opisthonotal gland chemistry has yet to be investigated. Euphthiracaroids are considered a group of highly derived mixonomatans that, together with phthiracaroids, constitute a possible sister group of *Collohmannia*. Thus, euphthiracaroids are likely candidates for producing astigmatid compounds. The study of the chemistry of opisthonotal glands in euphthiracaroids is important not only to further our understanding of the evolution of astigmatid compounds, but also because this group is a crucial link for tracing a possible evolutionary lineage from Oribatida to Astigmata. With respect to the latter, some (not all!) recent molecular studies (e.g., Murell et al. 2005; Domes et al. 2007) conflict with morphological data. Therefore, studying the opisthonotal gland chemistry may resolve this problem.

In the present paper, we provide the first chemical analysis of the opisthonotal gland secretion of *Oribotritia berlesei*, a large-sized oribatid mite from the near-basal euphthiracaroid family Oribotritiidae.

Methods and Materials

Mites and Mite Extracts Specimens of *O. berlesei* (Michael 1898) (mixonomatan Oribatida: Euphthiracaroidea, Oribotritiidae) were collected from the litter and fermentation layer of a mixed forest near Ferlach (Carinthia, Austria). Individuals were collected by hand or extracted from soil samples by using Berlese-Tullgren funnels. In all, 35 adults (all in the course of one collection in September 2006) were used for chemical analyses, five adults were used for scanning electron microscopy, and 30 adults were transferred to Petri dishes (equipped with Plaster of Paris and dead wood) and kept in the dark for a period of about 12 months either at room temperature (one Petri dish) or at 10°C (second Petri dish), respectively. Individuals of both Petri dishes laid eggs, and the offspring were reared to deutonymphal and tritonymphal instars. Since immatures are burrowers in dead wood, pieces of dead wood (provided in the rearing dishes) were checked for immatures at weekly intervals. Ten deutonymphs and eight tritonymphs were used in the chemical studies.

Extracts (containing opisthonotal gland secretions) of adults were prepared by immersing freshly collected, living, individuals in hexane (one individual per 100 μl) for 30 min (Raspotnig et al. 2001, 2005a, b). For juveniles, individual and pooled extracts (containing two to six individuals) were prepared similarly. The extracts were used without further cleanup.

Chemical Analysis: Gas Chromatography—Mass Spectrometry A trace gas chromatograph (GC) coupled to a Voyager mass spectrometer (MS; both from Thermo, Vienna, Austria) and equipped with a ZB-5MS fused silica capillary column (30 m×0.25 mm i.d., 0.25 μm film thickness, Phenomenex, Germany) was used for the analyses. Injection was splitless with helium (at a constant flow rate of 1.5 ml min^{-1}) as a carrier gas. The column temperature was programmed from 50°C (held for 1 min) to 200°C at 10°C min^{-1}, and then to 300°C at 15°C min^{-1}. The ion source of the mass spectrometer and the transfer line were kept at 150°C and 310°C, respectively. Electron impact (EI) spectra were recorded at 70 eV.

Derivatization, Syntheses, Reference Compounds, and Other Chemicals Tri-, penta-, and heptadecane, as well as citral (60% geranial, 40% neral) and selenium dioxide for the preparation of oxocitral (see Bellesia et al. 1986), were purchased from Aldrich (Vienna, Austria). For determination of double bonds in the heptadecadiene, dimethyl-disulfide (DMDS) adducts were generated according to the method described by Vincenti et al. (1987). The opisthonotal gland secretion of *Platynothrus peltifer* (Raspotnig et al. 2005b) was used as a natural source for 6,9-heptadecadiene and 8-heptadecene for chromatographic comparisons. In order to clarify further the structure of chrysomelidial, the *O*-methyl-oxime derivatives were prepared with methoxylamine hydrochloride (MOX.HCl; 2% in pyridine): 200 µl of MOX was added to 50 µl of extract and left at 70°C for 1 h. Products were cleaned in H_2O, extracted with hexane, and analyzed. Stereoisomers of chrysomelidial and dehydroiridodial were synthesized according to Bellesia et al. (1986). Secretions from eversible defense glands of larvae of the chrysomelid beetles, *Gastrophya viridula* and *Plagiodera versicolora* (collected in the surroundings of Graz, Austria), known to contain natural (3*S*,8*S*)-chrysomelidial (e.g., Pasteels et al. 1982), were collected on small filter paper pieces, extracted in hexane (one individual in 100 µl), and used as sources for authentic reference compounds. Hexane extracts of fresh leaves of *Actinidia polygama* (obtained from the botanical garden in Tübingen, Germany) were taken as a natural source for (3*R*,8*S*)-dehydroiridodial (Yoshihara et al. 1978).

Scanning Electron Microscopy Air-dried mites were mounted on aluminum stubs, sputtercoated (AGAR sputtercoater, Gröpl, Tulln, Austria), and examined with a Philips XL30 ESEM (Philips/FEI, Vienna, Austria) at high vacuum mode and 20 kV accelerating voltage.

Results

Extracts of Adult O. berlesei A total of ten components (peaks a–j) were detected in extracts of adult *O. berlesei* (Fig. 1a). The main component in extracts, component c (RT=13.36 min; ca. 45% of the total extract, based on peak areas) had an EI fragmentation pattern resembling a methyl-cyclopentene monoterpene (Fig. 2a), with a molecular ion at *m*/*z* 166 (7) and characteristic fragments at *m*/*z* 151 (6), 148 (46), 138 (25), 133 (9), 123 (15), 120 (12), 119 (10), 109 (49), 108 (40), 105 (30), 95 (19), 91 (27), 81 (100), 79 (78), and 77 (38). The spectrum was consistent with that of chrysomelidial from the literature (e.g., Blum et al. 1978; Oldham et al. 1996) and with a compound from the NIST library ("2-formyl-α,3-dimethyl-2-cyclopentene-1-acetaldehyde"; CAS no. 75332-42-2: "2-methyl-5(1-methyl-2-oxoethyl)-1-cyclopentene-1-carbaldehyde"). In order to prove the dialdehyde structure, we generated *O*-methyl-oxime derivatives, resulting in the elimination of peak c and the formation of two new peaks, c′ and c″, with longer retention times (RT=14.92 and 15.11 min). The two compounds had identical mass spectra, with molecular ions at *m*/*z* 224 (4), and fragment ions at *m*/*z* 193 (13), 178 (22), 146 (25), 138 (100), 107 (38), 106 (95), 91 (18), 87 (21), 79 (47), and 77 (29). This pattern indicated *O*-methyl-oximation of compound c, forming two isomeric dimethyloximes, which confirmed the proposed dialdehydic structure (addition of two *O*-methyl-oxime groups to compound c= 166 plus 2×29=new molecular ion at *m*/*z* 224).

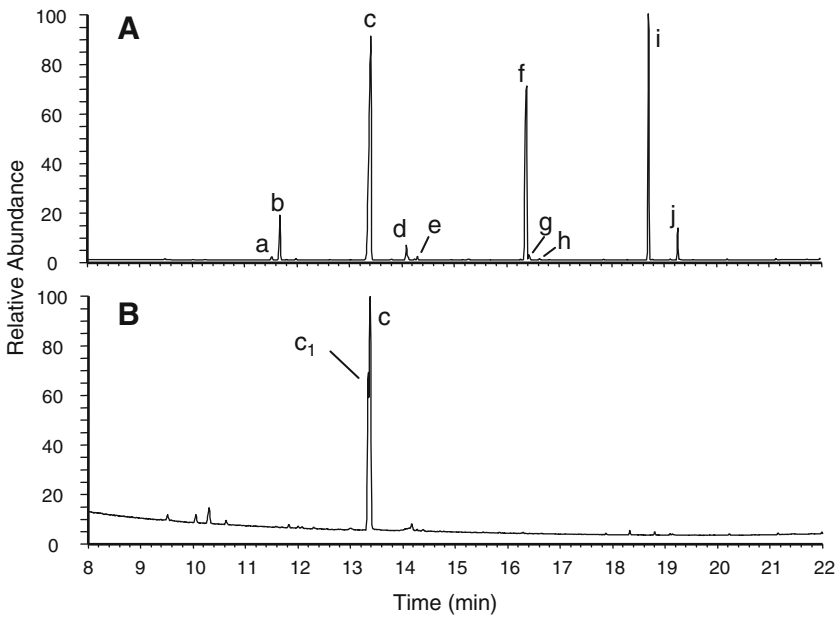

Fig. 1 Typical mass chromatograms of extracts of *Oribotritia berlesei*: **a** adult individual. **b** Deutonymph. Peaks *a* (tridecene), *b* (tridecane), c_1 ("epi-chrysomelidial" = 3*S*,8*R*-chrysomelidial), *c* ("chrysomelidial" = 3*S*,8*S*-chrysomelidial), *d* (pentadecene), *e* (pentadecane), *f* (6,9-heptadecadiene), *g* (8-heptadecene), *h* (heptadecane), *i* (tentatively, β-springene), *j* (tentatively, 9,17-octadecadienal)

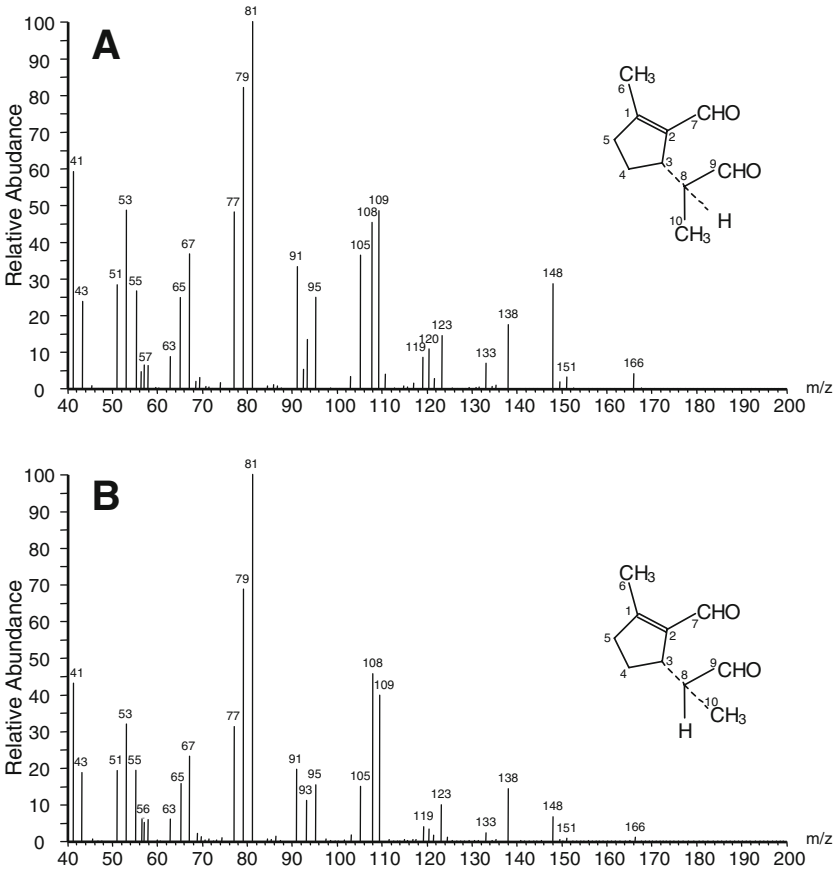

Fig. 2 Electron impact mass spectra of chrysomelidials in extracts of *Oribotria berlesei*. **a** Compound c, tentatively identified as (3*S*,8*S*)-chrysomelidial. **b** Compound c_1, tentatively identified as epi-chrysomelidial = (3*S*,8*R*)-chrysomelidial. Note the differences in relative intensities of ions at *m*/*z* 148 (M^+–H_2O) and *m*/*z* 138 (M^+–CO). Further diagnostic fragments are assigned to: M^+–H_2O–CO (*m*/*z* 120); $C_7H_9O^+$ (*m*/*z* 109), $C_7H_7^+$ (*m*/*z* 91), and $C_6H_9^+$ (*m*/*z* 81: base peak = the methyl-cyclopentene moiety)

For a comparison of compound c to authentic chrysomelidial and its characterization, we used (1) a synthetic mixture of chrysomelidial-stereoisomers, and (2) naturally occurring (3*S*,8*S*)-chrysomelidial. The synthetic mixture was assumed to contain four stereoisomers of chrysomelidial and dehydroiridodial (according to Bellesia et al. 1986), namely two diastereomeric pairs of enantiomers, i.e., (3*S*,8*S*)-chrysomelidial, (3*R*,8*R*)-dehydroiridodial, and (3*S*,8*R*)-chrysomelidial (= epi-chrysomelidial), (3*R*,8*S*)-dehydroiridodial. However, the mixture exhibited only two, poorly separable peaks at RT=13.32 and 13.36 min, each peak corresponding to one pair of enantiomers, with single enantiomers not separable under the chromatographic conditions. The later-eluting peak of the mixture corresponded to compound c from the *Oribotritia* extract, with respect to both retention time and EI fragmentation pattern. Larval secretions of the leaf beetle *Gastrophysa viridula* (see "Methods and Materials"), contain mainly (3*S*,8*S*)-chrysomelidial ("original chrysomelidial"). Analysis of this secretion showed only one abundant component that eluted at RT=13.36 min, corresponding to the later-eluting peak of the synthetic mixture and to compound c of the mite extracts. Thus, the later-eluting peak of the synthetic mixture was assigned as either (3*S*,8*S*)-chrysomelidial or its enantiomer, (3*R*,8*R*)-dehydroiridodial. Since dehydroiridodials are considered of plant origin only (see "Discussion"), compound c was tentatively identified as (3*S*,8*S*)-chrysomelidial.

The mass spectrum of peak *f* (RT=16.36 min), the second most abundant compound in extracts (about 25% of total), suggested a doubly unsaturated C17-hydrocarbon, based on a molecular ion at *m*/*z* 236 and a series of unsaturated hydrocarbon fragments. The DMDS adduct of the heptadecadiene showed a molecular ion at *m*/*z* 362 and diagnostic ions at *m*/*z* 231, 203, 183, 159, 155 (base peak), and 131, consistent with 6,9-heptadecadiene (see Raspotnig et al. 2005b). Compound i (RT=18.70 min; ca. 20% of the total) showed a terpene fragmentation pattern with a molecular ion at *m*/*z* 272 (4) and further *m*/*z* of 257 (3), 229 (4), 203 (5), 187 (10), 161 (17), 133 (31), 120 (15), 119 (17), 107 (20), 93 (50), 81 (37), 69 (100), 55 (16), 53 (17), and 41 (59). The compound was tentatively identified as β-springene on the basis of mass spectral comparison to β-springene spectra from literature and from the NIST library. The remaining peaks a, b, d, e, g, and h, all of them minor components, appeared to be a series of hydrocarbons, with compounds b (RT=11.69), e (RT=14.32 min), and h (RT=16.65 min) exhibiting the spectra of saturated *n*-alkanes with molecular ions at *m*/*z* 184, 212, and 240, respectively. The compounds were identified as tri-, penta-, and heptadecane by comparison of retention times to authentic

standards. Compounds a (RT=11.54), d (RT=14.10 min), and g (RT=16.44 min) had mass spectra of mono-unsaturated analogs, with molecular ions at *m*/*z* 182, 210, and 238, corresponding to tri-, penta-, and heptadecene. The position of the double bonds was not determined because, due to their low amounts, the compounds were not recovered after DMDS derivatization. The retention time of compound g was the same as that of 8-heptadecene (from extracts of *P. peltifer*, see "Methods and Materials"). Compound j (RT=19.27 min), also a minor component, had the following mass spectrum: *m*/*z* 264 (11), 235 (3), 221 (4), 207 (4), 165 (4), 151 (7), 137 (10), 123 (10), 109 (22), 95 (61), 81 (16), 79 (52), 67 (100), 55 (38), 54 (32), 44 (32), and 41 (69). The compound was tentatively identified as 9,17-octadecadienal on the basis of these mass spectral data.

The pattern of the ten compounds was consistently detected in 30 of the 35 individuals of adult *O. berlesei* (Fig. 1a), with relatively low inter-individual variation with respect to the relative abundance of components (data not shown). The other five extracts did not contain any of the components.

Extracts of Juvenile O. berlesei Extracts of juveniles (exclusively deuto- and tritonymphs) contained only two compounds in a 1:2 ratio (peak c_1 and c in Fig. 1b): The major component c corresponded to tentatively identified (3*S*,8*S*)-chrysomelidial from the adult extracts, based on identical retention time and mass spectrum. The minor component c_1 eluted earlier (RT=13.32 min) but was not fully separable from (3*S*,8*S*)-chrysomelidial. The mass spectrum of compound c_1 appeared to be similar to that of (3*S*,8*S*)-chrysomelidial (Fig. 2); one difference, however, was the greater intensity of the ion at *m*/*z* 138 in relation to the ion at *m*/*z* 148 [c.f., in (3*S*,8*S*)-chryomelidial the ion at *m*/*z* 148 is more intense]. These data suggested the compound to be one of the other possible stereoisomers of chrysomelidial, most likely an epimeric diastereomer of (3*S*,8*S*)-chrysomelidial. Moreover, the retention time and mass spectrum of compound c_1 fully corresponded with those of the earlier-eluting peak of the synthetic chrysomelidial mixture, limiting the possibilities to epi-chrysomelidial (3*S*,8*R*-chrysomelidial) and its enantiomer, (*3R*,*8S*)-dehydroiridodial. In addition, its retention time and mass spectrum matched those of plant-derived (*3R*,*8S*)-dehydroiridodial. Thus, again assuming that dehydroiridodials are exclusively plant-derived compounds, compound c_1 was tentatively identified as epi-chrysomelidial (3*S*,8*R*-chrysomelidial).

From a total of eight extracts of juvenile *O. berlesei* investigated (derived from 18 individuals), seven extracts consistently showed these two stereoisomers only; the other extract (an extract of a single individual) apparently did not contain any compounds at all.

Potential Secretory Glands The most conspicuous exocrine gland system of *O. berlesei* is the paired opisthonotal glands that, in light microscopic observations, are visible through the cuticle of the notogaster as kidney-shaped sacs of considerable size (maximal diameter about 0.5 mm: i.e., ca. 1/3 of body length; see Fig. 3b). The glands open to the notogastral surface via one single flap-provided pore orifice on either side of the body (Fig. 3a,c,d). The flap,

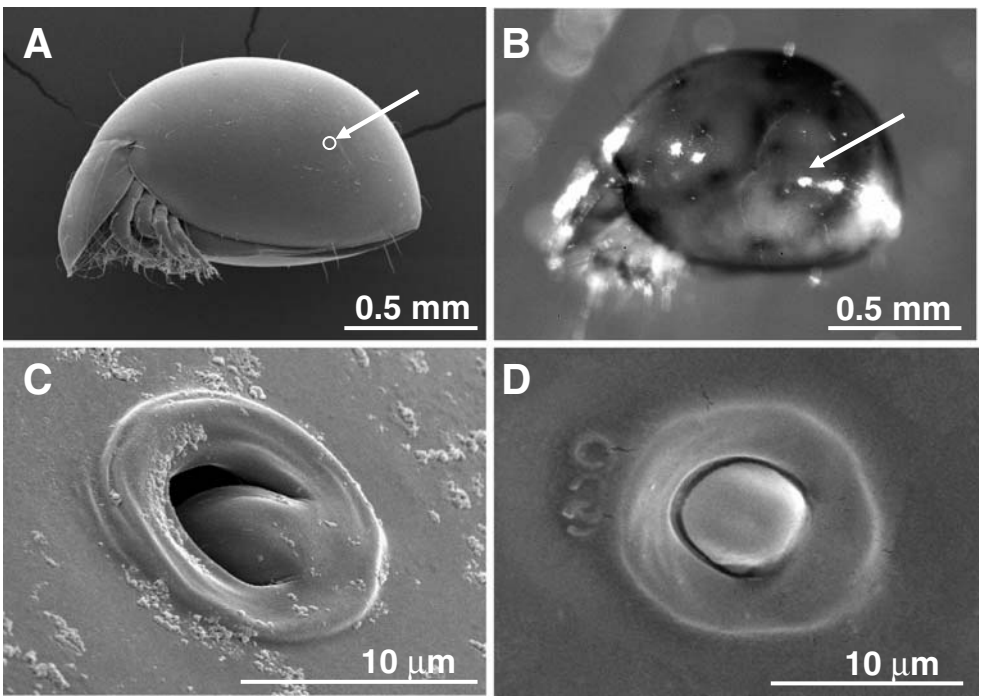

Fig. 3 Topography and external morphology of opisthonotal glands in *Oribotritia berlesei*. **a** Scanning electron micrograph (SEM) of an adult individual, *lateral view*. The *arrow marks* the orifice of the left opisthonotal gland. **b** Opisthonotal glands are visible through the integument under light microscopy. **c** SEM of an opisthonotal gland pore with flap open. **d** SEM of the pore of another individual, orifice closed

presumably an external closing mechanism, was found either in an opened (Fig. 3c) or a closed position (Fig. 3d) in different individuals. No other comparably large glands or pore orifices are present in this species. Opisthonotal glands are present in adults and in all juvenile stages.

Discussion

Occurrence of Chrysomelidial Even though iridoid monoterpenes are widely distributed in exocrine secretions of a diverse range of arthropods, chrysomelidial, so far, has only been reported from leaf beetle larvae (Chrysomelidae) and from certain rove beetles (e.g., Pasteels et al. 1982; Weibel et al. 2001). Originally, chrysomelidial was found as a major constituent of the larval eversible glands of the chrysomelids, *Gastrophysa cyanea* (Blum et al. 1978) and *Plagiodera versicolora* (Meinwald et al. 1977), and its stereochemistry later elucidated by Meinwald and Jones (1978). According to these studies, and to current knowledge, chrysomelidial in leaf beetles is a mixture of two diastereomers, (3*S*,8*S*-chrysomelidial (= the original chrysomelidial) and (3*S*,8*R*)-chrysomelidial (= epi-chysomelidial), with the former being the predominant isomer in the majority of chrysomelidial-producing species (e.g., Sugawara et al. 1979; Pasteels et al. 1982). In contrast, the remaining two possible stereoisomers (there are four stereoisomers due to the two asymmetric carbon atoms at C_3 and C_8) are termed "dehydroiridodials" and are considered of plant origin only (Yoshihara et al. 1978; Sugawara et al. 1979). Under the conditions used in this study, we were able to separate the diastereomers but not the respective enantiomers of chrysomelidial and dehydroiridodial. Assignments of chrysomelidials and dehydroiridodials are often misinterpreted in the literature. For instance, Bellesia et al. (1986) described the chrysomelidials as a pair of enantiomers, whereas they are in fact diastereomers. In the present paper, we use the classification of Sugawara et al. (1979) for these compounds, but also use the more common nomenclature of Bellesia et al. (1986). Thus, assuming dehydroiridodials are found exclusively in plants, the compounds in *O. berlesei* extracts are (3*S*,8*S*)-chrysomelidial and (3*S*,8*R*)-chrysomelidial (epi-chrysomelidial).

Our analysis of *O. berlesei* is not only the first example of the chemistry of exocrine glands of Euphthiracaroidea but also the only report of chrysomelidials found outside the Coleoptera. In *O. berlesei*, chrysomelidials most likely originate from the opisthonotal glands: these glands are quite large in *O. berlesei*, and thus probably capable of producing compounds in the amount detected. Furthermore, some of the components, especially 6,9-heptadecadiene and some other hydrocarbons, found in the extract are characteristic constituents of opisthonotal glands of many oribatid (and astigmatid) mite species (Kuwahara 2004; Raspotnig 2006). Functionally, at least in Chrysomelidae, iridoid monoterpenes such as chrysomelidials are considered defensive or for microbial protection; these functions are also generally attributed to opisthonotal glands of Oribatida (Raspotnig et al. 2003).

Chemosystematic Impact With respect to chemosystematic studies that use opisthonotal gland profiles of Oribatida, the finding of chrysomelidials in *O. berlesei* is significant. In preliminary investigations, chrysomelidials have been found also in other species of Euphthiracaroidea (data unpublished), suggesting that this chemical class may be characteristic for species of Oribotritiidae, possibly representing an important chemical "synapomorphy" of this family. Also, two further compounds in extracts of *O. berlesei*, if our tentative identifications are correct, would be novel for oribatid and astigmatid mite opisthonotal gland secretions. The diterpene, β-springene, which we tentatively identified as one of the three main components of the secretion, was originally found in secretions of springboks (Burger et al. 1978), and has also been detected in exocrine secretions of further vertebrates, especially mammals and reptiles (e.g., Waterhouse et al. 1996; Schulz et al. 2003), and invertebrates such as certain hymenopterans (e.g., Howard et al. 2003; Cruz-Lopez et al. 2005), but not in arachnids. The other novel compound (for oribatid and astigmatid mite opisthonotal gland secretions) is the tentatively identified 9,17-octadecadienal, a minor constituent of the *O. berlesei* extract. Both tentatively identified compounds, β-springene and 9,17-octadecadienal, may be of chemosystematic value in Oribatida. Their unequivocal identification and possible distribution across other species of Euphthiracaroidea remain to be studied.

Astigmatid Compounds Perhaps the most surprising result of our study is the lack of "astigmatid compounds" in the opisthonotal gland extract of *O. berlesei*. These compounds are common secretion constituents in Mixonomata–Desmonomata and Astigmata, and are regarded as characteristic of all groups above middle-derived mixonomatans. In Collohmanniidae (proposed close relatives of euphthiracaroids), astigmatid compounds dominate opisthonotal gland secretions. Astigmatid compounds, at least in some higher-derived desmonomatans and in brachypylines, may be reduced and replaced by other (novel) compounds in adult secretions of certain groups as in, for example, *Hermannia convexa* (Raspotnig et al. 2005a) and *Scheloribates* ssp. (Takada et al. 2005). Thus, there is a chemical dimorphism between juveniles and adults, with juvenile secretions being distinguished by the plesiomorphic, astigmatid compounds-rich condition. This chemical dimorphism was tested in *O. berlesei*; however, neither adults nor juveniles contained any astigmatid compounds.

Even though the lack of astigmatid compounds in *O. berlesei* may be explained by complete evolutionary reduction, this situation may suggest a possible polyphyletic origin of astigmatid compounds in opisthonotal glands in general. The lack of astigmatid compounds is even more surprising in light of the classification system proposed by Haumann (1991), in which oribotritiids are regarded as basal euphthiracaroids. Astigmatid compounds-free opisthonotal gland secretions in adults and juveniles also occur in several groups of brachypylines (Raspotnig, unpublished data), but these groups are considered highly derived. The lack of astigmatid compounds in Oribotritiidae could be reconciled by a systematic replacement of euphthiracaroids below the Collohmanniidae, corresponding to a possible split from the oribatid stem-line before astigmatid compounds evolved. However, this is not supported by morphological characters (Grandjean 1969). The lack of astigmatid compounds may not necessarily be true for all Oribotritiidae, and any extrapolation from data for *O. berlesei* remains speculative. Our major aim for future studies is the search for astigmatid compounds in other representatives of euphthiracaroid families.

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References

BELLESIA, F., GUELFI, F., PAGNONI, U. M., and PINETTI, A. 1986. The base-catalyzed cyclization of 10-oxocitral. Synthesis of chrysomelidial and dehydroiridodial. *Tetrahedron Lett.* 27:381–382.

BLUM, M. S., WALLACE, J. B., DUFFIELD, R. M., BRAND, J. M., FALES, H. M., and SOKOLOSKI, E. A. 1978. Chrysomelidial in the defensive secretion of the leaf beetle *Gastrophysa cyanea* Melsheimer. *J. Chem. Ecol.* 4:47–53.

BURGER, B. V., LE ROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R. C. 1978. Mammalian pheromone studies. III. (E,E)-7,11,15-Trimethyl-3-methylenehexadeca-1,6,10,14-tetraene, a new diterpene analogue of b-farnesene from the dorsal gland of the springbok, *Antidorcas marsupialis*. *Tetrahedron Lett.* 52:5221–5224.

CRUZ-LOPEZ, L., MALO, E. A., MORGAN, E. D., RINCON, M., GUZMAN, M., and ROJAS, J. C. 2005. Mandibular gland secretion of *Melipona beecheii*: chemistry and behavior. *J. Chem. Ecol.* 31:1621–1632.

DOMES, K., ALTHAMMER, M., NORTON, R. A., SCHEU, S., and MARAUN, M. 2007. The phylogenetic relationship between Astigmata and Oribatida (Acari) as indicated by molecular markers. *Exp. Appl. Acarol.* 42:159–171.

GRANDJEAN, F. 1969. Considerations sur le classement des Oribates. Leur division en 6 groupes majeurs. I. Les affinites de *Collohmannia gigantea* Sellnick, 1922. *Acarologia* 11:127–153.

HAUMANN, G. 1991. Zur Phylogenie primitiver Oribatiden (Acari: Oribatida). DBV-Verlag, Graz.

HOWARD, R. W., BAKER, J. E., and Morgan, E. D. 2003. Novel diterpenoids and hydrocarbons in the Dufour gland of the ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae). *Arch. Insect Biochem. Physiol.* 54:95–109.

KUWAHARA, Y. 2004. Chemical ecology of astigmatid mites, pp. 76–109, in R. T. Cardé, and J. G. Millar (eds.). Advances in Insect Chemical EcologyCambridge University Press, Cambridge.

MEINWALD, J., and JONES, T. H. 1978. Synthesis and stereochemistry of chrysomelidial and plagiolactone. *J. Am. Chem. Soc.* 100:1883–1886.

MEINWALD, J., JONES, T. H., EISNER, T., and HICKS, K. 1977. New methylcyclopentanoid terpenes from larval defensive secretion of a chrysomelid beetle (*Plagiodera versicolora*). *Proc. Natl. Acad. Sci. U. S. A.* 74:2189–2193.

MURELL, A., DOBSON, S. J., WALTER, D. E., CAMPBELL, N. J. H., SHAO, R., and BARKER, S. C. 2005. Relationships among the three major lineages of the Acari (Arthropoda: Arachnida) inferred from small subunit rRNA: paraphyly of the Parasitiformes with respect to the Opilioacariformes and relative rates of nucleotide substitution. *Invertebrate Systematics.* 19:383–389.

NORTON, R. A. 1998. Morphological evidence for the evolutionary origin of Astigmata. *Exp. Appl. Acarol.* 22:559–594.

OLDHAM, N. J., VEITH, M., BOLAND, W., and DETTNER, K. 1996. Iridoid monoterpene biosynthesis in insects: evidence for a de novo pathway occurring in the defensive glands of *Phaedon armoraciae* (Chrysomelidae) leaf beetle larvae. *Naturwissenschaften* 83:470–473.

PASTEELS, J. M., BREAKMAN, J.-C., DALOZE, D., and OTTINGER, R. 1982. Chemical defence in chrysomelid larvae and adults. *Tetrahedron* 38:1891–1897.

RASPOTNIG, G. 2006. Characterisation of monophyletic oribatid groups by oil gland chemistry—a novel systematic approach in Oribatida (Acari). *Abh. Ber. Naturkundemus. Görlitz* 78:31–46.

RASPOTNIG, G., SCHUSTER, R., KRISPER, G., FAULER, G., and LEIS, H. J. 2001. Chemistry of the oil gland secretion of *Collohmannia gigantea* (Acari: Oribatida). *Exp. Appl. Acarol.* 25:933–946.

RASPOTNIG, G., SCHUSTER, R., and KRISPER, G. 2003. Functional anatomy of oil glands in *Collohmannia gigantea* (Acari, Oribatida). *Zoomorpholgy* 122:105–112.

RASPOTNIG, G., KRISPER, G., and SCHUSTER, R. 2005a. Ontogenetic changes in the chemistry and morphology of oil glands in *Hermannia convexa* (Acari: Oribatida). *Exp. Appl. Acarol.* 35:47–58.

RASPOTNIG, G., KRISPER, G., SCHUSTER, R., FAULER, G., and LEIS, H. J. 2005b. Volatile exudates from the oribatid mite, *Platynothrus peltifer*. *J. Chem. Ecol*. 31:419–430.

SAKATA, T., and NORTON, R. A. 2001. Opisthonotal gland chemistry of early-derivative oribatid mites (Acari) and its relevance to systematic relationships of Astigmata. *Int. J. Acarol.* 27:281–291.

SAKATA, T., and NORTON, R. A. 2003. Opisthonotal gland chemistry of a middle-derivative oribatid mite, *Archegozetes longisetosus* (Acari: Trhypochthoniidae). *Int. J. Acarol.* 29:345–350.

SAKATA, T., TAGAMI, K., and KUWAHARA, Y. 1995. Chemical ecology of oribatid mites. I. Oil gland components of *Hydronothrus crispus* Aoki. *J. Acarol. Soc. Jpn*. 4:69–75.

SAKATA, T., SHIMANO, S., and KUWAHARA, Y. 2003. Chemical ecology of oribatid mites. III. Chemical composition of oil gland exudates from *Trhypochthoniellus* sp. and *Trhypochthonius japonicus* (Acari: Trhypochthoniidae). *Exp. Appl. Acarol*. 29:279–291.

SAPORITO, R., DONELLY, M. A., NORTON, R. A., GARAFFO, H. M., SPANDE, T. F., and DALY, J. W. 2007. Oribatid mites as a major

source for alkaloids in poison frogs. *Proc. Natl. Acad. Sci. U. S. A.* 104:8885–8890.

SCHULZ, S., KRÜCKERT, K., and WELDON, P. J. 2003. New terpene hydrocarbons from the Alligatoridae (Crocodylia, Reptilia). *J. Nat. Prod.* 66:34–38.

SHIMANO, S., SAKATA, T., MIZUTANI, Y., KUWAHARA, Y., and AOKI, J.-I. 2002. Geranial: the alarm pheromone in the nymphal stage of the oribatid mite *Nothrus palustris*. *J. Chem. Ecol.* 28:1831–1837.

SUGAWARA, F., MATSUDA, K., KOBAYASHI, A., and YAMASHITA, K. 1979. Defensive secretion of chrysomelid larvae *Gastrophysa atrocyanea* Motschulsky and *Phaedon brassicae* Baly. *J. Chem. Ecol.* 5:635–641.

TAKADA, W., SAKATA, T., SHIMANO, S., ENAMI, Y., MORI, N., NISHIDA, R., and KUWAHARA, Y. 2005. Scheloribatid mites as the source of pumiliotoxins in dendrobatid frogs. *J. Chem. Ecol.* 31:2403–2415.

VINCENTI, M., GUGLIELMETTI, G., CASSANI, G., and TONINI, C. 1987. Determination of double bond position in diunsaturated compounds by mass spectrometry of dimethyl disulfide derivatives. *Anal. Chem*. 59:694–699.

WATERHOUSE, J. S., KE, J., PICKETT, J. A., and WELDON, P. J. 1996. Volatile components in the dorsal gland secretion of the collared peccary, *Tayassu tajacu* (Tayassuidae, Mammalia). *J. Chem. Ecol.* 22:1307–1314.

WEIBEL, D. B., OLDHAM, N. J., FELD, B., GLOMBITZA, G., DETTNER, K., and BOLAND, W. 2001. Iridoid biosynthesis in staphylinid rove beetles (Coleoptera: Staphylinidae, Philonthinae). *Insect Biochem. Mol. Biol.* 31:583–591.

YOSHIHARA, K., SAKAI, T., and SAKAN, T. 1978. Dehydroiridodial, the pungent principle of *Actinidia polygama* Miq. *Chem. Lett.* 1978:433–434.

of hand-applied rope dispensers for season-long control of this pest by grape growers has been low, and no companies currently produce sprayable pheromones for *P. viteana* control. Among the reasons for the low adoption of this technology is the 2- to 3-week duration of pheromone release by sprayable formulations (R. Isaacs, unpublished data) and the labor required to apply rope dispensers to the crop.

Recently, a paraffin wax emulsion has been developed, into which insect sex pheromones can be mixed at the required concentration (Atterholt 1996; Atterholt et al. 1998; de Lame 2003). This wax is a viscous homogenate that hardens once applied to crop foliage or branches and it can act as a long-lasting discrete source of pheromone emission. The wax can be applied into crop systems by using manual methods that are more rapid than manual application of twist ties (de Lame 2003; Stelinski et al. 2005; Epstein et al. 2006), or it can be applied by mechanical methods (Stelinski et al. 2005) that enable application to large orchards or vineyards from motorized vehicles (Stelinski et al. 2006).

The optimization of mating disruption requires that the density and size of droplets, as well as the pheromone release rate and duration, are appropriate for the biology of the targeted pest (Miller et al. 2006a, b). Because the size, density, and distribution of wax droplets can be easily manipulated, this delivery system also provides researchers with a flexible tool to aid investigations of how moths can be optimally disrupted. For example, Stelinski et al. (2005) documented increased mating disruption of male oriental fruit moth, *Grapholitha molesta*, in apple orchards as droplet density increased, thus leading to increased suppression of mating in virgin female moths. The authors compared mating disruption in plots treated with wax droplets or the standard twist-tie dispenser, and found superior performance when wax was used. This suggests that the typical deployment density of pheromone twist ties may not provide sufficient point sources of pheromone for optimal disruption of *G. molesta*. More recent studies that evaluated mating disruption of the codling moth, *Cydia pomonella*, in apple orchards treated with the codlemone sex pheromone in wax droplets provide further evidence that higher densities of pheromone release sources improve disruption of mating (Epstein et al. 2006). The success of these studies and others in cotton, pear, and walnut crops has led to the development of a commercial product called SPLAT™ (Specialized Pheromone and Lure Application Technology) that acts as the carrier for sex pheromones or other semiochemicals.

To ascertain the potential for wax-based formulations for mating disruption of *P. viteana*, we determined the duration and efficacy of pheromone disruption of *P. viteana* in Michigan vineyards over two growing seasons by using the GBM-SPLAT™ commercial formulation of the wax matrix. We also tested the hypotheses that orientation of *P. viteana* to droplets would decrease with increasing pheromone release, and that mating disruption would be improved by increasing the density of droplets.

Methods and Materials

Study Sites All studies were conducted in mature *Vitis labrusca* var. 'Concord' vineyards in Van Buren Co., Michigan. These vineyards received standard fungicide and insecticide programs applied by the growers, but low crop levels due to a late spring freeze in 2006 led to a reduced pesticide program and higher activity of *P. viteana* in the second year.

Large-scale Mating Disruption Study This study was conducted during 2005 and 2006 in four vineyards at the same farm, each of which was split into two equal-sized plots [ca. 1.3 ha (100×130 m)]. The plots were assigned to be treated with pheromone in wax, or not treated, in a randomized complete block design with four replicates. Plant spacing in these plots was 2.7 m between vines and 3 m between rows, with posts distributed every three vines, resulting in 1200 vines and 400 posts per hectare. In both years, 1-ml droplets of GBM-SPLAT™ (ISCA Technologies, Inc., Riverside, CA, USA) were deployed by hand on the north side of each wooden post in the treated plots with a 30-ml syringe. The applications were made on 9 May in 2005 and 1 May in 2006, with the same plots treated in both years with wax containing 3% (*v*/*v*) of *P. viteana* pheromone (12 ml AI/ha) at a 10:1 ratio of (*Z*)-9 dodecenyl acetate: (*Z*)-11 tetradecenyl acetate (Shin-Etsu Chemical Co., Tokyo, Japan). In 2005, the formulation also contained 3% (*v*/*v*) cypermethrin, but no moths were trapped near these droplets when placed on sticky traps, suggesting that *P. viteana* moths did not contact the droplets (R. Isaacs, unpublished data).

Adult male *P. viteana* were monitored in each plot with large plastic delta traps (Suterra LLC, Bend, OR, USA) baited with rubber septa that contained female sex pheromone (90:10 ratio of (*Z*)-9–12Ac and (*Z*)-11–14Ac) (Suterra LLC, Bend, OR). Two traps were suspended from the top trellis wire at a height of 1.5 m at both the border and the interior of each vineyard, with at least 33 m between traps. Those in the interior were 65 m from the border, in the same rows as the border traps. The number of male *P. viteana* captured was monitored weekly, and moths were removed or sticky inserts were replaced. Pheromone lures were replaced every 4 wk, with lures from the same lot throughout each season.

Infestation by *P. viteana* was quantified by visually examining 50 clusters (25 clusters at two sampling sites) at

the border and interior of each plot. Both the number of clusters, and the number of berries infested were recorded in the first generation of GBM on 29 June 2005 and 6 July 2006, and during the second generation on 15 August 2005 and 4 August 2006.

Droplet Release Rates To determine the release of pheromone from wax droplets, five 1-ml droplets of GBM-SPLAT™ were applied to each of five 30.5×2.9 cm untreated wood garden stakes (Dayton Garden Labels, Dayton, OH, USA) and deployed on 24 May 2006 at one corner of each of the four pheromone-treated plots described above. Garden stakes were scored every 3.8 cm with a utility knife to allow for ease of individual drop collection. Each stake was nailed to the north face of a post located farthest from monitoring traps. One drop was collected from a randomly selected stake in each of the four treated plots each week until the termination of the experiment. Each sample was placed into a 60-ml glass bottle (Qorpak, Bridgeville, PA, USA) and stored at −20°C until it was extracted using the procedure of Stelinski et al. (2005), which was modified from Meissner et al. (2000). Fifty milliliters of an internal standard solution of 232 m1/l methyl myristate (99%, Acros Organics, Geel, Belgium) in acetonitrile (HPLC grade, EMD Chemicals Inc., Gibbstown, NJ, USA), were added to each sample. The bottles were placed into a water bath shaker (model 406015, AO Scientific Instruments, Keene, NH) at 70–75°C without shaking for 10 min, followed by 10 min of shaking, then briskly agitated by hand for 10 sec, before an additional 3 min of shaking in the water bath. Samples were then hand-agitated for 10 sec and frozen at −20°C to precipitate the wax. After 20 hr, samples were thawed, vortexed, and 1 ml of the solution was removed and filtered into a 2-ml GC vial (Agilent Technologies, Santa Clara, CA, USA) through a disposable glass Pasteur pipette fitted with a paper plug (Kimberly-Clark Corp., Roswell, GA, USA) at the tapered end. The pheromone in each sample was quantified by gas chromatography (GC) (HP-6890, Hewlett-Packard, Palo Alto, CA, USA) by using a 30-m HP DB-23 polar column (model 122-2332, J&W Scientific, Folsom, CA, USA) with the internal standard method (McNair and Miller 1998).

Droplet Density Study This study was conducted during 2006 within one large vineyard to determine the effect of droplet density on disruption of male *P. viteana* flight to pheromone traps. The experiment consisted of a randomized complete block design replicated six times. Treatment plots were 15.2×16.5 m with 80 m between plots and between blocks. Plots received either 0, 1, 3, 10, or 30 0.2 ml droplets of wax containing 3% (*v*/*v*) *P. viteana* pheromone per vine, applied by hand with a 1-ml syringe, or Isomate GBM Plus rope dispensers (Shin-Etsu Chemical Co., Tokyo, Japan) at a density of 450/ha. Treatments were applied on 1 and 3 June 2006. A large plastic delta trap (Suterra LLC, Bend, OR, USA), baited with rubber septa that contained *P. viteana* sex pheromone (Suterra LLC, Bend, OR) was placed in the middle of each plot. Traps were monitored weekly until 24 August 2006 to record the number of male *P. viteana* captured, at which time the moths were removed or sticky inserts replaced. Pheromone lures were replaced every 4 wk with lures from the same lot.

Droplet Size Study This experiment used the same protocol as the droplet density study, but in this case the traps were baited with 0-, 0.2-, 0.5-, or 1.0-ml wax droplets (approximately 6, 15.9, and 30 mg *P. viteana* sex pheromone, respectively) applied to aluminum foil suspended inside the traps, or a rubber septum loaded with 0.1 mg *P. viteana* sex pheromone. All treatments were applied on 24 May 2006, and the traps were monitored weekly until 24 August 2006. To help minimize the effect of pest pressure within experimental blocks, treatments were rotated within each block every week, and droplets were changed every 4 wk.

Statistical Analyses In the mating disruption study, the significance of treatment effect on the number of moths captured for 13 and 15 wk after application in 2005 and 2006, respectively, was compared among treatments for both locations (plot interior and border) by using ANOVA (SAS/STAT 2003). For each sample date, the average number of moths per trap was compared among treatments within each vineyard position by using ANOVA (SAS/STAT 2003). All data were log-transformed ($n+1$) for normality prior to analysis, and Tukey's HSD test was used to determine differences between means at $\alpha=0.05$. Percent disruption values were calculated as the proportional reduction in the number of moths caught in the treated plots compared to the untreated plots. Infestation data were arcsine squareroot transformed prior to analysis and compared between treatments at the border and at the interior positions by using ANOVA for each sampling date (SAS/STAT 2003).

To determine the relationship between time after application and the amount of pheromone released, residual concentration of pheromone droplets in the release rate study was analyzed by regression analysis (SAS/STAT 2003). These values also were compared to the percent disruption values calculated from moth captures, to determine a critical release rate for disruption of *P. viteana*.

The significance of treatment effect on total moths captured in the droplet density and droplet size study was compared among treatments by using analysis of variance (ANOVA) (SAS/STAT 2003). Data were log-transformed ($n+1$) prior to analysis and Tukey's HSD test was used to determine differences between means at $\alpha=0.05$.

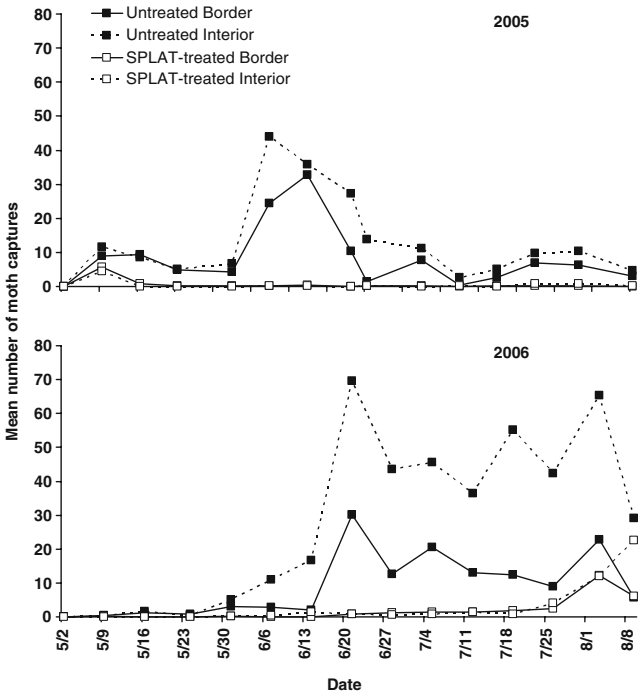

Fig. 1 Average number of adult male *Paralobesia viteana* captured in pheromone traps at the vineyard border or vineyard interior of vineyard plots that were either untreated or treated with 1-ml droplets of SPLAT-GBM™ containing 3% pheromone

Results

Large-scale Mating Disruption Study Before application of treatments in 2005, there was no significant difference in the number of moths trapped between treatments (F=3.67; df=1, 2; P=0.20) or positions (F=0.05; df=1, 8; P=0.83), indicating that pest pressure was similar across vineyards (Fig. 1). However, following the SPLAT-GBM™ treatments, the seasonal total number of moths per trap was lower in treated (2.6±0.7) than untreated (148.9±28.1) plots (F=96.76; df=1, 3; P=0.002) (Fig. 1), a pattern observed both within (F=126.6; df=1, 3; P=0.002) and at the borders (F=37.55; df=1, 3; P=0.009) of plots (Fig. 1).

In 2006, *P. viteana* were not trapped before treatment applications (moths had been trapped at nearby vineyards), but post-treatment the captures of *P. viteana* were significantly lower in the SPLAT-treated (36.5±9.3) than the untreated (279.6±47.2) vineyards (F=39.82; df=1, 3; P=0.008). Most moth captures in the treated plots occurred in the last 2 wk of the trial as the disruption efficiency of the formulations declined (Fig. 1). In contrast to 2005, in 2006 more male *P. viteana* were captured at the interior compared to the border (F=35.4; df=1, 12; P<0.001) within plots. However, similar to 2005, fewer moths were captured at the interior (F=33.97; df=1, 3; P=0.01) and the border (F=21.22; df=1, 3; P=0.019) of treated plots compared to untreated plots.

Percent disruption exceeded 90% at the vineyard interior for approximately 12 wk in 2005 and 11 weeks in 2006. In 2006, when there were no insecticide applications to the vineyards, the level of disruption had declined to 22.7% after 13 wk. In both years, disruption was greater at the interior traps than in traps placed at the borders.

In 2005, neither the total number of larvae found in each sample (F≤0.53; df=1, 3; P≥0.52) nor the proportion of clusters infested with *P. viteana* larvae (F≤3.71; df=1, 3; P≥0.15) varied significantly between treatments or vineyard positions for either sampling date (Table 1). During the first generation samples of 2006, there was no significant difference between treatments in the proportion of clusters infested or the number of larvae in cluster samples at the border (F=2.32; df=1, 3; P=0.23) or interior (F=6.91; df=1,3; P=0.078) of the vineyard (Table 1). However, during the second generation, there were differences in these parameters between the SPLAT-treated and untreated borders (F=12.76; df=1, 3; P=0.038).

Droplet Release Rates Droplets of SPLAT-GBM™ released pheromone as a first-order exponential decay function

Table 1 Average number of *Paralobesia viteana* larval berry infestations and the percentage of clusters infested with *P. viteana* (±S.E.) at the borders and interiors of vineyard plots that were untreated or treated with SPLAT-GBM™ during 2005 and 2006

Date	Position	No. of infestations per 25 clusters			Percent of clusters infested		
		Untreated	SPLAT	*P* value	Untreated	SPLAT	*P* value
6/29/2005	Border	6.1±1.3	7.4±2.2	0.62	7.8±1.0	10.0±1.8	0.15
	Interior	1.9±0.7	1.4±0.9	0.52	2.8±0.9	2.0±1.1	0.48
8/15/2005	Border	_	_	_	20.0±2.0	21.5±3.2	0.64
	Interior	_	_	_	5.8±3.4	3.5±1.6	0.33
6/27/2006	Border	11.1±2.5	7.5±1.4	0.23	15.8±4.5	9.5±1.3	0.23
	Interior	6.8±0.6	3.4±1.1	0.078	9.3±1.0	6.5±2.2	0.27
8/4/2006	Border	22.5±1.9	18.4±1.5	0.038	35.5±3.5	25.8±2.4	0.004
	Interior	11.8±3.9	8.6±1.2	0.43	18.0±5.9	15.3±2.2	0.70

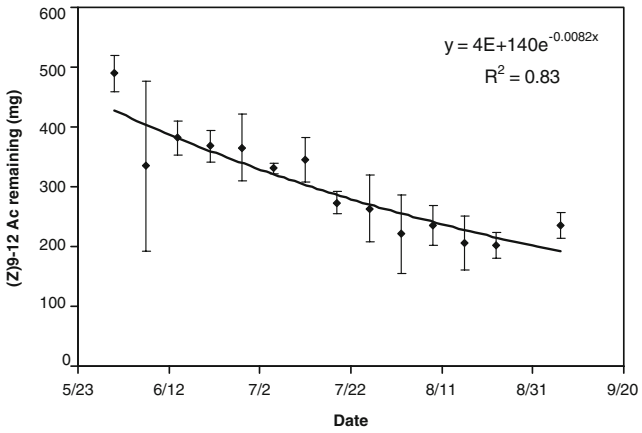

Fig. 2 Release of pheromone from 1-ml droplets of SPLAT-GBM™ during 2006. The release profile best fits an exponential decay function, and the best-fit curve and equation are presented on the graph

(Fig. 2), although the high variability among replicates (shown by the high standard error values), resulted in a relatively low predictive power of the exponential equation. The release function suggests that almost 50% of the pheromone in the wax droplets was still bound in the wax in late July when captures of *P. viteana* were first observed in the SPLAT-treated plots.

Droplet Density Study Significantly more moths were captured in the untreated control plots compared to all other treatments, but the one and three drops per vine treatments had higher moth captures than the 10 drops per vine, 30 drops per vine, and Isomate treatments (F=27.06; df=5, 25; P<0.001) (Fig. 3).

Droplet Size Study The size of each droplet affected how many moths were trapped (F=66.1; df=4, 20; P<0.001) (Fig. 4). The 0.2- and 0.5-ml droplets trapped low numbers

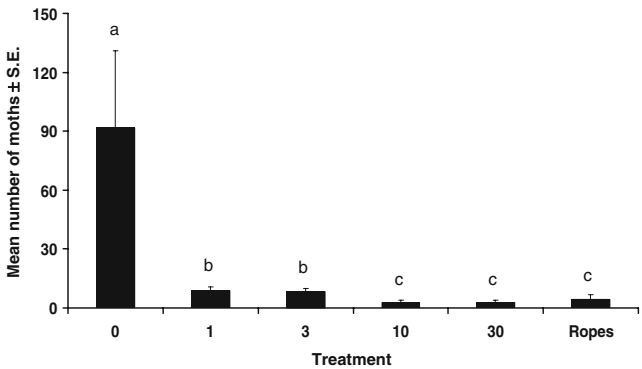

Fig. 3 Average (+SE) number of adult male *Paralobesia viteana* captured in pheromone traps in vineyard plots treated with varying densities of 0.2-ml droplets of SPLAT-GBM™ wax that contained 3% sex pheromone, or pheromone ropes. Bars with the same letter are not significantly different at α=0.05

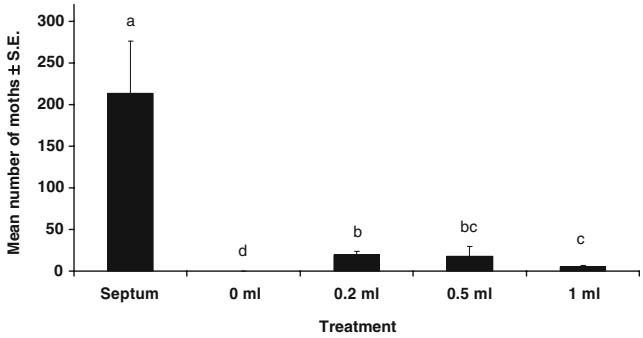

Fig. 4 Average (+SE) number of adult male *Paralobesia viteana* captured in pheromone traps baited with different sizes of SPLAT-GBM™ wax droplets that contained 3% sex pheromone, or a commercial pheromone lure containing 0.1 mg of sex pheromone. Bars with the same letter are not significantly different at α=0.05

of moths, and increasing the droplet size to 1.0 ml caused a slight decrease in the number of moths trapped (Fig. 4). The greatest captures of moths were in traps baited with the lure that contained the standard 0.1 mg of sex pheromone, an amount much lower than that in any of the applied droplets (approximately 6 mg or greater). No moths were caught when the traps were unbaited.

Discussion

Application of 1-ml droplets of SPLAT-GBM™ that contained 3% pheromone to vineyards before the first generation flight of *P. viteana* resulted in high levels of disruption of male moth orientation to monitoring traps during two growing seasons, with reduced crop infestation in treated plots in the second growing season. This first report of using a wax formulation for pheromone deployment in vineyards provides evidence for the potential of this formulation to control *P. viteana*.

Wooden vineyard posts provided a practical target for application of the wax, and droplets applied on the north side of these structures in early May each year remained in place throughout the growing season. These droplets were easy to apply by hand with a 30-ml syringe, and their application was faster than that of Isomate twist ties at 450/ha (Isaacs, unpublished data). The large droplets used in this study released pheromone until late July. The longevity of these droplets is lower compared with those used for mating disruption of *G. molesta* (Stelinski et al. 2006, 2007).

Pheromone dispenser density affects the distribution of pheromone that permeates the crop habitat, thus influencing the degree of mating disruption achieved (Rothschild 1975; Flint and Merkle 1983; Lawson et al. 1996; Stelinski et al. 2005; Miller et al. 2006a, b). A similar pattern was found in this study, as trap catches in untreated plots were greater than one or three droplets per vine (0.2-ml volume) >10 and 30 droplets per vine. The higher doses gave similar results

as Isomate GBM ropes applied once every 0.4 vine. Overall, our results are similar to those observed for *G. molesta* (Stelinski et al. 2005), although they reported higher densities of droplets outperformed twist ties and completely prevented mating of tethered female moths. A profile analysis of variation in moth catch with dispenser density, similar to that conducted by Miller et al. (2006a), was not possible in our study because of an insufficient range of pheromone point-source densities.

A pheromone formulation lasting >20 wk, as achieved with Isomate-GBM twist ties, is necessary to cover the full activity period of *P. viteana* in the Great Lakes region of the US and Canada. Our results suggest that this cannot be currently achieved by using SPLAT-GBM™, so one approach to would be to re-treat vineyards in late July. Alternatively, one could apply an appropriate Lepidoptera-specific insecticide for control of first-generation *P. viteana*, followed by application of SPLAT-GBM™ in early July to provide protection against this pest until harvest. Another promising line of research would be the development of a wax formulation that provides a pheromone release system lasting >20 wk.

Development of formulations that slowly release the pheromone is an important goal for mating disruption, not only to provide a long period of activity but also to ensure that expensive pheromone is not wasted by remaining bound in the wax matrix (de Lame 2003). Residual analysis of wax revealed that approximately 50% of the *P. viteana* pheromone was still bound in the drops when disruption performance declined in late July. The 1-ml droplets used in this study are larger than those deployed in many recent studies (Stelinski et al. 2005, 2006, 2007), so the use of more, smaller-sized droplets should result in a greater proportion of the pheromone being released (Stelinski et al. 2005). This is currently under investigation, especially as the amount of pheromone released per hectare in the 10-drops-per-vine treatment (58.1 mg/Ha) was lower than the Isomate treatment (99.8 mg/Ha).

Evaluations of sex pheromone formulations for control of *P. viteana* have shown that mating disruption can help control this pest (Dennehy et al. 1990; Trimble 1993), yet there has been relatively low adoption of this approach in vineyard IPM programs in eastern North America where vineyards are infested by *P. viteana*. Primary impediments to adoption are the perception that pheromone formulations have lower efficacy, and the cost of pheromone products and their application is greater when compared with insecticides. An additional challenge to controlling infestation of *P. viteana* is that this insect typically causes higher levels of crop infestation at the vineyard borders than in the interiors (Hoffman and Dennehy 1989; Botero-Garcés and Isaacs 2003). Our results showed that while application of SPLAT-GBM™ caused an immediate shutdown of traps, a significant reduction in cluster infestation at the vineyard borders was observed only in the second year of treatment, suggesting that multiyear application of pheromone may be required for mating disruption of GBM.

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References

ATTERHOLT, C. A. 1996. Controlled release of insect sex pheromones from sprayable biodegradable materials for mating disruption. Ph.D. dissertation, University of California-Davis.

ATTERHOLT, C. A., DELWICHE, M. J., RICE, R. E., and KROCHTA, J. M. 1998. Study of biopolymers and paraffin as potential controlled-release carriers for insect pheromones. *J. Agric. Food Chem.* 46:4429–4434.

BOTERO-GARCÉS, N., and ISAACS, R. 2003. Distribution of grape berry moth, *Endopiza viteana* (Lepidoptera: Tortricidae), in natural and cultivated habitats. *Environ. Entomol.* 32:1187–1195.

de LAME, F. M. 2003. Improving mating disruption programs for the Oriental fruit moth, *Grapholita molesta* (Busck): Efficacy of new wax-based formulations and effects of dispenser application height and density. M.S. thesis, Michigan State University.

DENNEHY, T. J., HOFFMAN, C. J., NYROP, J. P., and SAUNDERS, M. C. 1990. Development of low-spray, biological, and pheromone approaches for control of grape berry moth, *Endopiza viteana* Clemens, in the eastern United States., pp. 261–282, in N. J. Bostanian, L. T. Wilson, and T. J. Dennehy (eds.). Monitoring and Integrated Management of Arthropod Pests of Small Fruit CropsIntercept Ltd., Andover, NH.

EPSTEIN, D. L., STELINSKI, L. L., REED, T. P., MILLER, J. R., and GUT, L. J. 2006. Higher densities of distributed pheromone sources provide disruption of codling moth (Lepidoptera: Tortricidae) superior to that of lower densities of clumped sources. *J. Econ. Entomol.* 99:1327–1333.

FLINT, H. M., and MERKLE, J. R. 1983. Pink bollworm (Lepidoptera: Gelechidae): Communication disruption by pheromone composition imbalance. *J. Econ. Entomol.* 76:40–46.

HOFFMAN, C. J., and DENNEHY, T. J. 1989. Phenology, movement, and within-field distribution of the grape berry moth, *Endopiza viteana* (Clemens) (Lepidoptera: Tortricidae), in New York vineyards. *Can. Entomol.* 121:325–335.

JENKINS, P. E., and ISAACS, R. 2007a. Cutting wild grapevines as a cultural control strategy for grape berry moth (Lepidoptera: Tortricidae). *Environ. Entomol.* 36:187–194.

JENKINS, P. E., and ISAACS, R. 2007b. Redued-risk insecticides for control of grape berry moth (Lepidoptera: Tortricidae) and conservation of natural enemies. *J. Econ. Entomol.* 100:855–865.

LAWSON, D. S., REISSIG, W. H., AGNELLO, A. M., NYROP, J. P., and ROELOFS, W. L. 1996. Interference with the mate-finding communication system of the obliquebanded leafroller (Lepidoptera: Tortricidae) using synthetic sex pheromones. *Environ. Entomol.* 25:895–905.

MCNAIR, H. M., and MILLER, J. M. 1998. Basic Gas Chromatography. Wiley, New York, NY.

MEISSNER, H. E., ATTERHOLT, C. A., WALGENBACH, J. F., and KENNEDY, G. G. 2000. Comparison of pheromone application rates, point source densities, and dispensing methods for mating disruption of tufted apple bud moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 93:820–827.

MILLER, J. R., GUT, L. J., de LAME, F. M., and STELINSKI, L. L. 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part I): Theory. *J. Chem. Ecol.* 32:2089–2114.

MILLER, J. R., GUT, L. J., de LAME, F. M., and STELINSKI, L. L. 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part II): Case studies. *J. Chem. Ecol.* 32:2115–2143.

ROELOFS, W. L., TETTE, J. P., TASCHENBERG, E. F., and COMEAU, A. 1971. Sex pheromone of the grape berry moth: Identification by classical and electroantennogram methods, and field tests. *J. Insect. Sci.* 17:2235–2243.

ROTHSCHILD, G. H. L. 1975. Control of the Oriental fruit moth (Cydia molesta) (Busck) (Lepidoptera: Tortricidae) with synthetic female pheromone. *Bull. Entomol. Res.* 65:473–490.

SAS INSTITUTE. 2003. SAS/STAT User's Manual, Version 9.1. SAS Institute, Cary, NC.

STELINSKI, L. L., GUT, L. J., MALLINGER, R. E., EPSTEIN, D. L., REED, T. P., and MILLER, J. R. 2005. Small plot trials documenting effective mating disruption of oriental fruit moth by using high densities of wax-drop pheromone dispensers. *J. Econ. Entomol.* 98:1267–1274.

STELINSKI, L. L., MILLER, J. R., LEDEBUHR, R., and GUT, L. J. 2006. Mechanized applicator for large scale field deployment of paraffin wax dispensers of pheromone for mating disruption in tree fruit. *J. Econ. Entomol.* 99:1705–1710.

STELINSKI, L. L., MILLER, J. R., LEDEBUHR, R., SIEGERT, P., and GUT, L. J. 2007. Season-long mating disruption of *Grapholita molesta* (Lepidoptera: Tortricidae) by one machine application of pheromone in wax drops (SPLAT-OFM). *Journal of Pesticide Science* 80:109–117.

TOBIN, P. C., NAGARKATTI, S., and SAUNDERS, M. C. 2003. Phenology of grape berry moth (Lepidoptera: Tortricidae) in cultivated grape at selected geographic locations. *Environ. Entomol.* 32:340–346.

TRIMBLE, R. M. 1993. Efficacy of mating disruption for controlling the grape berry moth, *Endopiza viteana* (Clemens) (Lepidoptera: Tortricidae), a case study over three consecutive growing seasons. *Can. Entomol.* 125:1–9.

TRIMBLE, R. M., PREE, D. J., VICKERS, P. M., and KER, K. W. 1991. Potential of mating disruption using sex-pheromone for controlling the grape berry moth, *Endopiza viteana* (Clemens) (Lepidoptera: Tortricidae), in Niagara peninsula, Ontario vineyards. *Can. Entomol.* 123:451–460.

TRIMBLE, R. M., VICKERS, P. M., NIELSEN, K. E., and BARINSHTEYN, G. 2003. Sprayable pheromone for the controlling the North American grape berry moth by mating disruption. *Agric. For. Entomol.* 5:263–268.

WITZGALL, P., BENGTSSON, M., and TRIMBLE, R. M. 2000. Sex pheromone of grape berry moth (Lepidoptera: Tortricidae). *Environ. Entomol.* 29:433–436.

better understanding of how they initially disrupt communication could provide important insights for optimizing this flexible technology (Knight and Larson 2004; Stelinski et al. 2005).

The major mechanisms of mating disruption, as postulated by Bartell (1982) and discussed by Cardé (1990), fall into two discrete categories, competitive vs. non-competitive (sensu Miller et al. 2006a). Competitive attraction, or false-plume following, occurs when males spend a portion of the female calling period orienting to and following attractive synthetic pheromone plumes from dispensers, thereby reducing the odds of successful mate location (Miller et al. 2006a). Major non-competitive mechanisms include: (1) camouflage of the female-produced pheromone plume by relatively constant atmospheric levels of synthetic pheromone; (2) adaptation of the male peripheral nervous system due to a reduction in the sensitivity of antennal receptors; (3) habituation at any level of the male central nervous system resulting from exposure to pheromone, which leads to a decrease or cessation of appropriate sexual response; and (4) sensory imbalance of the signal processed by males as a result of one or more synthetic pheromone components (Bartell 1982; Cardé 1990) being released in a ratio that differs from the female-produced blend (Flint and Merkle 1983; Judd et al. 1995).

Despite precise definitions of each mechanism, it is often difficult to demonstrate clearly the importance of one mechanism over another, as several are probably involved under most mating disruption regimes (Sanders and Lucuik 1996; Cardé et al. 1998; Miller et al. 2006a, b). Under certain circumstances, however, one can eliminate the importance of competitive mechanisms a priori. For example, MEC formulations that release an unattractive pheromone blend and are applied uniformly to crop foliage over large areas should not result in significant competitive attraction, as a relatively diffuse cloud of unattractive pheromone may be produced (Weatherston 1990; Doane 1999). In such cases, disruption of male mate-finding behavior should stem solely from non-competitive mechanisms that are concentration dependent (Miller et al. 2006a) and can be individually examined under experimental conditions.

MEC formulations characteristically have high initial pheromone release rates that quickly decline, and it is this rapid drop that is thought to reduce formulation efficacy over time (Hall and Marrs 1989; Polavarapu et al. 2001; Albajes et al. 2002). This has led to the conclusion that MEC pheromone technology fails to achieve significant disruption because it does not produce the sustained atmospheric concentrations of pheromone necessary to camouflage natural female plumes or invoke significant sensory effects. However, insects alighting or resting on foliage treated with MEC pheromone may be in continuous contact with undiluted pheromone at the leaf surface–air interface, exposing them to far greater pheromone concentrations than the average atmospheric concentration (Judd et al. 2005a). Despite this possibility, the degree to which surface contact exposure to pheromone might induce various non-competitive mechanisms of disruption has received almost no attention.

This study used *C. rosaceana*, a significant lepidopteran pest of pome fruit in North America (Knight et al. 1998; Lawson et al. 1996), to achieve two objectives. The first was to use behavioral and electrophysiological assays to examine the non-competitive mechanisms of communication disruption involved when male *C. rosaceana* were exposed through direct contact to formulations of MEC (*Z*)-11-tetradecenyl acetate (Z11-14:OAc). Z11-14:OAc is the major pheromone component in the four-component blend of *C. rosaceana* (El-Sayed et al. 2003). The second objective was to examine whether the addition of horticultural oil as an adjuvant to a MEC formulation affected its efficacy and any communication disruption mechanisms involved. This idea arose from earlier work that showed that the addition of horticultural oil stabilized the release rate of Z11-14:OAc from microcapsules over several weeks (Judd et al. 2006), potentially increasing formulation longevity. Specifically, we tested the hypothesis that the formulation of a MEC pheromone with a 2% horticultural oil adjuvant would result in stronger and more sustained disruption of male mate-finding behavior over time than the formulation of a MEC pheromone in water.

Methods and Materials

Insects *C. rosaceana* used in assays came from a 10-year-old colony originally collected in the Okanagan and Similkameen valleys (British Columbia, Canada) and periodically restocked with wild insects. Larvae were reared individually in 25-ml Solo® cups on a modified pinto bean diet (Shorey and Hale 1965) at 23°C under a 16:8 h (L:D) photoregime (scotophase began at 1400 hours). Pupae were collected weekly, separated by sex, and 190 of each sex were returned to the colony. The remaining pupae were placed in separate 10-l buckets for each sex. Eclosed adults were collected each day, transferred to a different 10-l bucket, and held under the same conditions in separate chambers until assays were conducted. Care was taken to ensure that males were not exposed to pheromone before assays.

Treatment Formulations 3M MEC-LR® containing 20% microencapsulated Z11-14:OAc by weight was obtained from 3M Canada (London, ON, Canada) and stored at 4°C between experiments. This formulation of pheromone is no

longer commercially available. Purespray Green® horticultural oil (batch # 655-0602, Petro-Canada, Mississauga, ON, Canada) is a highly purified, *n*C23 mineral oil, with a molecular weight of 325, and an average boiling point of 223.9°C (ASTM D 1160; Petro-Canada, technical data sheet). This oil is prepared with a proprietary emulsifier and approved for use by organic producers in the USA (Organic Materials Review Institute, Eugene, OR, USA). Purespray Green® was held at room temperature before use in formulations.

Treatment Application and Ageing For behavioral and electrophysiological assays, male moths were exposed to treatment formulations while resting on 8.5 cm (diam.)-treated galvanized sheet metal disks (Fehling's Sheet Metal, Penticton, BC, Canada). Each disk received spray applications of one of the following four treatments: (1) distilled water control; (2) 2% (*v*/*v*) Purespray Green® in distilled water; (3) MEC-LR® mixed in distilled water and applied at 1 μg a.i. cm^{-2}; and (4) MEC-LR® applied at 1 μg a.i. cm^{-2} in a 2% (*v*/*v*) Purespray Green® plus distilled water mixture. Hereafter, these treatments are referred to as: water, oil, MEC, and MEC + oil, respectively. One hundred milliliter of each solution was dispensed into 160 ml glass bottles attached to hand-held, pressurized, disposable spray guns (Preval® Sprayer, Precision Valve Corporation, Yonkers, NY, USA). Metal disks were placed on a disposable plastic sheet within a 40×50-cm marked area. The area was sprayed with 20 ml of solution at a distance of 30 cm in successive horizontal and vertical transects. The resulting application rate of 1 μg a.i. cm^{-2} per disk for MEC-LR® best approximated both label-recommended field application rates of 100 g a.i. Z11-14:OAc ha^{-1} for MEC-LR® and 1,000 l water ha^{-1} for Purespray Green®. Disks were air-dried and stored in a fume hood where the sprayed formulations were allowed to age for different time periods before use.

Flight Tunnel Bioassay Protocol Flights were conducted in a flight tunnel described in detail by Evenden et al. (1999, 2000). Air was pushed through the 2.45-m-long×1-m-high tunnel by a variable speed fan and exhausted out of the building by a centrally located exhaust fan. The tunnel was illuminated from above by six 25-W incandescent bulbs that produced uniform, diffuse white light (1 lux). The wind speed was maintained at 0.3–0.4 m s^{-1}, the tunnel temperature was 22–24°C, and all flight experiments started at 1400 hours.

In each replicate of each flight tunnel experiment, a single, calling virgin female (4–48 h old) served as the pheromone source. Female *C. rosaceana* in this age group are similarly attractive to males (Delisle 1992) and have similar pheromone titer (Delisle and Royer 1994). The female was presented in a wire mesh cage (3 cm^3), positioned on a small metal platform 45 cm above the tunnel floor and 20 cm from the upwind end. A cylindrical trap (13 cm long×11 cm diam.) constructed from clear, 1-mm thick polyester (GE Polymershapes, Coquitlam, BC, Canada), was suspended from a ring stand directly downwind of the female's cage so that its long axis was parallel to the tunnel floor, and the pheromone plume traveled directly through the center of the cylinder. The trap was coated internally with STP Oil (First Brands Corporation, Scarborough, ON, Canada) to capture males that flew upwind in close proximity to the female.

Male moths used were aged 24–96 h old, an age range shown to have consistent mating success (Delisle 1995). Males were briefly chilled at 2°C, randomly divided into groups of ten, and each group was lightly dusted with a uniquely colored Day-Glo® Daylight Fluorescent Powder to identify each of the four different treatments (Switzer Brothers Inc., Cleveland, OH, USA). One hour before the start of flights, a group of ten males was placed on a treated disk in a wire mesh release cage (9 cm diam.×2 cm height) with a removable wire mesh lid. Males initially placed on the treated disks often moved onto the sides and inner mesh lid of the release cage; however, individuals were always at a distance of <2 cm from the disk. Males on treated disks were transferred immediately into the bioassay room, placed in a small fume hood adjacent to the tunnel, and held under test conditions. This process was repeated every 15 min until all treatments were established. Clear plastic dividers within the fume hood separated males in different treatments to ensure no pheromone cross contamination among treatments. Flights were staggered at 15 min intervals to maintain a 1-h treatment exposure period for each group. After each flight day, the flight tunnel was wiped down with ethanol, and the wire mesh release devices, female holding cages, and release platform were rinsed with acetone and heated for ca. 12 h at 200°C.

Experiment 1: Disruption of Mate-finding Behavior Through Continuous Exposure with a Pheromone-treated Surface In this experiment, we tested the hypothesis that continuous contact with MEC- and MEC + oil-treated surfaces would disrupt male orientation to a calling female. This experiment also tested the hypothesis that the disruptive efficacy of the MEC treatment would decrease more rapidly over time than the MEC + oil treatment. Disks were sprayed with the four treatments as described above, and aged in the fume hood for 1, 6, or 13 days prior to use in the bioassay. Following the 1-h treatment exposure, the wire mesh release cage that contained the first test group of ten males on the treated disk was introduced into the downwind end of the tunnel on a sliding cart. An externally operated line was used to lift the lid off the release device

5 s after cage introduction. Each group of males was left to respond to the calling female for 5 min, after which the release device and trap were removed. Males caught in each trap were examined under UV light to identify from which group they originated, to ensure that only males caught from the appropriate group were used in subsequent analyses. A fresh trap was inserted after each group flight, with subsequent flights initiated every 15 min. Forty males (four groups of ten) were tested at each treatment and disk age combination (Table 1).

Experiments 2 and 3: Disruption of Mate-finding Behavior After Exposure to Pheromone-treated Disks Experiments 2 and 3 eliminated the effect of camouflage and tested the hypothesis that prolonged sensory effects from pheromone exposure caused disruption in males. In experiment 2, males in groups of ten were exposed for 1 h to disk treatments as above. Disks were aged in the fume hood for increasing lengths of time (1, 6, 13, 20, 33, and 47 days) to further test the hypothesis that the disruptive efficacy of the MEC treatment would decrease more rapidly over time than the MEC + oil treatment (Table 1). After the 1-h exposure, males were chilled briefly in a cooler of ice and quickly transferred to a clean release device before introduction into the flight tunnel exactly 75 s after removal from the treated disk. A preliminary experiment showed no effect of the brief cooling protocol on pheromone responses of naive males, and the 75-s recovery period was chosen to correspond with the electroantennogram (EAG) measurements (experiments 5 and 6). Sixty to 70 males (six to seven groups of ten) were tested at each treatment and disk age combination (Table 1).

Experiment 3 tested the effect of a 60-min clean air recovery period after exposure to treated disks. Experiments 3–6 only used disks aged in the fume hood for 6 days (Table 1). Flight protocol for experiment 3 was identical to experiment 2 except that after the 1-h exposure period, chilled males were transferred to a clean release device and placed in a clear Plexiglas® box equipped with a fan that blew charcoal-filtered air over males under bioassay conditions for 1 h before flights. Seven groups of ten males were tested per treatment.

Experiment 4: Disruption of Mate-finding Behavior at Increasing Times After Exposure to Pheromone-treated Disks In this experiment, we flew individual males to

Table 1 Bioassay descriptions for flight tunnel and electroantennogram (EAG) assays conducted on male *Choristoneura rosaceana*

Experiment	Assay	Treatment[a]	Formulation age[b] (days)	Males tested per treatment × age combination	Exposure time[c]	Recovery time[d]
1	Group flights	Water	1, 6, 13	40	60 min	None
		Oil	1, 6, 13	40	60 min	None
		MEC	1, 6, 13	40	60 min	None
		MEC + oil	1, 6, 13	40	60 min	None
2	Group flights	Water	1, 6, 13, 20, 33, 47	60–70	60 min	75 s
		Oil	1, 6, 13, 20, 33, 47	60–70	60 min	75 s
		MEC	1, 6, 13, 20, 33, 47	60–70	60 min	75 s
		MEC + oil	1, 6, 13, 20, 33, 47	60–70	60 min	75 s
3	Group flights	Water	6	70	60 min	60 min
		Oil	6	70	60 min	60 min
		MEC	6	70	60 min	60 min
		MEC + oil	6	70	60 min	60 min
4	Individual flights	Water	6	98	60 min	1–50 min
		MEC	6	103	60 min	1–50 min
		MEC + oil	6	95	60 min	1–50 min
5	EAG	Water	6	10	60 min	75 s
		Oil	6	10	60 min	75 s
		MEC	6	10	60 min	75 s
		MEC + oil	6	10	60 min	75 s
6	EAG	Water	6	9	24 h	75 s
		Oil	6	9	24 h	75 s
		MEC	6	9	24 h	75 s
		MEC + oil	6	9	24 h	75 s

[a] Treatment formulations: (1) water = distilled water; (2) oil = 2% Purespray Green® in water; (3) MEC = MEC-LR® applied at 1 μg a.i.$^{-1}$ cm^{-2}; (4) MEC + oil = MEC-LR® at 1 μg a.i.$^{-1}$ cm^{-2} + 2% Purespray Green®

[b] Formulation age after spray application on disk at time of moth exposure

[c] Time spent by each group of male moths on treated disks prior to the initiation of the bioassay

[d] Time spent by each group of male moths in clean air after removal from treated disks prior to the initiation of the bioassay

examine the impact of exposure to MEC formulations on mate-finding behavior at increasing times (between 1 and 50 min) after exposure. The oil treatment was excluded from this experiment (Table 1) as earlier group flight assays (experiments 1–3) indicated no differences in behavior of males exposed on oil-treated disks compared to males exposed on water-treated disks. Each replicate consisted of males (24–96 h old) exposed for 1 h on a metal disk treated with either water, MEC, or MEC + oil. After the 1-h exposure, the wire mesh release cages that contained males on treated disks were briefly chilled in a cooler of ice, and males were transferred into clean, individual wire mesh release cages (3.5 cm diam.×1.5 cm height) with a sheet metal lid. Individual release cages were positioned in the clear Plexiglas® box adjacent to the flight tunnel, where charcoal-filtered air was continually blown over them until use in the bioassay. Individual flights were initiated every 2 min, alternating males from the three treatments, so that males were assayed at various times between 1 and 50 min after removal from treated disks. The sequence of treatments was re-randomized each day. Males were flown individually to the calling female by inserting the individual release cage into the center of the pheromone plume on the sliding cart at the downwind end of the tunnel. Female handling and placement was identical to that of experiments 1–3, except no cylindrical trap was used. Males were given 5 s in the plume before release. Male responses were scored as positive (+) or negative (−) for contact with the cage housing the female (source contact). Males were quickly aspirated out of the tunnel after each flight.

Experiments 5 and 6: Electrophysiological Effects After Exposure to Pheromone-treated Disks We used EAGs to measure directly and compare potential adaptation of antennal receptors following exposure to MEC- and MEC + oil-treated disks. The effect of 1- and 24-h exposure periods to the four treatment formulations aged for 6 days in the fume hood on subsequent antennal sensitivity to Z11-14:OAc was tested in experiments 5 and 6, respectively. Our EAG system consisted of an IDAC-02 data acquisition interface board, an INR-02 EAG-SSR system, and EAG 2000 software from Syntech (Hilversum, The Netherlands). Each antenna was attached to a gold-plated Syntech PRG-2 probe with a small quantity of Spectra 360 conductive gel (Parker Laboratories Inc., Orange, NJ, USA). Pheromone-loading doses consisted of neat Z11-14:OAc (99% purity, Pherobank, Wageningen, The Netherlands) serially diluted in HPLC-grade hexane to obtain decade solutions between 0.2 ng and 20 μg Z11-14:OAc μl^{-1} hexane. Fifty microliters of each solution, and 50 μl of a hexane control, were pipetted individually onto 1×2-cm strips of folded Whatman no. 1 filter paper and allowed to evaporate in a fume hood for 5 min. As a standard, 10 μl of the plant volatile (*E*)-2-hexenal (1 μg/μl paraffin oil) also were pipetted onto filter paper and allowed to evaporate. Treated strips were inserted into disposable Pasteur pipettes and allowed to equilibrate for 30 min before use. Two-seconds stimulus puffs were generated with a Syntech CS-05 pulse generator at a flow rate of 10 ml/s. EAG responses were measured as the maximum amplitude of depolarization elicited by the stimulus applied. An initial dose-response experiment on naive male *C. rosaceana* antennae determined an optimal range of responsiveness to stimulus puffs between 10 and 1,000 μg Z11-14:OAc in 50 μl hexane; therefore, this stimulus range was used in subsequent experiments.

Male moths (24–96 h old) were exposed individually on metal disks sprayed with one of the four treatments as above. Exposure occurred in standard release devices placed in a fume hood where air was continuously drawn over them for either 1 or 24 h (Table 1). After exposure, a male was removed from a treated disk, and one antenna excised and mounted on the EAG. A stimulus puff of 10 μg Z11-14:OAc was administered to the antenna exactly 75 s after removal from the treated disk, and each antenna received a series of puffs delivered every 30 s in the following order: 10 μg Z11-14:OAc, 10 μg (*E*)-2-hexenal, 100 μg Z11-14:OAc, 10 μg (*E*)-2-hexenal, 1,000 μg Z11-14:OAc, 10 μg (*E*)-2-hexenal, hexane control, and 10 μg (*E*)-2-hexenal. The plant volatile was used to normalize antennal response over one dose series by dividing mV response to each Z11-14:OAc stimulus by the average mV response for (*E*)-2-hexenal for one antenna (Karg et al. 1994; Judd et al. 2005b). We used ten and nine male antennae per treatment for the 1 and 24 h exposure experiments, respectively.

Experiment 7: Microcapsule Density Microcapsules on MEC- and MEC + oil-sprayed disks were counted to determine the rates of microcapsule deposition from each formulation. The disk spraying protocol was identical to experiments 1–6, however, formulations included 0.2% Rose Bengal dye (Aldrich Inc., Milwaukee, WI, USA) to increase microcapsule visibility. After disks had dried, microcapsules were counted with an Olympus SZX12 stereo microscope (×72 magnification; 9.1 mm^2 field of view). Six fields of view were examined per disk, with N= 16 disks/treatment.

Statistical Analyses For both experiments 1 and 2, mean proportion of males captured in group flights was analyzed with a two-way analysis of variance (ANOVA) with formulation treatment and disk age as factors. Significant treatment differences were separated by using the Student–Newman–Keuls (SNK) multiple comparison procedure. In experiment 3, mean proportion trap catch of males was analyzed with a one-way ANOVA followed by a SNK test

to separate significant treatment differences. Experiments 1–3 were analyzed in Sigmastat® 3.0.1 (SYSTAT Software Inc., San Jose, CA, USA). For individual flights (experiment 4), the relationship between time post-exposure and male ability to make source contact was analyzed separately for each treatment by using logistic regression due to the binomial nature of the data (i.e., males scored as (+) or (−) for contacting the female). As both MEC-exposed and MEC + oil-exposed males showed a significant effect of time after exposure on ability to contact the female, a logistic regression model with treatment as a categorical variable and time post-exposure as a continuous variable was used to test the effect of treatment on male ability to contact the female. For experiments 5 and 6, differences in normalized mV antennal response between treatments at each stimulus dose were compared by using a one-way ANOVA. The number of microcapsules deposited on metal disks by application of MEC and MEC + oil in Exp. 7 was compared by using a one-way nested ANOVA with the number of microcapsules within each sampled field of view nested within each sampled disk. Experiments 4–7 were analyzed with SYSTAT 11 (SYSTAT Software Inc., San Jose, CA, USA). All proportion data were arcsine square-root-transformed prior to analysis, and significance set at α=0.05 for all tests.

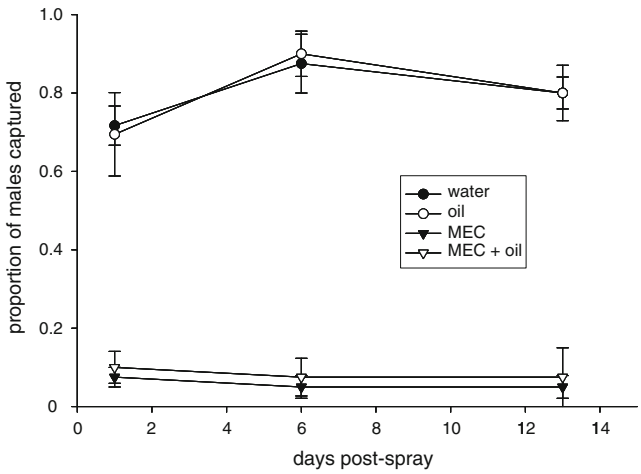

Fig. 1 Mean (±SE) proportion of male *Choristoneura rosaceana* caught in female-baited traps in a flight tunnel following a 1-h exposure on a metal disk sprayed with either water, 2% Purespray Green® (oil), MEC-LR® applied at 1 μg a.i.$^{-1}$ cm^{-2} (MEC), or MEC-LR® at 1 μg a.i.$^{-1}$ cm^{-2} + 2% Purespray Green® (MEC + oil). Groups of ten males were introduced into a female-produced pheromone plume while resting on treated disks, with formulations aged on disks for 1, 6, or 13 days (N=4 groups of ten males/treatment). The effect of treatment was significant ($F_{3,\ 36}$=74.73, P<0.001) while the effect of formulation age was not significant ($F_{2,\ 36}$=1.36, P=0.268) by two-way analysis of variance (ANOVA). Treatment means were further separated by Student–Newman–Keuls multiple comparison test: water and oil treatments were equal, and significantly greater than MEC and MEC + oil treatments, which were equal

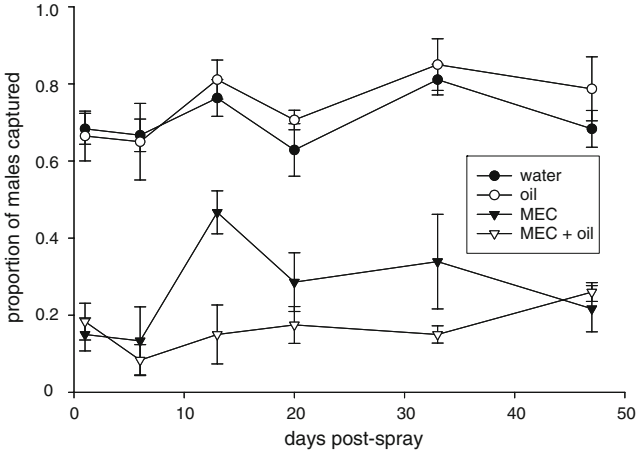

Fig. 2 Mean (±SE) proportion of male *Choristoneura rosaceana* caught in female-baited traps when males were released 75 s after a 1-h treatment exposure on a metal disk sprayed with either water, 2% Purespray Green® (oil), MEC-LR® applied at 1 μg a.i.$^{-1}$ cm^{-2} (MEC), or MEC LR® at 1 μg a.i.$^{-1}$ cm^{-2} + 2% Purespray Green® (MEC + oil). Formulations were aged on disks for 1, 6, 13, 20, 33, or 47 days (N=6–7 groups of ten males/treatment). The effect of both treatment ($F_{3,\ 124}$=95.69, P<0.001) and formulation age ($F_{5,\ 124}$=4.35, P=0.001) were significant by two-way analysis of variance (ANOVA). Treatment means were further separated by Student–Newman–Keuls multiple comparison test: water and oil treatments were equal, and significantly greater than the MEC treatment, which was significantly greater than the MEC + oil treatment

Results

Experiment 1: Disruption of Mate-finding Behavior Through Continuous Exposure with a Pheromone-treated Surface The proportion of males captured in female-baited traps was reduced when males were continuously exposed to pheromone while resting on MEC- or MEC + oil-treated disks as compared to control disks ($F_{3,\ 36}$=74.73, P< 0.001). The proportion of males captured was unaffected ($F_{2,\ 36}$=1.36, P=0.268) by formulation aging up to 13 days after application (Fig. 1). Mate finding was equally disrupted by exposure to MEC alone and MEC + oil treatments (Fig. 1).

Experiments 2 and 3: Disruption of Mate-finding Behavior After Exposure to Pheromone-treated Disks In experiment 2, the effect of a 1-h exposure treatment was significant ($F_{3,\ 124}$=95.69, P<0.001; Fig. 2). The proportion of males captured over the 47-day period was not significantly different between the water (70.4±2.2%) and oil (74.4± 2.8%) treatments. However, exposure to the MEC and MEC + oil treatments caused a significant reduction in trap catch compared to control treatments. In addition, the mean proportion of MEC + oil-exposed males captured (16.8± 1.9%) was significantly lower than MEC-exposed males (26.6±3.5%; Fig. 2). There was an effect of formulation age ($F_{5,\ 124}$=4.35, P=0.001), but there was no significant

interaction between treatment and formulation age ($F_{15, 124}$= 1.29, P=0.215). Although pheromone release rates should have dropped as formulations aged, the effect of exposure to both MEC and MEC + oil treatments on mate finding was still strong on day 47 (Fig. 2).

Even when males were given a 1-h recovery period following exposure to 6-day-old formulations (Fig. 3), there was a significant difference in male trap catch among treatments ($F_{3, 18}$=4.5, P=0.016). The proportion of males captured was significantly reduced in the MEC + oil treatment compared to either control treatment. However, while MEC was similar to MEC + oil, there was no difference between MEC and control treatments (Fig. 3).

Experiment 4: Disruption of Mate-finding Behavior at Increasing Times After Exposure to Pheromone-treated Disks For males exposed to water-treated disks, there was no relationship between flight time post-exposure (1–50 min) and the probability of source contact (N=98, slope=0.012, P=0.545). In contrast, there was a positive relationship between the length of the recovery period and the proportion of males that reached the calling female for both MEC (N=103, slope=0.04, P=0.006) and MEC + oil treatments (N=95, slope=0.04, P=0.010). However, eight of 20 MEC- and nine of 21 MEC + oil-exposed males flown within 5 min of removal from treated disks made contact with the female. When MEC- and MEC + oil-exposed males were included in a pooled model, there was no difference in male recovery time between the two treatments (N=198, P=0.349).

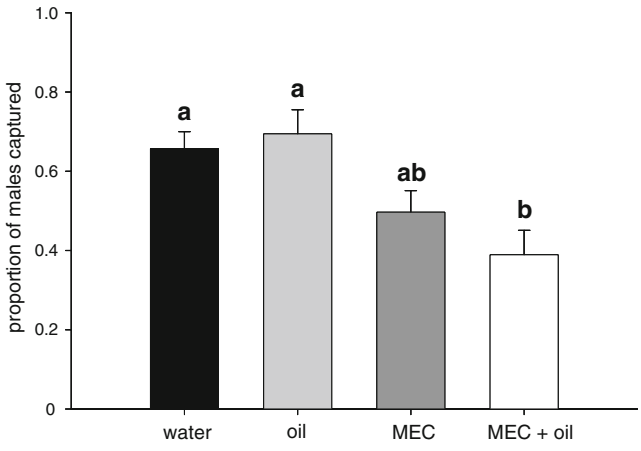

Fig. 3 Mean (+SE) proportion of male *Choristoneura rosaceana* caught in female-baited traps tested 1 h after a 1-h exposure on a metal disk sprayed with either water, 2% Purespray Green® (oil), MEC-LR® applied at 1 μg a.i.$^{-1}$ cm^{-2} (MEC), or MEC-LR® at 1 μg a.i.$^{-1}$ cm^{-2} + 2% Purespray Green® (MEC + oil). Formulations were aged on disks for 6 days (N=7 groups of ten males/treatment). Means with a *different letter* are significantly different by Student–Newman–Keuls multiple comparison test following significant ANOVA (P<0.05)

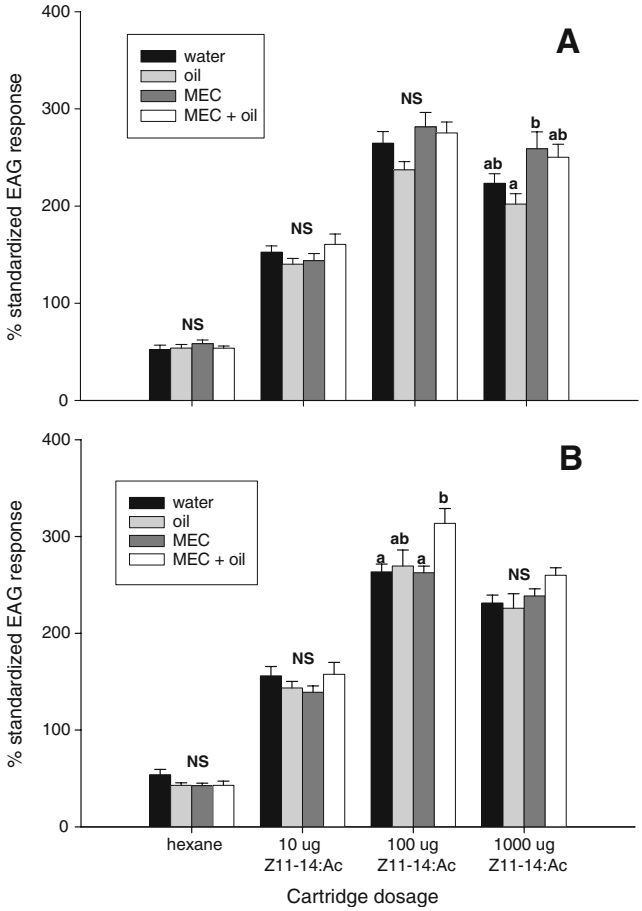

Fig. 4 Mean (+SE) standardized EAG responses generated from excised antenna of male *Choristoneura rosaceana* assayed 75 s after a 1-h (**a**) or 24-h (**b**) exposure on a metal disk sprayed with either water, 2% Purespray Green® (oil), MEC-LR® applied at 1 μg a.i.$^{-1}$ cm^{-2} (MEC), or MEC-LR® at 1 μg a.i.$^{-1}$ cm^{-2} + 2% Purespray Green® (MEC + oil) treatments. Formulations were aged on disks for 6 days before male exposure treatments, N=9–10 antennae/treatment. Means with a *different letter* are significantly different by Student–Newman–Keuls multiple comparison test following significant ANOVA (P< 0.05)

Experiments 5 and 6: Electrophysiological Effects After Exposure to Pheromone-treated Disks Neither 1- nor 24-h exposure periods to formulations of MEC and MEC + oil reduced male antennal response to Z11-14:OAc compared to controls (Fig. 4a, b). Interestingly, after a 1-h exposure, MEC-exposed males showed a small but significantly greater antennal response to the 1,000-μg Z11-14:OAc stimulus ($F_{3, 36}$=3.92, P=0.016) than did oil-exposed males (Fig. 4a), and after a 24-h exposure (Fig. 4b) MEC + oil-exposed males showed enhanced response to the 100-μg Z11-14:OAc stimulus ($F_{3, 32}$=3.66, P=0.018).

Experiment 7: Microcapsule Density Direct counts of microcapsules showed that MEC-treated disks had fewer microcapsules deposited on them compared to MEC + oil-

treated ones (64.8±5.1 microcapsules cm^{-2} vs. 95.1±8.2 microcapsules cm^{-2}: $F_{1, 30}$=4.45, P=0.04).

Discussion

Our data demonstrate that surface exposure to formulations of MEC pheromones in water or oil has a strong effect on mate-finding behavior in *C. rosaceana*, but causes no reduction of antennal response to Z11-14:OAc tested immediately after exposure. Taken collectively, our behavioral and electrophysiological results suggest that habituation of the male central nervous system may be a major mechanism of disruption in this study, and that the disruptive effect of MEC formulations may be moderately enhanced by the addition of 2% oil.

Experiments 1 and 2 showed that exposure to MEC-LR® formulated in oil or water disrupts mate-finding behavior compared to controls up to 47 days after application. Males placed on pheromone-treated disks were generally more quiescent before the flight assays than those placed on control disks, which eliminates exhaustion as a possible explanation for our results. Experiments 2–4 provided behavioral evidence that habituation may be an important mating disruption mechanism because the experimental protocol eliminated any possibility of camouflage of the female plume as a potential mechanism. When males were assayed in the flight tunnel 75 s after removal from the treated surface in experiment 2, a similar reduction in trap catch to experiment 1 was observed. This indicates that the role of physical camouflage of the pheromone plume (Judd et al. 2005a) may be less important once significant habituation has been induced. In experiment 2, there was no significant interaction between treatment and formulation age, which indicates that the relative efficacy of treatments did not change as they aged in the fume hood.

Our finding that pheromone exposure resulted in a significant reduction in subsequent mate-finding behavior up to 1 h after exposure is consistent with previous studies on other moth species such as *G. molesta* (Figueredo and Baker 1992; Rumbo and Vickers 1997), *Trichoplusia ni* Hübner (Kuenen and Baker 1981; Liu and Haynes 1993), and *Heliothis virescens* Fabricius (Daly and Figueredo 2000). However, our results contrast with those of Evenden et al. (2000), in which a 1-h exposure to atmospheric pheromone caused no reduction in male *C. rosaceana* response to a calling female 10–30 min after exposure. It is possible that the atmospheric concentration produced from a pheromone-treated septa (Evenden et al. 2000) was insufficient to induce habituation as compared to males resting directly on a MEC-treated disk.

In experiment 4, both MEC- and MEC + oil-exposed males exhibited a significant positive relationship between the recovery time after exposure and probability of contacting the female. However, this relationship was not as strong as expected given the results from experiments 2 and 3. This suggests considerable phenotypic variability among individual male *C. rosaceana*, which is supported by the fact that 17 of 40 males (42.5%) were able to locate the calling female within 5 min of being removed from the MEC- and MEC + oil-treated disks. Alternatively, it is possible that all males were not exposed identically to pheromone. Males that spent more time on the cage that housed the treated disk may have received lower pheromone exposure than males that consistently remained on the disk surface.

The results of the electrophysiological experiments also indicate that central nervous system habituation is the important mechanism of mating disruption in our experiments, as no significant antennal adaptation was found when EAG assays were performed 75 s after removal of males from treated disks. It is possible that males experienced some transient antennal adaptation within the 75 s immediately following removal from the treated disk. However, release rates of Z11-14:OAc from MEC-LR® in water and oil at 6 days post-spray were ≈8 pg Z11-14:OAc cm^{-2} min^{-1} (Judd et al. 2006), which are well below the threshold concentration of 500 pg Z11-14:OAc ml^{-1} air necessary to induce significant "long-lasting" antennal adaptation in male *C. rosaceana* (Stelinski et al. 2003a, b). Interestingly, in two instances, our EAG experiments showed a marginal increase in antennal response to a stimulus of Z11-14:OAc following exposure to MEC-LR® formulations, possibly a result of antennal sensitization similar to that described by Stelinski et al. (2003c).

The addition of oil to the MEC formulation moderately enhanced disruption over time (Fig. 2). One possible reason is that MEC + oil maintains an elevated release rate (≈8 pg cm^{-2} min^{-1} between 13 and 30 days after application) compared to MEC alone, which maintains a release rate of ≈2 pg cm^{-2} min^{-1} between day 13 and 30 after application (Judd et al. 2006). The greater efficacy of the MEC + oil treatment also could be related to the increased number of microcapsules deposited on the disks compared with MEC. Although the reasons for this remain unclear, the oil may increase the deposition of spray droplets by affecting the surface tension properties and spreading coefficient of the spray (Anderson et al. 1987; Streibig and Kudsk 1992). When these same treatments were applied to apple foliage in the field, an equal level of disruption of mate-finding behavior in *C. rosaceana* was found between MEC and MEC + oil treatments up to 42 days after treatment (Wins-Purdy et al. 2007).

After a 1-h recovery period, the mate-finding ability of MEC-exposed males was no different from control males,

while the mate-finding ability of MEC + oil-exposed males was still significantly reduced (Fig. 3). If pheromone release rates were nearly equal from MEC and MEC + oil disks aged 6 days (Judd et al. 2006), then the difference may be related to small amounts of pheromone adsorbed or absorbed by the male. Pheromone and oil could have been picked up by the male tarsi as a result of direct contact with the treated disks, and potentially transferred to the antennae through preening. Since horticultural oils can penetrate insect tissues (Taverner et al. 2001), the oil could increase the physical adsorption or absorption of pheromone by the male (Krupke et al. 2002; Evenden et al. 2005).

The 47-day period of sustained treatment efficacy in this study is in sharp contrast to the results of Judd et al. (2005a), who found that MEC-LR® applied as an atmospheric treatment was an effective disruptant of mate finding in *C. rosaceana* for <55 h. The degree of formulation longevity in our study highlights the possible importance of male proximity to the treated surface in maintaining a significant disruptive effect, even when pheromone release rates from microcapsules drop to low levels. Atmospheric pheromone concentration declines with the inverse square root of the distance from the pheromone source (Karg et al. 1994). Therefore, pheromone concentrations in the boundary layer (e.g., <1 cm from the treated surface), where an insect may alight or rest, may be many times greater than the average atmospheric concentration throughout a treated area. However, the amount of time that male *C. rosaceana* rest on MEC-treated foliage in the field, where they could be exposed to these higher pheromone concentrations, needs to be determined experimentally.

We applied the same amount of MEC and MEC + oil per unit area to metal disks that would be applied per unit area to an orchard, using the label-recommended application rate for MEC-LR® and Purespray Green®. However, the rate of deposition of microcapsules on metal disks may not equal that on plant tissues under field conditions (Knight and Larson 2004), and we were unable to develop a reliable method for counting microcapsules applied to apple foliage (Wins-Purdy et al. 2007). It is also likely that release rates of Z11-14:OAc from the surface differ between these substrates. Despite these differences, there is a consistency between the results of the current study and our previous findings that used apple foliage over a 21-day period (Wins-Purdy et al. 2007). This suggests that the use of metal disks as a treatment surface is appropriate for an examination of mechanisms of mating disruption with MEC formulations. Wins-Purdy et al. (2007) found that both MEC and MEC + oil treatments lost efficacy after 21 days when foliage was treated and aged in the field, thus suggesting environmental factors such as UV light, high temperatures, and rainfall (Waldstein and Gut 2004) may play a significant role in reducing the efficacy of MEC and MEC + oil formulations over time.

C. rosaceana is a difficult pest to control successfully through mating disruption alone, although the reasons for this remain unclear (Agnello et al. 1996; Lawson et al. 1996; Knight et al. 1998; Stelinski et al. 2003a; Trimble and Appleby 2004). Evenden et al. (2000) concluded that habituation did not occur in this species as a result of exposure to their atmospheric pheromone treatment, and Stelinski et al. (2004) demonstrated an increase in male responsiveness to pheromone 24 h after pheromone exposure. The current study is the first to demonstrate significant nervous system habituation in *C. rosaceana*, although in no trial did pheromone exposure completely eliminate response of all males to calling virgin females. Stelinski et al. (2003a) suggested that significant long-lasting antennal adaptation found in *C. rosaceana* could act to shield the central nervous system from the effects of habituation and allow some males to overcome mating disruption. While this seems an unlikely explanation for our results given the lack of any significant antennal adaptation after exposure, it is possible that transient adaptation of antennal receptors occurred while males rested on the MEC-treated surface. Transient antennal adaptation was demonstrated in *T. ni* (Kuenen and Baker 1981), and would allow for a rapid recovery of sexual behavior once the male leaves an area of high pheromone concentration. Other authors have demonstrated that the time male moths are exposed to pheromone influences the duration of habituation and the subsequent disruption to mate-finding behavior (Rumbo and Vickers 1997; Stelinski et al. 2003a). Further study is necessary to determine how long male *C. rosaceana* need to be exposed to MEC-treated surfaces to achieve significant disruption.

The lack of efficacy and longevity of MEC formulations remains a significant barrier to their widespread adoption. However, the fact that physical contact with MEC- and MEC + oil-treated surfaces in this study induced significant habituation but not adaptation in *C. rosaceana* suggests that the central nervous system of males was not shielded (sensu Stelinski et al. 2003a). As central nervous system habituation should be a more effective mechanism of disruption than antennal adaptation (Kuenen and Baker 1981), this could represent an important benefit of MEC formulations over attractive, high-dosage dispensers, where males may be exposed to much greater concentrations of pheromone that could induce a protective antennal adaptation.

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References

ANDERSON, N. H., HALL, D. J., and SEAMAN, D. 1987. Spray retention: effects of surfactants and plant species. *Asp. Appl. Biol.* 14:233–243.

AGNELLO, A. M., REISSIG, W. H., SPANGLER, S. M., CHARLTON, R. E., and KAIN, D. P. 1996. Trap response and fruit damage by obliquebanded leafroller (Lepidoptera: Tortricidae) in pheromone-treated apple orchards in New York. *Environ. Entomol.* 25:268–282.

ALBAJES, R., KONSTANTOPOULOU, M., ETCHEPARE, O., EIZAGUIRRE, M., FREROT, B., SANS, A., KROKOS, F., AMELINE, A., and MAZOMENOS, B. 2002. Mating disruption of the corn borer *Sesamia nonagrioides* (Lepidoptera: Noctuidae) using sprayable formulations of pheromone. *Crop Prot.* 21:217–225.

BARTELL, R. J. 1982. Mechanisms of communication disruption by pheromone in the control of Lepidoptera: a review. *Physiol. Entomol.* 7:353–364.

BEROZA, M., HOOD, C. S., TREFREY, D., LEONARD, D. E., KNIPLING, E. F., KLASSEN, W., and STEVENS, L. J. 1974. Large field trial with microencapsulated sex pheromone to prevent mating of the gypsy moth. *J. Econ. Entomol.* 67:659–664.

CARDÉ, R. T. 1990. Principles of mating disruption, pp. 47–71, in R. L. Ridgway, R. M. Silverstein, and M. N. Inscoe (eds.). Behaviour-modifying Chemicals for Insect Management: Applications of Pheromone and Other AttractantsMarcel Dekker, New York.

CARDÉ, R. T., STATEN, R. T., MAFRA, and NETO, A. 1998. Behaviour of pink bollworm males near high-dose, point sources of pheromone in field wind tunnels: insights into mechanisms of mating disruption. *Entomol. Exp. Appl.* 89:35–46.

DALY, K. C., and FIGUEREDO, A. J. 2000. Habituation of sexual response in male *Heliothis* moths. *Physiol. Entomol.* 25:180–190.

DELISLE, J. 1992. Age related changes in the calling behaviour and the attractiveness of obliquebanded leafroller virgin females, *Choristoneura rosaceana*, under different constant and fluctuating temperature conditions. *Entomol. Exp. Appl.* 63:55–62.

DELISLE, J. 1995. Effect of male and female age on the mating success of the obliquebanded leafroller *Choristoneura rosaceana* (Lepidoptera: Tortricidae) under different ecological conditions. *J. Insect Behav.* 8:781–799.

DELISLE, J., and ROYER, L. 1994. Changes in pheromone titre of oblique-banded leafroller, *Choristoneura rosaceana*, virgin females as a function of time of day, age, and temperature. *J. Chem. Ecol.* 20:45–69.

DOANE, C. C. 1999. Controlled-release devices for pheromones, p. 295, in H. B. Scher (ed.). Controlled-release Delivery Systems for PesticidesMarcel Dekker, New York.

EL-SAYED, A. M., DELISLE, J., DE, LURY, N., GUT, L. J., JUDD, G. J. R., LEGRAND, S., REISSIG, W. H., ROELOFS, W. L., UNELIUS, C. R., and TRIMBLE, R. M. 2003. Geographic variation in pheromone chemistry, antennal electrophysiology, and pheromone-mediated trap catch of North American populations of the obliquebanded leafroller. *Environ. Entomol.* 32:470–476.

EVENDEN, M. L., JUDD, G. J. R., and BORDEN, J. H. 1999. A synomone imparting distinct sex pheromone communication channels for *Choristoneura rosaceana* (Harris) and *Pandemis limitata* (Robinson) (Lepidoptera: Tortricidae). *Chemoecology* 9:73–80.

EVENDEN, M. L., JUDD, G. J. R., and BORDEN, J. H. 2000. Investigations of mechanisms of pheromone communication disruption of *Choristoneura rosaceana* (Harris) in a wind tunnel. *J. Insect Behav.* 13:499–510.

EVENDEN, M. L., MCLAUGHLIN, J. R., and CZOKAJLO, D. 2005. Effects of exposure to pheromone and insecticide constituents of an attracticide formulation on reproductive behaviour of oriental fruit moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 98:334–341.

FIGUEREDO, A. J., and BAKER, T. C. 1992. Reduction of the response to sex pheromone in the oriental fruit moth, *Grapholita molesta* (Lepidoptera: Tortricidae) following successive pheromonal exposures. *J. Insect Behav.* 5:347–363.

FLINT, H. M., and MERKLE, J. R. 1983. Pink bollworm (Lepidoptera: Gelechiidae): communication disruption by pheromone composition imbalance. *J. Econ. Entomol.* 76:40–46.

HALL, D. J., and MARRS, G. J. 1989. Microcapsules, p. 199, in A. R. Jutsum, and R. F. S. Gordon (eds.). Insect Pheromones in Plant ProtectionWiley, New York.

JUDD, G. J. R., MCBRIEN, H. L., and BORDEN, J. H. 1995. Modification of responses by *Campylomma verbasci* (Heteroptera: Miridae) to pheromone blends in atmospheres permeated with synthetic sex pheromone or individual components. *J. Chem. Ecol.* 21:1991–2002.

JUDD, G. J. R., DE LURY, N. C., and GARDINER, M. G. T. 2005a. Examining disruption of pheromone communication in *Choristoneura rosaceana* and *Pandemis limitata* using microencapsulated (*Z*)-11-tetradecenyl acetate applied in a laboratory flight tunnel. *Entomol. Exp. Appl.* 114:35–45.

JUDD, G. J. R., GARDINER, M. G. T., DELURY, N. C., and KARG, G. 2005b. Reduced antennal sensitivity, behavioural response, and attraction of male codling moths, *Cydia pomonella*, to their pheromone (*E,E*)-8,10-dodecadien-1-ol following various pre-exposure regimes. *Entomol. Exp. Appl.* 114:65–78.

JUDD, G. J. R., GARDINER, M. G. T., and WINS-PURDY, A. H. 2006. Formulating microencapsulated pheromones with horticultural oil improves release-rate characteristics and efficacy of disruption, p. 15, *in* Proceedings of 80th Annual Western Orchard Pest & Disease Management Conference, Portland, OR, USA January 11–13, 2006.

KARG, G., SUCKLING, D. M., and BRADLEY, S. J. 1994. Absorption and release of pheromone of *Ephiphyas postvittana* (Lepidoptera: Tortricidae) by apple leaves. *J. Chem. Ecol.* 20:1825–1841.

KNIGHT, A. L., and LARSON, T. E. 2004. Improved deposition and performance of a microencapsulated sex pheromone formulation for codling moth (Lepidoptera: Tortricidae) with a low volume application. *J. Entomol. Soc. B.C.* 101:79–86.

KNIGHT, A. L., THOMSON, D. R., and COCKFIELD, S. D. 1998. Developing mating disruption of obliquebanded leafroller (Lepidoptera: Tortricidae) in Washington State. *Environ. Entomol.* 27:1080–1088.

KOVANCI, O. B., WALGENBACH, J. F., KENNEDY, G. G., and SCHAL, C. 2005. Effects of application rate and interval on the efficacy of sprayable pheromone for mating disruption of the oriental fruit moth *Grapholita molesta*. *Phytoparasitica* 33:334–342.

KRUPKE, C. H., ROITBERG, B. D., and JUDD, G. J. R. 2002. Field and laboratory responses of male codling moth (Lepidoptera: Tortricidae) to a pheromone-based attract-and-kill strategy. *Environ. Entomol.* 31:189–197.

KUENEN, L. P. S., and BAKER, T. C. 1981. Habituation versus sensory adaptation as the cause of reduced attraction following pulsed and constant sex pheromone pre-exposure in *Trichoplusia ni*. *J. Insect Physiol.* 27:721–726.

LAWSON, D. S., REISSIG, W. H., AGNELLO, A. M., NYROP, J. P., and ROELOFS, W. L. 1996. Interference with the mate-finding communication system of the obliquebanded leafroller (Lepidoptera: Tortricidae) using synthetic sex pheromones. *Environ. Entomol.* 25:895–905.

LIU, Y.-B., and HAYNES, K. F. 1993. Pheromone-mediated responses of male cabbage looper moths, *Trichoplusia ni*, following various exposures to sex pheromone or (*Z*)-7-dodecenol. *J. Chem. Ecol.* 1993:503–515.

MILLER, J. R., GUT, L. J., DE, LAME, F. M., and STELINSKI, L. L. 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (part I): theory. *J. Chem. Ecol.* 32:2089–2114.

MILLER, J. R., GUT, L. J., DE, LAME, F. M., and STELINSKI, L. L. 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (part 2): case studies. *J. Chem. Ecol.* 32:2115–2143.

POLAVARAPU, S., LONERGAN, G., PENG, H., and NEILSEN, K. 2001. Potential for mating disruption of *Sparganothis sulfureana* (Lepidoptera: Tortricidae) in cranberries. *J. Econ. Entomol.* 94:658–665.

RUMBO, E. R., and VICKERS, R. A. 1997. Prolonged adaptation as possible mating disruption mechanisms in oriental fruit moth, *Cydia* (=*Grapholita*) *molesta*. *J. Chem. Ecol.* 23:445–457.

SANDERS, C. J., and LUCUIK, G. S. 1996. Disruption of male oriental fruit moth to calling females in a wind tunnel by different concentrations of synthetic pheromone. *J. Chem. Ecol.* 22:1971–1986.

SHOREY, H. H., and HALE, R. L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

STELINSKI, L. L., GUT, L. J., and MILLER, J. R. 2003a. Concentration of air-borne pheromone required for long-lasting peripheral adaptation in the obliquebanded leafroller, *Choristoneura rosaceana*. *Physiol. Entomol.* 28:97–107.

STELINSKI, L. L., MILLER, J. R., and GUT, L. J. 2003b. Presence of long-lasting peripheral adaptation in oblique-banded leafroller, *Choristoneura rosaceana* and absence of such adaptation in redbanded leafroller, *Argyrotaenia velutinana*. *J. Chem. Ecol.* 29:405–423.

STELINSKI, L. L., MILLER, J. R., RESSA, N. E., and GUT, L. J. 2003c. Increased EAG responses of tortricid moths after prolonged exposure to plant volatiles: evidence for octopamine-mediated sensitization. *J. Insect Physiol.* 49:845–856.

STELINSKI, L. L., GUT, L. J., VOGEL, K. J., and MILLER, J. R. 2004. Behaviors of naive vs. pheromone-exposed leafroller moths in plumes from high-dosage pheromone dispensers in a sustained-flight wind tunnel: implications for mating disruption of these species. *J. Insect Behav.* 17:533–554.

STELINSKI, L. L., GUT, L. J., KETNER, K. C., and MILLER, J. R. 2005. Orientational disruption of codling moth, *Cydia pomonella* (L.) (Lep., Tortricidae), by concentrated formulations of microencapsulated pheromone in flight tunnel assays. *J. Appl. Entomol.* 129:481–488.

STREIBIG, J. C., and KUDSK, P. 1992. The influence of adjuvants on herbicidal activity of alloxydim-sodium, fluazifop-butyl, and tribenuron, pp. 513–524, in C. L. Foy (ed.). Adjuvants for AgrichemicalsCRC, Boca Raton.

TASCHENBERG, E. F., and ROELOFS, W. L. 1976. Pheromone communication disruption of the grape berry moth with microencapsulated and hollow fiber systems. *Environ. Entomol.* 5:688–691.

TAVERNER, P. D., GUNNING, R. V., KOLESIK, P., BAILEY, P. T., INCEOGLU, A. B., HAMMOCK, B., and ROUSH, R. T. 2001. Evidence for direct neural toxicity of a "light" oil on the peripheral nerves of lightbrown apple moth. *Pestic. Biochem. Physiol.* 69:153–165.

TRIMBLE, R. M., and APPLEBY, M. E. 2004. Comparison of efficacy of programs using insecticide and insecticide plus mating disruption for controlling the obliquebanded leafroller in apple (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 97:518–524.

TRIMBLE, R. M., VICKERS, P. M., NIELSEN, K. E., and BARINSHTEYN, G. 2003. Sprayable pheromone for controlling the North American grape berry moth by mating disruption. *Agric. For. Entomol.* 5:263–268.

TRIMBLE, R. M., PREE, D. J., BARSZCZ, E. S., and CARTER, N. J. 2004. Comparison of a sprayable pheromone formulation and two hand-applied pheromone dispensers for use in the integrated control of oriental fruit moth (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 97:482–489.

WALDSTEIN, D. E., and GUT, L. J. 2004. Effects of rain and sunlight on oriental fruit moth (Lepidoptera: Tortricidae) pheromone microcapsules applied to apple foliage. *J. Agric. Urban Entomol.* 21:117–128.

WEATHERSTON, I. 1990. Principles of design of controlled-release formulations, pp. 93–112, in R. L. Ridgeway, R. M. Silverstein, and M. N. Inscoe (eds.). Behaviour-modifying Chemicals for Insect Management: Applications of Pheromone and Other AttractantsMarcel Dekker, New York.

WINS-PURDY, A. H., Judd, G. J. R., and Evenden, M. L. 2007. Disruption of pheromone communication of *Choristoneura rosaceana* (Lepidoptera: Tortricidae) using microencapsulated sex pheromones formulated with horticultural oil. *Environ. Entomol.* 36:1189–1198.

ing citrus, is of greater importance than its impact as an herbivore. Infection by *X. axonopodis* results in defoliation, premature fruit drop, and general decline in tree quality (Graham et al. 2004). Although this disease is spread primarily by wind-driven rain, the serpentine-feeding galleries of *P. citrella* are easily colonized by the bacterium, and the resulting canker lesions generally are larger and more infectious than those formed without the benefit of *P. citrella* leaf injury (Graham et al. 2004; Gottwald et al. 2007).

In Japan, (*Z*,*Z*)-7,11-hexadecadienal (*Z*7*Z*11-16Ald) was reported as the behaviorally active sex pheromone (Mafi et al. 2005). Brazilian and US populations of *P. citrella* produce a 30:10:1 mixture of (*Z*,*Z*,*E*)-7,11,13-hexadecatrienal (*Z*7*Z*11*E*13-16Ald), *Z*7*Z*11-16Ald, and (*Z*)-7-hexadecenal (Leal et al. 2006; Moreira et al. 2006). However, a 3:1 blend of *Z*7*Z*11*E*13-16Ald/*Z*7*Z*11-16Ald is sufficient for eliciting the full complement of male sexual behaviors (Leal et al. 2006; Moreira et al. 2006) and is now being exploited in management programs for this pest (Lapointe et al. 2006; Stelinski and Rogers 2008).

Recently, two mathematical transformations of moth catch *versus* dispenser density data were developed to differentiate between competitive and noncompetitive mechanisms of mating disruption (Miller et al. 2006a,b). Competitive disruption occurs when males are attracted to the source of synthetic pheromone, while noncompetitive mechanisms, such as camouflage or sensory desensitization, by definition, do not require attraction to the synthetic pheromone source (Miller et al. 2006a). If competitive attraction occurs, then plotting 1 over male visitation rate to a given attractant source against dispenser density (on the *Y*- and *X*-axes, respectively, Miller–Gut plot) yields a straight line with a positive slope, while plotting male visitation rate to a given attractant source against dispenser density×visitation rate (Miller–de Lame plot) results in a straight line with a negative slope (Miller et al. 2006a). In contrast, noncompetitive attraction predicts a characteristically concave Miller–Gut plot, as well as a recurved Miller–de Lame plot (Miller et al. 2006a). Post hoc analyses of 13 published mating disruption trials across a range of moth species were consistent with the hypothesis that competitive attraction is the mechanism that explains the majority of cases (Miller et al. 2006b).

The first objective of the current investigation was to evaluate the potential of using mating disruption as a management tactic for *P. citrella* in Florida citrus. The second was to gain insight into the behavioral mechanisms that underlie disruption of this species, which may help facilitate development of robust and economical management of *P. citrella* with pheromones. In addition, the current study was designed to test directly the predictions recently outlined by Miller et al. (2006a) by developing moth catch *versus* dispenser density profiles while concurrently observing moth behavior in the field.

Methods and Materials

Field plots The experiment was conducted in an 8-yr-old 10.2-ha orange grove (*Citus sinensis* [L.] var. "Valencia") in Clermont, FL, USA. Trees were planted on a 3×6-m spacing, and the average canopy height was 4 m. The grove was managed by the University of Florida according to commercial pruning, irrigation, herbicide, and fungicide management practices but without the input of insecticides.

Dispenser Density Treatments and Experimental Design Disruption trials were conducted by comparing four application densities of red rubber septa (The West Company, Lionville, PA, USA): 0 (0/ha, 0.0 g active ingredient [AI]/ha), 0.2 (one every fifth tree or 35/ha, 0.05 g AI/ha), 1 (215/ha, 0.29 g AI/ha), and 5 (1,100/ha, 1.5 g AI/ha) per tree loaded with 1.0 mg of *Z*7*Z*11*E*13-16Ald and 0.33 mg of *Z*7*Z*11-16Ald (ISCA Technologies, Riverside, CA, USA). Pheromone components were greater than or equal to 98% pure by gas chromatographic analysis. The experiment was arranged as a randomized complete block design with five replicates, each consisted of a 30-tree (0.14 ha) plot of five rows of six trees. Replicate plots were separated by 40 m, and blocks of treatments were separated by 50 m. Rubber septum dispensers were affixed to foliage via metal paper clips on the edge of tree canopies approximately 2.0 m above ground level, which is the location of greatest male *P. citrella* activity within trees approximately 4 m tall (Stelinski and Rogers 2008). Treatments were applied on 4 April 2007 and renewed on 15 June 2007 so that the experiment could be conducted throughout the *P. citrella* seasonal flight.

Orientational Disruption Measurements Disruption of male *P. citrella* orientation was quantified by using two pheromone traps (LPD Scenturion Guardpost, Suterra, Bend, OR, USA) deployed within each replicate plot. One trap was placed in the central tree of each plot and the second on the inside border row two trees from the plot edge. All traps were baited with a single red rubber septum lure loaded with 0.1 mg of *Z*7*Z*11*E*13-16Ald and 0.03 mg of *Z*7*Z*11-16Ald as this has been shown to be highly effective for trapping male *P. citrella* in Florida (Stelinski and Rogers 2008). Monitoring traps were hung at least 1.0 m from the nearest mating disruption dispenser, at approximately 1.5–2 m above ground level in the midcanopy (Stelinski and Rogers 2008). Pheromone lures were replaced a total of four times or approximately every 7.5 wk throughout the season based on known longevity of attractiveness (Lapointe and Leal 2007). Moths captured in traps were counted and removed weekly.

Flush Infestation Damage to newly flushed leaves was assessed weekly 15 April through 28 October, 2007, except during the weeks of 16 July, 6 and 13 August, 10 and 17

September, and 15 October because of insufficient new flush on those dates. Twenty shoots, ten from the midcanopy (2.5 m) and ten from the lower canopy (1.0 m), were inspected at random from 20 trees per replicate block (2,000 flush samples per treatment), and the number of shoots per tree containing live mining *P. citrella* larvae was recorded.

Field Observations Male *P. citrella* behavior was studied in the field to test the prediction that male moths approach mating disruption dispensers in pheromone-treated plots, as other studies have reported male moths of various species orienting to, closely approaching, and even touching pheromone dispensers of a wide range of release rates in the field (Stelinski et al. 2004; Epstein et al. 2006). Observations of dispensers in tree canopies were conducted for approximately 2 hr each night between 21:00 and 23:00 hours, the period of greatest male *P. citrella* response (Stelinski and Rogers 2008), on eight nights between 24 August and 13 September and on five subsequent nights between 27 September and October 11. An observer rotated among plots conducting 20 min observational bouts per treatment such that multiple treatments were observed on a given night. The order of observations across treatments was randomized nightly.

In addition, male moth orientation to pheromone traps (as described above) was observed for 2-hr periods on four separate nights (14–22 September 2007) in control plots to verify that male *P. citrella* could be observed orienting to an attractive point source in the field. The number of moths observed orienting to such traps, as well as the number caught in traps under observation, was assessed. During the observations of both dispensers and traps, data were dictated into a hand-held microcassette audio recorder by an investigator standing 0.75 m from the pheromone source under observation. Observations after dusk employed night-vision goggles (Rigel, Model 3250, DeWitt, IA, USA) as described by Stelinski et al. (2004).

Profile Analyses Moth catch vs. dispenser density data were analyzed according to the profile analyses developed by Miller et al. (2006a,b). In addition to examining the untransformed data, two types of transformations were performed on male catch vs. dispenser density data: (1) 1 over male catch per trap per night on the *y*-axis was plotted against dispenser density on the *x*-axis (Miller–Gut plot), and (2) male catch per trap per night on the *y*-axis was plotted against dispenser density×visitation rate on the *x*-axis (Miller–de Lame plot).

Trapping Evaluation of Pheromone Dispensers An experiment was conducted to compare the lure dose used to monitor the effect of disruption treatments (0.1 mg of *Z7Z11E*13-16Ald and 0.03 mg of *Z7Z*11-16Ald) with the dose used to induce mating disruption (1.0 mg of *Z7Z11E*13-16Ald and 0.33 mg of *Z7Z*11-16Ald). The objective was to determine whether the release rate of the pheromone from the mating disruption dispensers was attractive to *P. citrella* in pheromone-free air over the course of the mating disruption study. This test was conducted on 13 April through 1 November, 2007 in 4.0 ha of untreated plots of "Valencia" oranges. Fresh lures were installed every 7.5 wk into plastic delta traps. Unbaited delta traps were included as a negative control treatment. The experiment was arranged in a randomized complete block with five replicates. Traps, hung approximately 1.5–2 m above ground level in the upper third of the tree canopy, were spaced approximately 40 m apart within replicates, and replicate blocks were spaced by 60–70 m. Moths captured in traps were counted and removed weekly, at which time the position of traps was rotated.

Statistical Analyses For orientational disruption and trapping studies, data were transformed to $\ln(x+1)$ (which normalized the distributions) and then subjected to analysis of variance (ANOVA). Flush injury data were arcsine transformed prior to ANOVA. When significant ANOVAs were identified, differences among means were separated by using the least significant difference (LSD) test (SAS Institute 2000). In all cases, the significance level was $\alpha<0.05$. Percent disruption was calculated as $1-[(\text{mean moth catch per trap in the pheromone-treated block/mean moth catch per trap in the control block})]\times 100$.

Results

Moth Catches and Leaf Infestation Preliminary analysis revealed no significant difference in moth catch between traps placed at the centers and borders of plots. Thus, data from the two traps per plot were combined for subsequent analyses. Fewer ($F=29.3$, $df=3, 16$, $P<0.001$) male *P. citrella* were captured in traps in plots treated with 1,100 dispensers per hectare (five dispensers per tree) than in any other treatment (Fig. 1a). This highest dispenser density treatment resulted in 98% disruption of moth catch in traps relative to untreated control plots over the season. There also was a significant reduction in male catch in traps placed in plots treated with 215 dispensers per hectare (69% disruption) compared with plots treated with 35 dispensers per hectare and untreated control plots; however, there was no difference in male catch between the latter two treatments (Fig 1a).

There was a trend of declining infestation with increasing dispenser density with a significant reduction ($F=12.1$, $df=3, 16$, $P<0.01$) in infestation of flush by both the 1,100- and 215-dispensers-per-hectare treatments relative to untreated plots (Fig. 1b), and the lowest infestation occurred

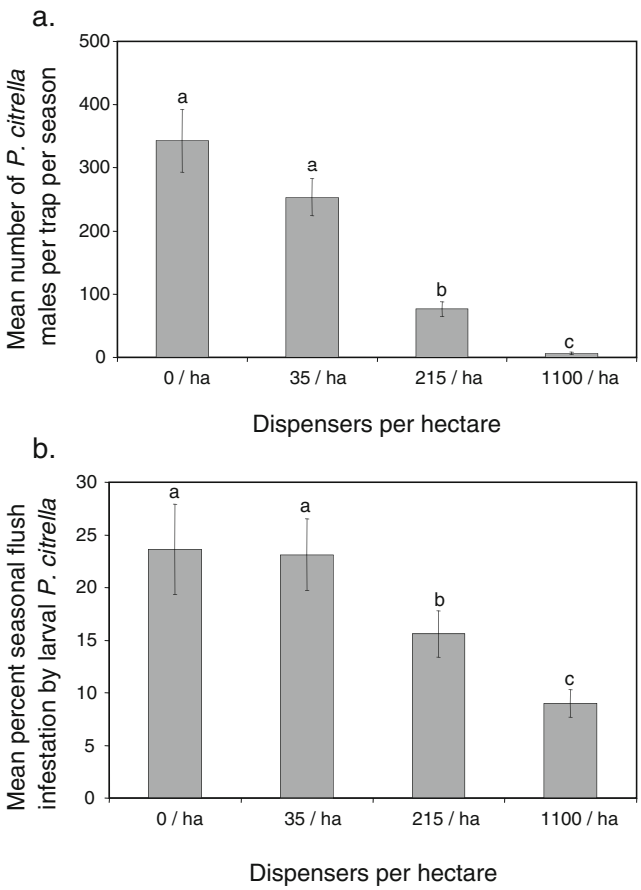

Fig. 1 Mean captures of male *Phyllocnistis citrella* in lure-baited delta traps in plots containing various densities of red septum dispensers of a 3:1 blend of *Z*7*Z*11*E*13-16Ald and *Z*7*Z*11-16Ald (**a**), Mean percent flush infestation by *P. citrella* larvae (**b**). Means followed by the same letter are not significantly different (AVOVA followed by LSD test at $\alpha<0.05$)

in plots treated with 1,100 dispensers per hectare. Flush infestation in plots treated with 35 dispensers per hectare was not significantly different from control plots.

Field Observations No male *P. citrella* were observed orienting to rubber septum disruption dispensers in any of the treatments during 26 hr of observation. In contrast, in control plots, 17.0±2.1 (mean±SE) males per night (N=4 nights) were observed orienting to baited monitoring traps, and 11.6±0.9 males were captured.

Profile Analysis The untransformed plots of dispenser density (Fig. 2a), as well as both secondary transformation plots (Fig. 2b,c), were consistent with the predictions of mating disruption by a noncompetitive mechanism. The untransformed plot was initially linear (Fig. 2a), the Miller–Gut plot was concave (Fig. 2b), and the Miller–de Lame plot was recurved (Fig. 2c). This set of traits is diagnostic of a noncompetitive mating disruption mechanism (Miller et al. 2006a).

Trapping Evaluation of Pheromone Dispensers In plots not under pheromone disruption, the number of male *P. citrella* captured per night in traps baited with the lower-loading monitoring lures (261.4±18.2, X±SEM) was significantly greater (F=15.7, df=2, 8, $P<0.01$) than the number captured with those baited with higher-dose mating disruption dispensers (179.3±16.9). Traps baited with both lure dosages captured significantly more moths over the course of the season than blank negative control traps (0.03±0.01).

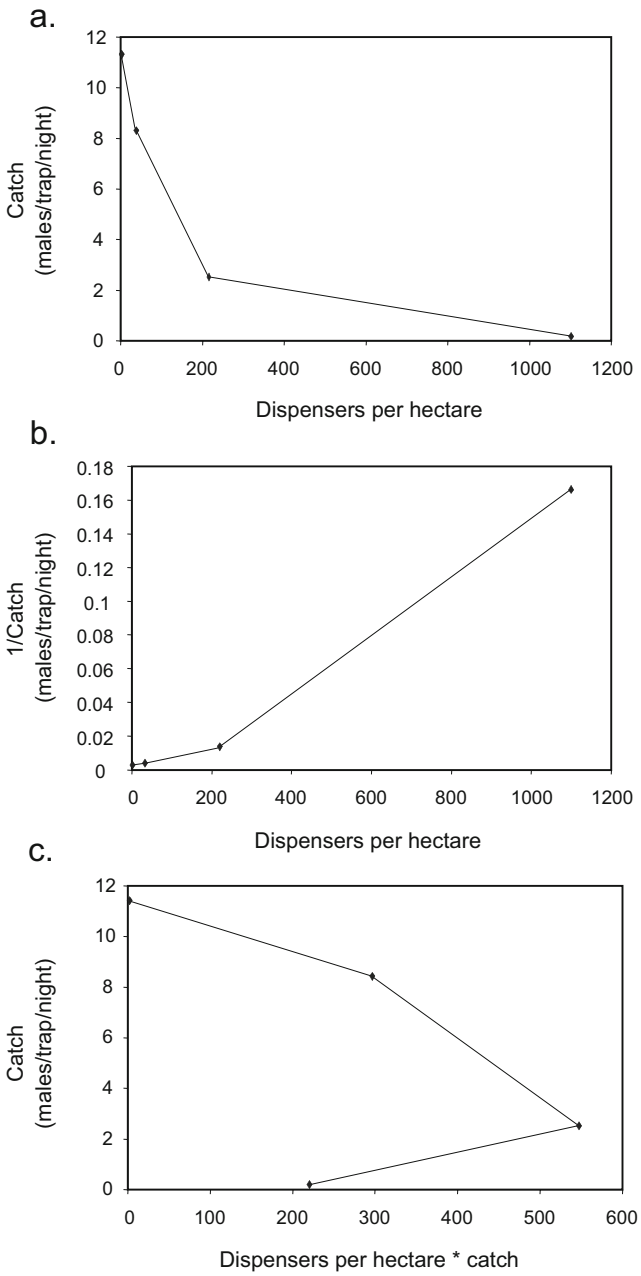

Fig. 2 Untransformed (**a**), Miller–Gut (**b**), and Miller–de Lame (**c**) plots of the disruption profile varying pheromone dispenser density for disruption of *P. citrella*

Discussion

Nearly complete disruption of *P. citrella* orientation to female-proxy-monitoring traps was achieved for 221 d with only two deployments of just 1.5 g AI/ha each. This would be sufficient for the season-long disruption of *P. citrella* males in Florida. Furthermore, nearly 70% disruption was achieved with only a 0.25-g/ha rate. The pheromone rate per hectare required for the disruption of *P. citrella* was well below 75–300 g of pheromone per hectare required for effective disruption of other species such as the Oriental fruit moth, *Grapholita molesta* (Rice and Kirsch 1990), the European grape moth, *Eupoecilia ambiguella* (Charmillot et al. 1987), the grape berry moth, *Paralobesia viteana* (Trimble et al. 1991), and the codling moth, *Cydia pomonella* (Howell et al. 1992). Ten grams of pheromone per hectare is one of the lowest effective rates for disruption of pink bollworm, *Pectinophora gossypiella*, when applied every 23 weeks as a microencapsulated sprayable formulation (Critchley et al. 1984). Short (2–3 wk) residual activity is characteristic of sprayable formulations in general (Gut et al. 2004). Effective disruption at a relatively low deployment rate (10 g of pheromone per hectare) was also achieved for a different leafmining species, the tomato pinworm (*Keiferia lycopersicella*; Jenkins et al. 1990). Thus, the low amount of pheromone required for effective season-long disruption of *P. citrella* is unique among moth species investigated to date and may be characteristic of Gracillariids and Gelechiids.

One of the objectives of this investigation was to test directly the predictions of Miller et al. (2006a,b) by conducting a disruption experiment where male moth catch in pheromone monitoring traps was quantified in plots with varying dispenser densities to differentiate between competitive and noncompetitive attraction. Furthermore, the outcomes of the theoretical predictions were compared with behavioral data collected directly in the field, thus providing evidence for a noncompetitive mechanism of disruption for *P. citrella* (Fig. 2a–c). As predicted, males did not approach mating disruption dispensers in any of the dispenser density treatments. Furthermore, we confirmed that male *P. citrella* could be observed orienting to attractive pheromone dispensers in plots not treated with pheromone. In addition, our trapping study confirmed that the dosage of pheromone loaded into the dispensers used as the mating disruption treatment attracts male *P. citrella* in control plots. These findings present a dilemma—how does a noncompetitive disruption profile arise from deployment of dispensers that, in a trapping study, were attractive? We speculate that the release rate of pheromone from the "disruptive dispensers" was well above normal but insufficient to preclude brief attraction events when such dispensers were sparse and hence rarely encountered. However, when these higher-dosage dispensers were packed together promoting numerous plume encounters, males enveloped in them for prolonged periods became desensitized and ceased plume following, or discrete plumes were enveloped within a miasma of background pheromone precluding orientation to an individual point source. Cessation of attraction under high dispenser density was supported both by the profile analysis and the failure to observe males arriving at dispensers in the disruption trials. Had attraction been a required first step mediating disruption of all males under these conditions, some males should have been observed approaching dispensers in pheromone-treated plots, and the profiles should have reflected competitive attraction. It appears that the disruption in this study was occurring mainly at some distance from the dispensers.

This reasoning raises the question of over what area each dispenser exerted a given effect. An examination of Fig. 2a reveals that approximately 100 dispensers per hectare were required to reduce male catch by 50% (11.5 to 5.7 males per trap per night). Since 1 ha=10,000 m^2, each dispenser can be calculated to have halved catch across approximately 100 m^2 of citrus grove (10,000 m^2 per 100 dispensers). If each dispenser had released all of its 1.33 mg of pheromone over the 14 weeks it was deployed, the average release rate per dispenser per hour would be 0.6 μg/hr, and the corresponding specific area for 1/2 trap suppression per microgram pheromone per hour would be 167 m^2. We suspect that this value underestimates pheromone specific activity for this study, because it is likely that pheromone load was not completely exhausted over the 14 wk of deployment, given that catch in traps was still being suppressed when the dispensers were replaced on 15 June. Nevertheless, this specific activity value for *P. citrella* pheromone is 40-fold higher than the 4 m/ μg pheromone/hr calculated to halve male catch of Oriental fruit moth based on the noncompetitive disruption profiles reported by Miller et al. (2006b).

Mating disruption could be an important contribution to *P. citrella* management programs in the USA and elsewhere. Spray programs for leafminers in citrus are often ineffective, require biweekly application given continual growth of new flush, and are detrimental to natural enemy populations of *P. citrella* and other citrus pests (Peña et al. 2002). In the current study, new flush infestation by larval *P. citrella* was reduced by more than half in the highest pheromone rate treatment, despite the small size of study plots (0.14 ha). It is well known that disruption outcomes are better in larger plots (see reviews by Gut et al. 2004; Witzgall et al. 2008), and thus, the current data suggest that the disruption of *P. citrella* should be effective on a larger scale. Furthermore, the control of this species by mating disruption should be density independent given the noncompetitive mechanism (Miller et al. 2006a,b). This bodes well for the development of commercial formulations that are economically feasible in the face of high *P. citrella* populations in Florida (Lapointe et al. 2006; Stelinski and Rogers 2008).

The primary challenge to developing a commercially viable mating disruption formulation for *P. citrella* is the complexity of the pheromone chemistry (Leal et al. 2006; Moreira et al. 2006), which currently renders the cost of synthesis economically prohibitive from a management perspective. Fortunately, little pheromone is required for effective disruption, and even lower rates of pheromone may prove effective for integrated programs that combine pheromone and reduced insecticide input. However, opportunities also exist to improve the economic feasibility of this pheromone technology if a less expensive synthesis protocol could be developed. In addition, even though it has been proven that a 3:1 blend of *Z*7*Z*11*E*13-16Ald/*Z*7*Z*11-16Ald is required for the attraction of *P. citrella* to traps (Leal et al. 2006; Moreira et al. 2006), the evidence for a noncompetitive mechanism of disruption, such as desensitization or camouflage, suggests that the diene or triene components alone might yield effective disruption. Indeed, *Z*7*Z*11-16Ald alone has been shown to effectively disrupt communication of *P. citrella* in Japan (Mafi et al. 2005). Finally, the large acreage of citrus in Florida will demand mechanical application for economic feasibility; therefore, long-lasting formulations amendable to mechanical application should be explored (Stelinski et al. 2007).

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References

CHARMILLOT, P. -J., BLOESCH, B., SCHMID, A., and NEUMANN, U. 1987. Lutte contre cochylis de la vigne *Eupoecilia ambiguella* Hb., par la technique de confusion sexuelle. *Rev. Suisse Vitic. Arboric. Hortic.* 19:155–164.

CRITCHLEY, B. R., CAMPION, D. G., MCVEIGH, E. M., MCVEIGH, L. J., JUSTUM, A. R., GORDON, R. F., MARKS, G. J., NASR EL-SAYED, A., and HOSNY, M. M. 1984. Microencapsulated pheromones in cotton pest management, pp. 241–245, in Proceedings of British Crop Protection Conference: Pests and Diseases. British Crop Protection Council, Croydon, Surrey, UK.

EPSTEIN, D., STELINSKI, L. L., REED, T. R., MILLER, J. R., and GUT, L. J. 2006. Higher densities of distributed pheromone sources provide disruption of codling moth, (Lepidoptera: Tortricidae) superior to that of lower densities of clumped sources. *J. Econ. Entomol.* 99:1327–1333.

GILL, R. J. 1999. Citrus leafminer found in California. *Cal. Dept. Food and Agric., Cal. Plant Pest & Disease Rep.* 18:79–80.

GOTTWALD, T. R., BASSANEZI, R. B., AMORIM, L., and BERGAMIN-FILHO, A. 2007. Spatial pattern analysis of citrus canker-infected plantings in São Paulo, Brazil, and augmentation of infection elicited by the Asian leafminer. *Phytopathology* 97:674–683.

GRAHAM, J. H., GOTTWALD, T. R., CUBERO, J., and ACHOR, D. S. 2004. *Xanthosmonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. *Mol. Plant Pathol.* 5:1–15.

GUT, L. J., STELINSKI, L. L., THOMPSON, D. R., and MILLER, J. R. 2004. Behavior modifying chemicals: prospects and constraints in IPM, pp. 73–121, in O. Koul, G. S. Dhaliwal, and G. Cuperus (eds.). Integrated Pest Management: Potential, Constraints, and ChallengesCABI, Wallingford, UK.

HALL, D. G., and ALBRIGO, L. G. 2007. Estimating the relative abundance of flush shoots in citrus, with implications on monitoring insects associated with flush. *HortScience* 42:364–368.

HEPPNER, J. B. 1993. Citrus leafminer, *Phyllocnistis citrella*, in Florida. *Trop. Lepid.* 4:49–64.

HOWELL, J. F., KNIGHT, A. L., UNRUH, T. R., BROWN, D. F., KRYSAN, J. L., SELL, C. R., and KIRSCH, P. A. 1992. Control of codling moth in apple and pear with sex pheromone-mediated mating disruption. *J. Econ. Entomol.* 85:918–925.

JENKINS, J. W., DOANE, C. C., SCHUSTER, D. J., and JIMENEZ, M. J. 1990. Development and commercial application of sex pheromone for control of the tomato pinworm, pp. 269–280, in R. L. Ridgway, R. M. Silverstein, and M. N. Inscoe (eds.). Behavior-modifying Chemicals for Pest Management: Applications of Pheromones and Other AttractantsMarcel Dekker, New York.

LAPOINTE, S. L., HALL, D. G., MURATA, Y., PARRA-PEDRAZZOLI, A. L., BENTO, J. M. S., VILELA, E. F., and LEAL, W. S. 2006. Field evaluation of a synthetic female sex pheromone for the leafmining moth *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) in Florida citrus. *Fla. Entomol.* 89:274–276.

LAPOINTE, S. L., and LEAL, W. S. 2007. Describing seasonal phenology of the leafminer *Phyllocnistis citrella* (Lepidoptera: Gracillariidae) with pheromone lures: controlling for lure degradation. *Fla. Entomol.* 90:710–714.

LEAL, W. S., PARRA-PEDRAZZOLI, A. L., COSSE, A. A., MURATA, Y., BENTO, J. M. S., and VILELA, E. F. 2006. Identification, synthesis, and field evaluation of the sex pheromone from citrus leafminer, *Phyllocnistis citrella*. *J. Chem. Ecol.* 32:155–168.

LEGASPI, J. C., and FRENCH, J. V. 2003. The citrus leafminer and its natural enemies. http://primara.tamu.edu/kcchome/pubs/leafminer.htm.

MAFI, S. A., VANG, L. V., NAKATA, Y., OHBAYASHI, N., YAMAMOTO, M., and ANDO, T. 2005. Identification of the sex pheromone of the citrus leafminer (*Phyllocnistis citrella* Stainton, Lepidoptera: Gracillariidae) with a trial of control by the communication disruption method. *J. Pestic. Sci.* 34:361–367.

MILLER, J. R., GUT, L. J., DE, LAME, F. M., and STELINSKI, L. L. 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone: (Part I) theory. *J. Chem. Ecol.* 32:2089–2114.

MILLER, J. R., GUT, L. J., DE, LAME, F. M., and STELINSKI, L. L. 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone: (Part 2) case studies. *J. Chem. Ecol.* 32:2115–2143.

MOREIRA, J. A., MCELFRESH, J. S., and MILLAR, J. G. 2006. Identification, synthesis, and field testing of the sex pheromone of the citrus leafminer, *Phyllocnistis citrella*. *J. Chem. Ecol.* 32:169–194.

NAGAMINE, W. T., and HEU, R. A. 2003. Citrus leafminer *Phyllocnistis citrella* Stainton (Lepidoptera: Gracillariidae). State of Hawaii, Dept. of Agric., New Pest Adv. no. 00-01.

PEÑA, J. E., HUNSBERGER, A., and SCHAFFER, B. 2000. Citrus leafminer (Lepidoptera: Gracillariidae) density: effect on yield of "Tahiti" lime. *J. Econ. Entomol.* 93:374–379.

PEÑA, J. E., SHARP, J. L., and WYSOKI, M. 2002. Tropical Fruit Pests and Pollinators: Biology, Economic Importance, Natural Enemies, and Control. CABI, Wallingford, UK.

RICE, R. E., and KITSCH, P. 1990. Mating disruption of the oriental fruit moth in the United States, pp. 193–211, in R. L. Ridgway,

R. M. Silverstein, and M. N. Inscoe (eds.). Behavior-modifying Chemicals for Pest Management: Applications of Pheromones and Other AttractantsMarcel Dekker, New York.

SAS Institute 2000. SAS/STAT User's Guide, Version 6, vol. 1. 4th edn.SAS Institute, Cary, NC.

STAINTON, T. H. 1856. Descriptions of three species of Indian microlepidoptera. *Trans. Entomol. Soc. Lond.* 3:301–304.

STELINSKI, L. L., GUT, L. J., PIERZCHALA, A. V., and MILLER, J. R. 2004. Field observations quantifying attraction of four tortricid moth species to high-dosage, polyethylene-tube pheromone dispensers in untreated and pheromone-treated orchards. *Entomol. Exp. Appl*. 113:187–196.

STELINSKI, L. L., MILLER, J. R., LEDEBUHR, R., and GUT, L. J. 2007. Season-long mating disruption of *Grapholita molesta* by one machine application of pheromone in wax drops (SPLAT-OFM). *J. Pest Sci.* 80:109–117.

STELINSKI, L. L., and ROGERS, M. E. 2008. Factors affecting captures of male citrus leafminer, *Phyllocnistis citrella* Stainton, in pheromone-baited traps. *J. Appl. Entomol.* 132:143–150.

TRIMBLE, R. M., PREE, D. J., VICKERS, P. M., and KER, K. W. 1991. Potential of mating disruption using sex pheromone for controlling the grape berry moth. *Endopiza viteana* (Clemens) (Lepidoptera: Tortricidae), in Niagara peninsula, Ontario vineyards. *Can. Entomol.* 123:451–460.

WITZGALL, P., STELINSKI, L., GUT, L., and THOMSON, D. 2008. Codling moth management and chemical ecology. *Annu. Rev. Entomol.* 53:503–522.

Adults of *H. burnsi* are similar in size to those of *H. electra* but are different in appearance. The forewings of *H. electra* are white, with black borders and markings, and the hind wings are a brilliant red, with black borders. *H. burnsi* more closely resembles *H. neumogeni*, being nearly completely white with two narrow black lines across the wings and a black spot on each wing. Eggs and larvae of the three species appear similar and the host range of *H. burnsi* overlaps with those of the other two species, with *H. neumogeni* feeding on *Rhus trilobata* (Nuttal) and *Prunus fasciculata* (Torrey) Gray, *H. electra* using solely *Eriogonum fasciculatum* Bentham, and *H. burnsi* using several hosts including *P. fasciculata*, *Tetradymia axillaris* A. Nelson, *Dalea spp*. Jussieu and, in the last instar, *E. fasciculatum* (Tuskes 1984; Tuskes et al. 1996). All three species are gregarious in the early instars and disperse after the third instar (Tuskes 1984; Tuskes et al. 1996). In the last instar, both *H. burnsi* and *H. electra* can be found together feeding on *E. fasciculatum*, after the primary hosts of *H. burnsi* have been defoliated.

In a study comparing the attraction of males of *H. electra mojavensis* to conspecific females or females of the allopatric *H. electra electra*, it was found that male *H. electra mojavensis* flew predominantly in the morning hours, usually from 0800 hours to as late as 1300 hours (JSM, unpublished data). In contrast, *H. burnsi* tends to fly later in the day, from 1530 hours to dusk (Tuskes et al. 1996). Despite this, males of both species exhibit pheromone-mediated flight beyond their normal flight times (JSM, unpublished observations). For example, males of *H. burnsi* have been collected in flight as early as 0900 hours, and males of *H. electra* have been attracted to a calling female as late as sunset (JSM, personal observations). Females of both species fly and oviposit after sunset (Tuskes et al. 1996).

Studies on the sex pheromones of the two subspecies of *H. electra* identified three differences between the subspecies, even though they are separated by a relatively short distance (100 km; McElfresh and Millar 1999d): (1) the ratio of sex pheromone components in pheromone gland extracts, (2) responses of male antennae to the pheromone components in coupled gas chromatography–electroantennogram detection (GC–EAD) analyses, and (3) behavioral responses of males to reconstructed synthetic pheromone blends in the field. Based on these results, we hypothesized that the differences between the two populations of *H. electra* might be due to interference in the pheromone channel from *H. burnsi* in the western Mojave Desert, which appeared to use a similar or, at least, overlapping pheromone blend to those used by the respective *H. electra* subspecies (McElfresh and Millar 1999d). That is, *H. electra mojavensis* might have altered its pheromone blend, thus minimizing cross-attraction of males to *H. burnsi* females. The objective of the study reported here was to identify the sex pheromone blend of *H. burnsi* fully, in order to assess the degree of similarity, and therefore the potential for interactions, between the sex pheromone blends of the two species.

Methods and Materials

Insects Last-instar *H. burnsi* were collected on host plants at Oro Grande Wash, 10 miles east of Phelan, or at Rock Corral, both in San Bernardino County, CA, USA. The principal host for the moth at Oro Grande Wash was *T. axillaris*, whereas larvae were collected from *Dalea spp*. at Rock Corral. Larval development was completed on *E. fasciculatum* for both populations, with larvae held inside 3.2-mm mesh screen cylinders (45 cm high × 60 cm diameter). The screen cylinders were wrapped around an *E. fasciculatum* plant and anchored with a wooden stake, driven into the ground, to which the screen was stapled. Loose sand was piled along the base of the screen enclosure to prevent escape, and fiberglass window screen mesh was placed over the top and stapled into position. To prevent ants from killing the larvae or prepupae, a barrier of granular Dursban® insecticide was placed around the outside of the enclosure. Pupae and prepupae were removed from cocoons in the leaf litter at the base of the plant once sufficient insects had completed larval development. Pupae were kept in screened cages (30 cm^2) outdoors to coordinate emergence; adults were removed prior to mating and either used immediately or placed in labeled glassine envelopes, in plastic bags with damp tissue, and held at 10–15°C until needed. Females were placed in 6×8-cm screen cages and allowed to call for at least 1 h prior to removal of the sex pheromone gland. Males were allowed to warm to room temperature for at least 1 h prior to use in coupled GC–EAD experiments.

For the GC–EAD experiments (see below), adult male *H. burnsi* were collected at the Oro Grande Wash field site using a lure containing five components: [(10*E*,12*Z*)-hexadecadienyl acetate (*E*10,*Z*12-16:Ac), (10*E*,12*Z*)-hexadecadienol (*E*10,*Z*12-16:OH), (10*E*,12*Z*)-hexadecadienal (*E*10,*Z*12-16:Ald), (10*E*,12*E*)-hexadecadienyl acetate (*E*10,*E*12-16:Ac), and hexadecyl acetate (16:Ac)] optimized for that species (in a ratio of, respectively, 100:10:1:50:350 μg, *E*10,*Z*12-16:Ac: *E*10,*Z*12-16:OH: *E*10,*Z*12-16:Ald: *E*10,*E*12-16:Ac:16:Ac). *H. electra mojavensis* males were caught after attraction to an optimized three-component blend (100:10:33 μg, *E*10,*Z*12-16:Ac: *E*10,*Z*12-16:OH: *E*10,*Z*12-16:Ald) at a site east of Hesperia, CA, USA, along the base of Ord Mountain, where field trials for that species had been conducted previously (McElfresh and Millar 1999d). *H. electra electra* males were captured after being attracted to a lure with an optimized four-component blend (100:10:1:0:333 μg, *E*10,*Z*12-16:Ac: *E*10,*Z*12-16:OH: *E*10,*Z*12-

16:Ald: *E*10,*E*12-16:Ac:16:Ac), west of Anza, Riverside County, where field trials had been conducted previously (McElfresh and Millar 1999d).

Preparation of Extracts The sex pheromone gland was removed from virgin, 1–2-day-old calling females by applying gentle pressure to the lateral sides of the abdomen, everting the ovipositor and sex pheromone gland, and excising the terminal segments of the abdomen with microscissors. The gland was placed in approximately 30 μl pentane and extracted for up to 60 min before the extract was decanted to a clean conical insert; to ensure complete transfer of the extract, the original tube was rinsed with two 10–20 μl aliquots of pentane. The sample was stored at −20°C and concentrated by passive evaporation, as needed, prior to analysis.

Gas Chromatography and Coupled GC–EAD Synthetic standards and aliquots of female extract were analyzed by using Hewlett-Packard 5890A or 5890 series II gas chromatographs (Hewlett-Packard, Palo Alto, CA, USA) with helium as carrier gas, splitless injection, and an injector temperature of 250°C. Columns (J&W Scientific, Folsom, CA, USA) and temperature programs used for the analyses were a DB-5 (30 m×0.32 mm ID, 0.25-μm film; temperature program 100°C for 0 or 1 min, increased by 10°C or 15°C min^{-1} to 275°C, and held for up to 33 min) and a DB-Wax (30 m×0.32 mm ID, 0.25-μm film; temperature program 100°C for 1 min, increased by 10°C min^{-1} to 240°C and held for up to 60 min, or 130°C to 240°C at 10°C min^{-1}, and held for 5 min). Aliquots of single and composite extracts of pheromone glands and synthetic standards were analyzed by coupled GC–EAD with methods and equipment as described previously (McElfresh and Millar 1999d). Tentative identifications of compounds were based upon retention time matches and responses of antennae of male moths in GC–EAD studies that used the two different columns described above. Identifications were confirmed by gas chromatography–mass spectrometry (GC–MS) with synthetic standards available from previous work (e.g., McElfresh and Millar 1999d).

Antennal response profiles of *H. burnsi*, *H. electra mojavensis*, and *H. electra electra* were generated by stimulating antennae of male moths with a mixture of the five components in the blend found to elicit behavioral responses from *H. burnsi*. The GC–EAD standards consisted of 10 ng/μl each of 10*E*,12*Z*-16:Ac, 10*E*,12*E*-16:Ac, 10*E*,12*Z*-16:Ald, 10*E*,12*Z*-16:OH, and 16:Ac in heptane. Each antennal preparation was stimulated with 1 μl of the mix, and antennal responses to each compound were recorded with PeakSimple software (version 2.83, SRI Instruments, CA, USA).

Gas Chromatography–Mass Spectrometry Electron impact mass spectra (70 eV) were recorded with an HP 6890 gas chromatograph interfaced to an HP 5973 mass selective detector. Injection was splitless; helium was the carrier gas, and injector and transfer line temperatures were 275°C and 280°C, respectively. An HP5-MS column (30 m×0.25 mm ID), programmed from 50°C (held for 1 min) to 250°C at 10°C min^{-1} (held for 20 min), was used for the analyses. Compounds from a composite pheromone gland extract were identified by comparison of their retention times and mass spectra with those of authentic standards.

Formation of Adducts with 4-Methyl-1,2,4-triazoline-3,5-dione Determination of double-bond positions in the diene acetates was accomplished by reaction with the powerful dienophile 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) to form Diels-Alder cycloadducts (Young et al. 1990). Thus, an extract of two females was evaporated just to dryness, and approximately 5 μl of MTAD in methylene chloride (2 mg/ml) was added at room temperature. The solution was analyzed immediately by GC–MS, with injector and detector temperatures set at 300°C and an oven temperature program of 100°C for 0 min, increased by 15°C min^{-1} to 300°C and held for 20 min.

Chemicals Synthetic compounds used in this study were prepared as previously described (McElfresh and Millar 1999a), with the exception of hexadecyl acetate and hexadecanol, which were purchased from Sigma Chemical Co. (St. Louis, MO, USA) and Avocado Research Chemicals (Hassham, UK), respectively. All compounds used were at least 98% chemically and isomerically pure. GC standards of all four isomers of the 10,12-16 acetates, aldehydes, and alcohols were also obtained from Darwin Reed, Plant Biotechnology Institute, National Research Council of Canada, Saskatoon, Saskatchewan, Canada.

Field Trials Field trials were conducted at Oro Grande Wash, 16 km east of Phelan, CA, USA. A single field trial consisted of three to four replicate blocks, at least 100 m apart, with a maximum of 12 treatments per block. Custom-made cylindrical screen traps were as previously described (McElfresh and Millar 1999d). Compounds were loaded on 11-mm rubber septa (Wheaton Scientific, Millville, NJ, USA) in hexane solutions (doses and ratios are listed in figure captions). For the final trial, the traps were modified by the addition of clips and rubber bands to hold the top, inward-pointing funnel more securely. Securing the top funnel was necessary because, in the trapping area, high winds occasionally overturned traps and, on several occasions, roadrunners, *Geococcyx californianus* (Lesson), dismantled traps to get at the trapped moths. Consequently, it was not possible to leave the traps unattended for more

Table 1 Relative amounts of compounds identified in single female extracts and relative responses elicited from antennae of male moths by these compounds

Compound	Amount in single female extracts (% relative to *E*10,*Z*12-16:Ac)			Male antennal responses[a]	
	Mean±SD	*N*	Range	Mean±SD	*N*
*E*10,*Z*12-16:Ac	100	45	–	100	19
*E*10,*Z*12-16:Ald	0.37±0.03	3	0.34–0.42	17.8±11.9	8
16:OH	60±81	44	10–395	0	0
*E*10,*Z*12-16:OH	14±13	33	1.8–55	21.3±13.3	16
*E/Z*11-16:Ac	9.1±7.3	38	2.5–44	20.2±7.9	18
16:Ac	232±256	45	76–480	17.4±7.5	15
*E*10,*E*12-16:Ac	23±11	43	6–58	38.0±13.1	16

All extracts were analyzed on a DB-5 column (30 m×0.32, 25-μm film).
E10,Z12-16:Ac (10*E*,12*Z*)-hexadecadienyl acetate, *E10,Z12-16:Ald* (10*E*,12*Z*)-hexadecadienal, *16:OH* hexadecanol, *E10,Z12-16:OH* (10*E*,12*Z*)-hexadecadienol, *E/Z11-16:Ac* (*E*)- and (*Z*)-11-hexadecenyl acetate, *16:Ac* hexadecyl acetate, *E10,E12-16:Ac* (10*E*,12*E*)-hexadecadienyl acetate
[a] Responses from antennae of male moths, expressed as a percent of the response to *E*10,*Z*12-16:Ac, calculated from EAD peak height, with antennae stimulated with equal amounts of each compound.

than 2 h. Thus, blocks of traps were set up in the early afternoon (1200–1400 hours) and taken down just before dusk (1630–1700 hours). Catches were counted from one to three times a day (during which the traps were emptied) and the traps rerandomized after each count. Because of fluctuations in the populations of the moths over the relatively short flight season (4 to 5 weeks), and from year to year, field trials were conducted over several years.

Statistical Analyses For statistical analyses of field trials, possible day and time effects were eliminated by pooling counts for a particular treatment within a block. This sum was then transformed ($\sqrt{x}+0.5$) prior to conducting a two-way analysis of variance (ANOVA), followed by Student–Neuman–Keuls tests (SNK), as required for separation of means (SigmaStat 1.0, Jandel Scientific Corporation, 1992–1994). Any treatments that failed to attract males were not included in the statistical analysis to avoid violating assumptions of ANOVA (i.e., zero values have no variance).

For statistical comparisons of antennal responses to synthetic standards, the antennal response peak heights of a given antenna, to each of the five compounds in the blend, were converted to the proportion of the combined responses for all five compounds. This was then √arcsin-transformed prior to a one-way ANOVA, followed by SNK tests as described above ($\alpha=0.5$). If the assumptions for ANOVA were not met, Kruskal–Wallis ANOVA of ranks was used, followed by Dunn's method to separate means ($\alpha=0.5$).

Results

Analysis of Pheromone Gland Extracts The pheromone gland component that elicited the largest and most consistent responses from antennae of male *H. burnsi* was identified as *E*10,*Z*12-16:Ac (Table 1; Fig. 1, peak 6). The mass spectrum had a molecular ion at *m/z* 280 (23% of base peak at *m/z* 67), consistent with a 16-carbon diunsaturated acetate. The relatively strong molecular ion in the mass spectrum and longer retention time than 16:Ac, on the DB-

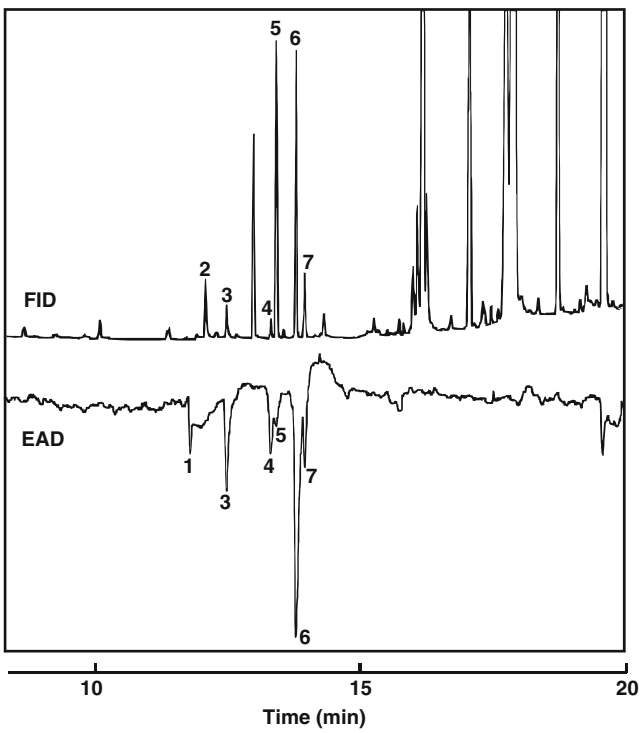

Fig. 1 Representative coupled gas chromatogram–electroantennogram of extract of female *H. burnsi* (Watson) pheromone gland (three female equiv) using a DB-5 column. *Top trace*: flame ionization detector (*FID*) response; *bottom*, *inverted trace* (*EAD*): response elicited from antenna of a male *H. burnsi* moth. Identification of peaks: *1*, (10*E*,12*Z*)-hexadecadienal; *2*, hexadecanol; *3*, (10*E*,12*Z*)-hexadecadienol; *4*, (*E*)- and (*Z*)-11-hexadecenyl acetate; *5*, hexadecyl acetate; *6*, (10*E*,12*Z*)-hexadecadienyl acetate; *7*, (10*E*,12*E*)-hexadecadienyl acetate

5 column, suggested a conjugated diene. Conjugation of the double bonds was confirmed by formation of the MTAD adduct, which showed a molecular ion at *m/z* 393 (5%) and diagnostic ions at *m/z* 208 (100%) and 350 (22%), indicating a 10,12 position for the double bonds. The *E*10,*Z*12 geometry was determined by exact retention time matches to a synthetic standard on both DB-5 and DB-Wax columns, which resolve all four isomers (McElfresh and Millar 1999a). In addition to the *EZ* isomer, a small amount of *E*10,*E*12-16:Ac was identified; based upon its mass spectrum (molecular ion at *m/z* 280, 27% of base peak at *m/z* 67), retention time matches to synthetic standards on both columns, and similar responses are elicited from male antennae in GC–EAD analyses (Table 1; Fig. 1, peak 7).

The identification of *E*10,*Z*12-16:OH was similarly based upon retention time matches on polar and nonpolar columns, matching of the mass spectrum with that of a standard, and similar GC–EAD responses elicited by the insect-produced compound and the standard (Table 1; Fig. 1, peak 3). The mass spectrum showed a comparatively strong molecular ion at *m/z* 238 (19% of base peak at *m/z* 67), suggesting a 16-carbon conjugated dienol. There was insufficient material in the extract to detect the MTAD adducts, but the other analytical data in support of this structure, its close similarity to the corresponding acetate, and its biological activity in field trials (see below) support its identity.

Two saturated 16-carbon compounds, 16:Ac and 16:OH, were also identified from pheromone gland extracts by tentative matches with database spectra. These identifications were confirmed by mass spectral and retention time matches with those of standards (Table 1; Fig. 1, peaks 5 and 2, respectively). Of the two, only 16:Ac elicited responses from antennae of male moths in GC–EAD analyses.

Trace quantities of *E*11- and *Z*11:16:Ac were identified, based upon retention time matches, during GC–EAD analyses (Table 1; Fig. 1, peak 4). These assignments were corroborated by GC–MS analysis (*m/z* 222, 22%, M^+–AcOH; significant ions at *m/z* 82, 96, 110, 124, 138, 152, 166 179/180, 196). The isomers were identified by comparison of retention indices to those of all possible C_{16} monounsaturated acetates, as described in McElfresh and Millar (1999a).

*E*10,*Z*12-16:Ald could not be conclusively identified in the extracts due to the very small amounts present. However, in GC–EAD analyses, responses were elicited from male moth antennae by a trace component eluting at the retention time of *E*10,*Z*12-16:Ald, and antennae of males also responded strongly when stimulated with synthetic *E*10,*Z*12-16:Ald (Table 1; Fig. 1, peak 1). Taken together, these data suggest that traces of this compound were present in the pheromone gland extract.

Because of the large response elicited from male antennae by *E*10,*Z*12-16:Ac from pheromone gland extracts, this component was considered to be the major component in the blend. The average ratio of the various components in pheromone gland extracts from individual females was 100:232:23:9:14:60:0.4 (*E*10,*Z*12-16:Ac: 16:Ac: *E*10,*E*12-16:Ac: *E/Z*12-16:Ac: *E*10,*Z*12-16:OH:16:OH: *E*10,*Z*12-16:Ald; Table 1).

Responses of Antennae of Male Moths to Synthetic Standards The profiles of antennal responses to the five compounds tested were remarkably consistent within populations, but there was considerable variation between species and populations (Fig. 2). In general, *E*10,*Z*12-16:Ac and *E*10,*Z*12-16:OH elicited the largest responses and, together, constituted over 50% of the summed total responses for each population. For *E*10,*Z*12-16:Ac, responses from antennae of *H. electra electra* were intermediate between those of *H. burnsi*, which had the largest proportional responses to this compound, and *H. e. mojavensis*, which had the smallest. For *E*10,*Z*12-16:OH, antennae of *H. burnsi* showed the smallest proportional responses, whereas antennae of *H. e. mojavensis* showed the largest, with *H. electra electra* once again being intermediate. Responses of *H. burnsi* to *E*10,*Z*12-16:Ald

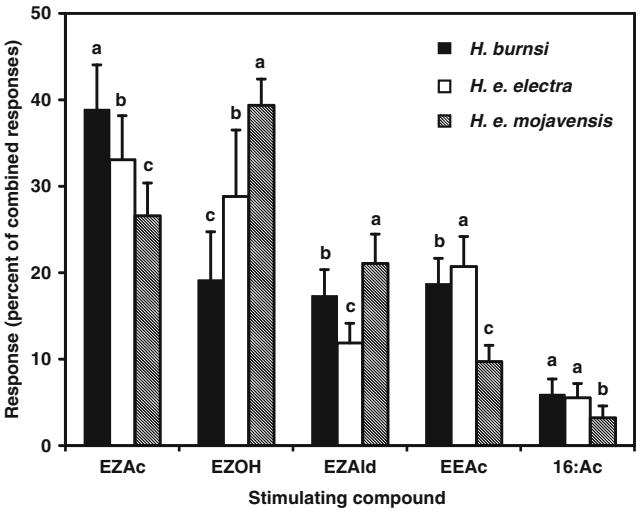

Fig. 2 Mean relative magnitudes (as a proportion of all responses) of antennal responses to synthetic standards [*EZAc*=(10*E*,12*Z*)-hexadecadienyl acetate, *EZOH*=(10*E*,12*Z*)-hexadecadienol, *EZAld*=(10*E*,12*Z*)-hexadecadienal, *EEAc*=(10*E*,12*E*)-hexadecadienyl acetate, *16:Ac*=hexadecyl acetate] of male *H. burnsi* (*N*=30), *H. electra electra* (*N*=23), and *H. electra mojavensis* (*N*=15). For each compound, *bars* surmounted with different *letters* are significantly different (SNK, α=0.05 or Dunn's method, α=0.05). For EZAc, one-way ANOVA, F=33.2; df=2, 67; P<0.001; for EZOH, Kruskal–Wallis ANOVA on ranks, H=41.1; df=2; P<0.001; for EZAld, one-way ANOVA, F=53.0; df=2, 67; P<0.001; for EEAc, one-way ANOVA, F=81.4; df=2, 67; P<0.001; and for 16:Ac, one-way ANOVA, F=17.24; df=2, 67; P<0.001

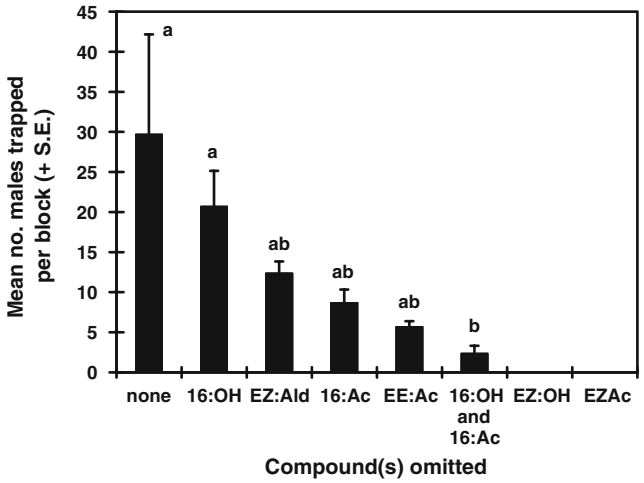

Fig. 3 Mean numbers of male *H. burnsi* caught in traps baited with a blend based upon ratios of compounds found in female *H. burnsi* extracts or deletions from that blend. Compounds and amounts (microgram per septum) used in the base blend were: hexadecanol (*16:OH*), 60; (10*E*,12*Z*)-hexadecadienal (*EZAld*), 1; hexadecyl acetate (*16:Ac*), 350; (10*E*,12*E*)-hexadecadienyl acetate (*EEAc*), 50; (10*E*,12*Z*)-hexadecadienol (*EZOH*), 10; and (10*E*,12*Z*)-hexadecadienyl acetate (*EZAc*), 100. Field trial was conducted on September 25 (three readings for blocks A and B, two for C) and 26 (two readings each block), 1994. Total moths trapped: 238. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=4.85; df=5, 17; P=0.016; for block effect F=2.14; df=2, 17; P=0.17

were intermediate between those of *H. e. mojavensis* (largest) and *H. electra electra* (smallest). Conversely, responses to *E*10,*E*12-16:Ac were largest for *H. electra electra* and smallest for *H. e. mojavensis*, with *H. burnsi* once again in the middle. Finally, antennae of both *H. burnsi* and *H. electra electra* exhibited small and similarly sized responses to 16:Ac, whereas *H. e. mojavensis* responses were smaller still.

Field Trials with Synthetic Compounds A preliminary field trial that used single components, binary blends, and a ternary blend (which attracted male *H. electra electra*) failed to attract *H. burnsi* males (data not shown). Consequently, a more complete reconstruction of the blend of compounds identified from pheromone gland extracts was tested. This six-component blend consisted of *E*10,*Z*12-16:Ac, *E*10,*E*12-16:Ac, *E*10,*Z*12-16:OH, 16:Ac, 16:OH, and *E*10,*Z*12-16:Ald in a ratio of 100:50:10:350:60:1 μg per septum, respectively. The *E*10,*Z*12-16:Ald dose used was an estimate because the actual amount in pheromone gland extracts could not be determined due to the small amounts present. Blends tested included the complete six-component blend, all blends with a deletion of one single component, and a blend in which the two saturated components were not present. There were no significant differences in the attractiveness of the complete blend or the blends lacking 16:OH, *E*10,*Z*12-16:Ald, 16:Ac, or *E*10,*E*12-16:Ac (Fig. 3). Blends lacking either *E*10,*Z*12-16:Ac or *E*10,*Z*12-16:OH caught no moths.

With this trial as a baseline, further studies were carried out to test the effects of different ratios of the various compounds and to verify the role of each compound as part of the attractant blend for male *H. burnsi*. Thus, a field trial testing different amounts of *E*10,*Z*12-16:OH (0, 3, 10, 33, and 100 μg per septum) showed a strong dose effect, with the 10 μg per septum treatment attracting the most males (Fig. 4). An increase to 33 μg or decrease to 3 μg per septum resulted in significantly fewer males being attracted than to the 10-μg treatment. Elimination of *E*10,*Z*12-16:OH from the base blend rendered the blend unattractive (three of 150 males trapped). These results confirmed the importance of both the presence and the ratio of *E*10,*Z*12-16:OH in the blend.

When *E*10,*E*12-16:Ac was varied over the same range of doses (0, 3, 10, 33, and 100 μg per septum), holding the ratio of the other components constant, trap catches increased with increasing amounts of *E*10,*E*12-16:Ac (Fig. 5), with the lowest dose attracting only nine males (out of 222 total), whereas the highest dose attracted 85 males. These results confirmed that *E*10,*E*12-16:Ac was necessary for maximum attraction of male *H. burnsi* and that the ratio again was important.

A broader range of doses was tested for 16:Ac (0, 1, 10, 100, 1,000 μg per lure) because this compound was the most abundant of the six compounds found in analyses of pheromone gland extracts (see above). Increasing amounts of 16:Ac resulted in greater numbers of males trapped

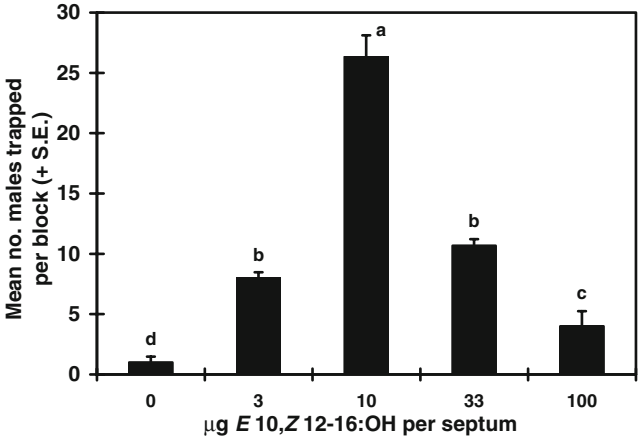

Fig. 4 Mean numbers of male *H. burnsi* caught in traps baited with 60 μg hexadecanol, 1 μg (10*E*,12*Z*)-hexadecadienal, 350 μg hexadecyl acetate, 50 μg (10*E*,12*E*)-hexadecadienyl acetate, 100 μg (10*E*,12*Z*)-hexadecadienyl acetate, and varying amounts of (10*E*,12*Z*)-hexadecadienol (*E*10,*Z*12-16:OH). Field trial conducted September 28 and 30, 1994, with two counts for blocks A and B and a single count for block C on each day. Total males trapped: 150. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=45.46; df=4, 14; P<0.001; for block effect F=2.01; df=2, 14; P=0.20

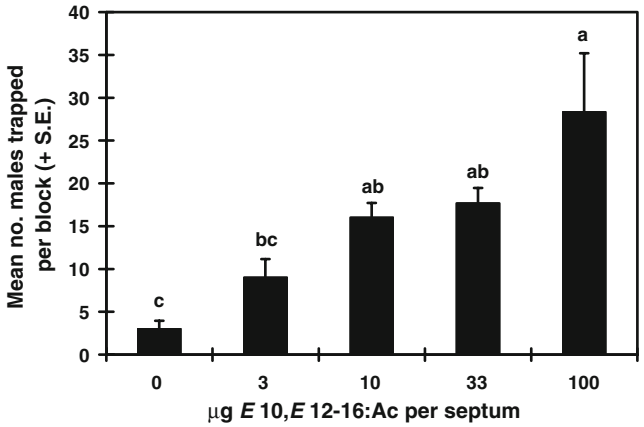

Fig. 5 Mean numbers of male *H. burnsi* caught in traps baited with 60 μg hexadecanol, 1 μg (10*E*,12*Z*)-hexadecadienal, 350 μg hexadecyl acetate, 10 μg (10*E*,12*Z*)-hexadecadienol, 100 μg (10*E*,12*Z*)-hexadecadienyl acetate, and varying amounts of (10*E*,12*E*)-hexadecadienyl acetate (*E*10,*E*12-16:Ac). Field trial conducted September 28 and 30, 1994, with two counts for blocks A and B and a single count for block C on each day. Total number males trapped: 222. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=8.05; df=4, 14; P=0.007; for block effect F=0.73; df=2, 14; P=0.51

(Fig. 6), but even blends that contained little or no 16:Ac still caught moths; traps baited with lures containing no 16:Ac captured approximately one fifth as many moths as those baited with the highest dose. Thus, the data suggested that this compound is an important, but not essential, component for attraction of male *H. burnsi*.

A field trial was conducted to assess further the possible role of *E*10,*Z*12-16:Ald in the pheromone blend, using *E*10,*Z*12-16:Ald doses of 0, 0.001, 0.01, 0.1, 1, 10, and 100 μg per lure. The data indicated that a 1 μg per septum dose of *E*10,*Z*12-16:Ald, in a base blend with the other components, was optimal (Fig. 7). Nevertheless, all of the other blends also attracted some moths, indicating that *E*10,*Z*12-16:Ald is a part of the attractive blend, but its presence is not essential for attraction.

Having investigated the roles of the various minor components, a dose response trial was conducted by varying the dose of the major component, *E*10,*Z*12-16:Ac, from 3.3 to 333 μg per lure, while holding the other components constant at 60 μg 16:OH, 1 μg *E*10,*Z*12-16:Ald, 50 μg *E*10,*E*12-16:Ac, 10 μg *E*10,*Z*12-16:OH, and 350 μg 16:Ac. Traps baited with lures that contained less than 10 μg per septum of *E*10,*Z*12-16:Ac failed to catch any males. However, the trap catch increased sharply as the dose of *E*10,*Z*12-16:Ac increased above 10 μg (Fig. 8). This confirmed the role of *E*10,*Z*12-16:Ac as an essential part of the pheromone blend, with higher doses giving greater trap catches.

Addition of 16:OH over a broad range of doses (0, 1, 10, 100, and 1,000 μg per septum) had no effect on the attractiveness of the basic blend (100:50:10:350:1 μg per septum, *E*10,*Z*12-16:Ac: *E*10,*E*12-16:Ac: *E*10,*Z*12-16:OH: 16:Ac: *E*10,*Z*12-16:Ald) in a field trial conducted between September 22 and 29, 1999 (189 males trapped; data not shown; two-way ANOVA for treatment effect, F=0.022;

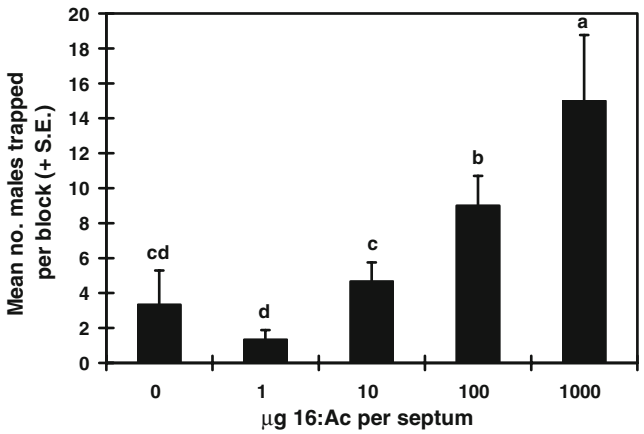

Fig. 6 Mean numbers of male *H. burnsi* caught in traps baited with 60 μg hexadecanol, 1 μg (10*E*,12*Z*)-hexadecadienal, 50 μg (10*E*,12*E*)-hexadecadienyl acetate, 10 μg (10*E*,12*Z*)-hexadecadienol, 100 μg (10*E*,12*Z*)-hexadecadienyl acetate, and varying amounts of hexadecyl acetate (16:Ac). Field trial conducted October 1, 2, and 8, 1994, with a total of four counts for each block over the 3-day period. Total number of males trapped: 100. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=22.00; df=4, 14; P<0.001; for block effect F=18.8; df=2, 14; P=0.001

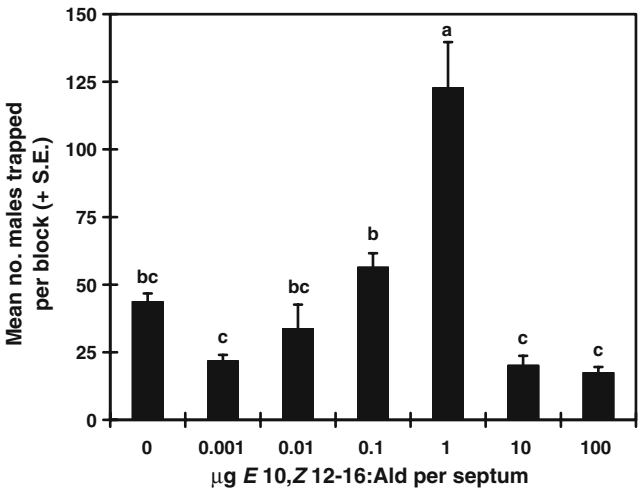

Fig. 7 Mean numbers of male *H. burnsi* caught in traps baited with 1,000 μg hexadecyl acetate, 60 μg hexadecanol, 100 μg (10*E*,12*E*)-hexadecadienyl acetate, 10 μg (10*E*,12*Z*)-hexadecadienol, and 100 μg (10*E*,12*Z*)-hexadecadienyl acetate per septum, and varying amounts of (10*E*,12*Z*)-hexadecadienal (*E*10,*Z*12-16:Ald). Field trials conducted October 7–8 and 14–15, 1995 for a total of four counts. Total males trapped: 946. Two-way ANOVA for treatment effect, F=19.09; df=6, 20; P<0.001; for block effect F=2.49; df=2, 20; P=0.125

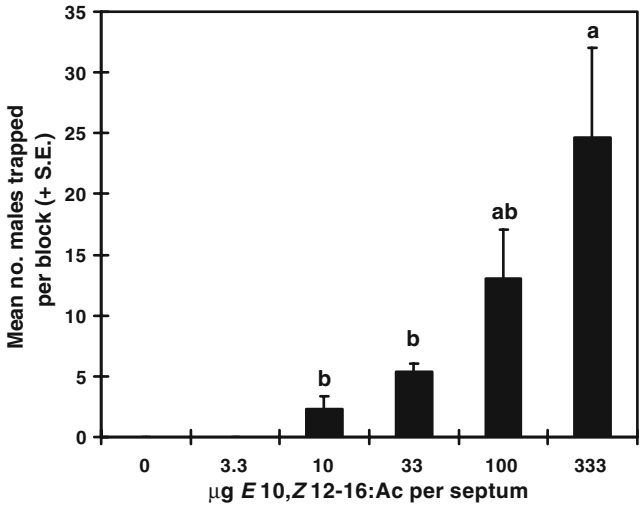

Fig. 8 Mean numbers of male *H. burnsi* caught in traps baited with 60 μg hexadecanol, 1 μg (10*E*,12*Z*)-hexadecadienal, 50 μg (10*E*,12*E*)-hexadecadienyl acetate, 10 μg (10*E*,12*Z*)-hexadecadienol, 350 μg hexadecyl acetate, and varying amounts of (10*E*,12*Z*)-hexadecadienyl acetate (*E*10,*Z*12-16:Ac). Field trial conducted September 22 and 28, 1999, with one count per block per day. Total males trapped: 136. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=6.83; df=3, 11; P=0.023; for block effect F=2.16; df=2, 11; P=0.20

df=4, 14; P=0.99; for block effect F=1.71; df=2, 14; P=0.24). Addition of *Z*- and *E*12-16:Ac at doses determined from a composite extract from nine pheromone glands (1.5 and 1.25 μg per septum, respectively) to a base blend (consisting of 100:100:10:1,000:60:1 μg per septum, *E*10,*Z*12-16:Ac: *E*10,*E*12-16:Ac: *E*10,*Z*12-16:OH: 16:Ac: 16:OH: *E*10,*Z*12-16:Ald) also had no affect on catches compared to the base blend (two-way ANOVA for treatment effect, F=0.65; df=3, 11; P=0.62; for block effect F=1.36; df=2, 11; P=0.33; 235 males trapped, data not shown).

A final field trial (Fig. 9) was conducted to compare the attractiveness of the basic five-component blend (100:50:10:350:1 μg per septum, *E*10,*Z*12-16:Ac: *E*10,*E*12-16:Ac: *E*10,*Z*12-16:OH: 16:Ac: *E*10,*Z*12-16:Ald, respectively) to single and multiple deletions from that blend and to a five-component blend with the optimal doses of *E*10,*E*12-16:Ac and 16:Ac (100 and 1,000 μg per septum, respectively), as determined from the field trials described above. The results confirmed that the blend of the two essential components alone (*E*10,*Z*12-16:Ac and *E*10,*Z*12-16:OH) was not attractive. Addition of any one, or any two, of the other three components to this two-component blend resulted in weak to moderate attraction. All five components were required for maximum trap catches. Somewhat surprisingly, the blend containing "optimized" ratios of *E*10,*E*12-16:Ac and 16:Ac caught similar numbers of moths as the nonoptimized blend.

Discussion

The five-component sex pheromone blend we have identified for *H. burnsi* is the most complex blend yet described for a hemileucine moth. None of the components are unique to *H. burnsi*; all have been identified from at least one other species of *Hemileuca* or its sister genus, *Coloradia*. Two components were found to be essential (*E*10,*Z*12-16:Ac and *E*10,*Z*12-16:OH), in that traps baited with blends missing either of these components caught no moths. *E*10,*Z*12-16:Ac has now been found to be a crucial pheromone component for all *Hemileuca* and *Coloradia* species examined to date, including *Hemileuca eglanterina*, *Hemileuca nuttalli*, and *H. electra*, *Coloradia velda* (McElfresh and Millar 1999a, b, c, d) and *Coloradia pandora* (McElfresh et al. 2000). However, although isomers of 10,12-16:Ac constitute the major pheromone component for at least one population of most of the *Hemileuca* and *Coloradia* species examined to date, this may be an artifact of sampling because we have been specifically studying species that may interact (Tuskes et al. 1996). *E*10,*Z*12-16:Ald is the major pheromone constituent of two of these species (McElfresh et al. 2000, 2001a, b) and has been found in others as a minor component (JSM, unpublished data).

*E*10,*Z*12-16:OH also has been found in all of the *Hemileuca* and *Coloradia* species examined to date and is

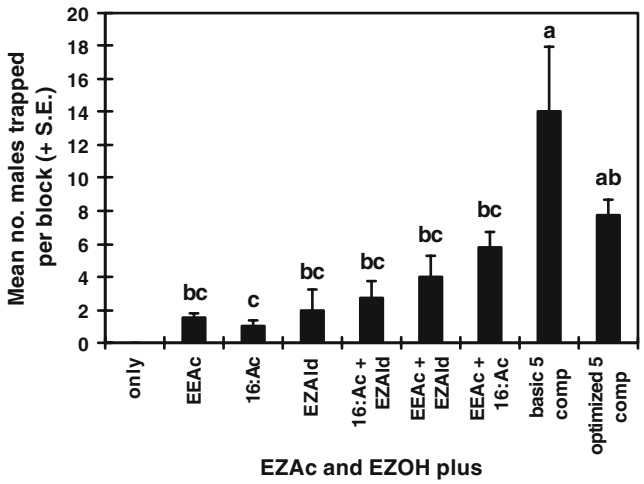

Fig. 9 Mean numbers of male *H. burnsi* caught in traps baited with 100 μg (10*E*,12*Z*)-hexadecadienyl acetate (EZAc) and 10 μg (10*E*,12*Z*)-hexadecadienol (EZOH) alone ("only") and in various combinations with 350 μg hexadecyl acetate (16:Ac), 50 μg (10*E*,12*E*)-hexadecadienyl acetate (EEAc), and 1 μg (10*E*,12*Z*)-hexadecadienal (EZAld) per septum. The "basic five-comp" blend included all five components in the amounts above. The "optimized five-comp" blend differed in that it contained 1,000 and 100 μg per septum of 16:Ac and EEAc, respectively. Field trial conducted October 2 and 7, 1999. Total males trapped: 155. Treatments with different *letters* are significantly different (SNK, α=0.05). Two-way ANOVA for treatment effect, F=6.81; df=7, 31; P<0.001; for block effect F=1.39; df=3, 31; P=0.27

an essential component of the sex pheromone blends of *H. electra*, *H. eglanterina*, and *Hemileuca maia*. It is less important for *C. velda* and not required at all by *H. nuttalli* or *C. pandora*. Thus, despite being present in pheromone gland extracts, it does not appear to be part of the attractive blend for some species and may even be antagonistic to other species at levels found in glands in *H. nuttalli* (McElfresh and Millar 1999b). In some cases, it may be present primarily as a biosynthetic intermediate–by-product, rather than a required pheromone component. Only one population of *H. eglanterina* has been shown to use *E*10, *Z*12-16:OH as a major pheromone component (McElfresh and Millar 2001).

Although *E*10,*E*12-16:Ac is frequently present in extracts of pheromone glands of hemileucine moth species, to date, only *H. nuttalli* appears to require this isomer as a component of its blend (McElfresh and Millar 1999b). It also appears to be a minor, but not essential, component for both species of *Coloradia* studied (McElfresh and Millar 1999c; McElfresh et al. 2000). Similarly, 16:Ac is a pheromone component in the blends of only two species studied, *H. burnsi* and the allopatric *H. electra electra* (McElfresh and Millar 1999d). *H. electra mojavensis*, which is sympatric with *H. burnsi*, does not use 16:Ac, either as an attractant or as an antagonist. For both species, inclusion of 16:Ac in synthetic pheromone blends is not essential. Nevertheless, addition of this compound, even at very high doses, consistently enhanced attraction (McElfresh and Millar 1999d).

It was not possible to prove conclusively the presence of *E*10,*Z*12-16:Ald in pheromone gland extracts of *H. burnsi* due to the trace amounts of the putative compound in extracts. Nevertheless, in toto, the available data make a strong case for its presence. In GC–EAD analyses of extracts, antennae of males showed strong responses to a trace component at the retention time of this compound and, when 1 μg of this compound was added to a six-component blend, trap catches increased significantly. It is interesting to note the degree of similarity between *H. burnsi* and *H. electra electra* in terms of their preferred doses–ratios of this aldehyde as compared to that of *H. electra mojavensis*. Specifically, *H. electra electra* and *H. burnsi* were more attracted to blends with low does of this compound (around 1% or less), whereas male *H. electra mojavensis* were more attracted to much higher levels (McElfresh and Millar 1999d). This alone could provide a strong reproductive isolation barrier between sympatric *H. burnsi* and *H. e. mojavensis*.

The response profiles of antennae of male moths to synthetic standards of the sex pheromone components showed that the antennae of the various species were differentially tuned to the various compounds. The relative antennal responses were consistent when expressed as a proportion of the combined responses to all five compounds. Antennae of male *H. burnsi* and *H. electra electra* showed large responses to *E*10,*Z*12-16:Ac, the major component in their respective blends. In contrast, antennae of male *H. electra mojavensis* responded less to *E*10,*Z*12-16:Ac and more to *E*10,*Z*12-16:Ald, mirroring the relative importance of these components in field bioassays (McElfresh and Millar 1999d). Antennae of both *H. burnsi* and *H. electra electra* responded similarly to 16:Ac, whereas antennae of *H. electra mojavensis*, which does not use this compound, showed relatively weak responses to this compound. Although the results from the GC–EAD comparisons appear clear-cut, one qualification is that the moths used in this study were collected in the field by using blends of synthetic pheromone components optimized for each population. This may have created a bias in the males collected and hence their EAD responses. This collection method was necessary because of the problems and time constraints (on average a 2-year life cycle) involved in rearing these insects. Nevertheless, such comparisons of antennal response profiles to a suite of possible pheromone components could provide a useful method of assessing differences in pheromone blends of populations.

Populations of *H. burnsi* and *H. electra mojavensis* co-occur throughout much of the Mojave Desert. Their distributions reflect the results of natural forces acting over millennia and have been minimally affected by human activities. We had previously hypothesized that the observed differences in the pheromone systems of *H. electra electra* and *H. electra mojavensis* were the result of the latter subspecies being sympatric with *H. burnsi* in the Mojave Desert, whereas the former subspecies had no apparent competition for its pheromone channel (McElfresh and Millar 1999d). The data presented here have allowed a more rigorous test of this hypothesis. The potential for interference was demonstrated by artificially placing females of the allopatric subspecies *H. electra electra* to attract wild males of both *H. burnsi* and *H. electra mojavensis* (McElfresh and Millar 1999d). This cross-attraction suggested that the pheromone blends of the latter two species–subspecies must be similar to that of *H. electra electra*, and this has now been verified. Specifically, both *H. burnsi* and *H. electra electra* use *E*10,*Z*12-16:Ac as their major component; both require *E*10,*Z*12-16:OH; both utilize 16:Ac as a minor component, and both prefer low doses of *E*10,*Z*12-16:Ald. In contrast, *H. electra mojavensis* is more divergent, using both *E*10,*Z*12-16:Ald and *E*10, *Z*12-16:Ac as major components and a specific ratio of *E*10,*Z*12-16:OH, with apparently no additional compounds. The new data reported here support our original hypothesis that the divergence between the two *H. electra* subspecies is a result of competition for, and interference with, a shared pheromone channel. This suggests that reproductive

character displacement has occurred with one subspecies, thus maximizing prezygotic mating barriers, thereby minimizing costly and unproductive interspecific mating.

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References

McElfresh, J. S., and Millar, J. G. 1999a. Sex pheromone of the common sheep moth, *Hemileuca eglanterina*, from the San Gabriel Mountains of California. *J. Chem. Ecol.* 25:687–709.

McElfresh, J. S., and Millar, J. G. 1999b. Sex pheromone of Nuttall's sheep moth, *Hemileuca nuttalli*, from the eastern Sierra Nevada mountains of California. *J. Chem. Ecol.* 25:711–726.

McElfresh, J. S., and Millar, J. G. 1999c. Sex pheromone of the saturniid moth, *Coloradia velda*. *J. Chem. Ecol.* 25:1067–1078.

McElfresh, J. S., and Millar, J. G. 1999d. Geographic variation in the sex pheromone blend of *Hemileuca electra* from southern California. *J. Chem. Ecol.* 25:2505–2525.

McElfresh, J. S., and Millar, J. G. 2001. Geographic variation in the pheromone system of the Saturniid moth *Hemileuca eglanterina*. *Ecology.* 82:3505–3518.

McElfresh, J. S., Chen, X., Ross, D. W., and Millar, J. G. 2000. Sex pheromone blend of the Pandora moth (Lepidoptera: Saturniidae), an outbreak pest in pine forests (Pinaceae). *Can. Entomol.* 132:775–787.

McElfresh, J. S., Millar, J. G., and Rubinoff, D. 2001a. (*E*4,*Z*9)-tetradecadienal, a sex pheromone for three North American moth species in the genus *Saturnia*. *J. Chem. Ecol.* 27:791–806.

McElfresh, J. S., Hammond, A. M., and Millar, J. G. 2001b. Sex pheromone components of the buck moth *Hemileuca maia*. *J. Chem. Ecol.* 27:1409–1422.

Tuskes, P. M. 1984. The biology and distribution of California Hemileucinae (Saturniidae). *J. Lepid. Soc.* 38:281–309.

Tuskes, P. M., Tuttle, J. P., and Collins, M. M. 1996. The Wild Silk Moths of North America: A Natural History of the Saturniidae of the United States and Canada. Cornell University Press, Ithaca.

Young, D. C., Vouros, P., and Holich, P. F. 1990. Gas chromatography-mass spectrometry of conjugated dienes by derivatization with 4-methyl-1,2,4-triazoline-3,5-dione. *J. Chromatog.* 522:295–302.

Keywords Australian gum leaf skeletonizer · *Uraba lugens* · *Nola* spp. · Nolinae · Nolidae · *Eucalyptus* defoliator · (10*E*,12*Z*)-hexadecadien-1-yl acetate · (10*E*,12*Z*)-hexadecadien-1-ol · GC-EAD · GC-MS · DMDS and MTAD derivatizations · Pheromone titer · Female calling behavior · Trapping bioassays

Introduction

The Australian gum leaf skeletonizer, *Uraba lugens* (Walker 1863) (Lepidoptera, Nolinae, Nolidae), arrived recently in New Zealand. In New Zealand, this insect has few natural enemies present (Mansfield et al. 2005) and is a significant threat to New Zealand eucalyptus forestry and amenity plantings (Kriticos et al. 2007). The larvae of *U. lugens* feed gregariously on *Eucalyptus* and allied Myrtaceae, skeletonizing the leaves and, at times, causing considerable damage (Common 1990; Allen and Keller 1991; Potter and Stephens 2005. Outbreaks of *U. lugens* in urban trees and school playground shade trees in Auckland have led to medical complaints about the urticating hairs of the larvae (Derraik 2006). A classical biological control program for this species has commenced, with testing of host range of parasitoids with respect to native New Zealand species (Bernt 2006; Berry and Mansfield 2006). In Australia, *U. lugens* occurs from Cooktown to the Atherton Tableland and from southern Queensland to Victoria, South and south-western Australia, and Tasmania (Common 1990). Outbreaks of *U. lugens* have been reported from Queensland (Brimblecombe 1962), New South Wales (Campbell 1962), Victoria (Harris et al. 1977), and Western Australia (Strelein 1988; Farr 2002; Farr et al. 2004). Between 1997 and 2001, infestations of *U. lugens* were discovered in New Zealand on three separate occasions at localities in the northern part of the North Island (Hoare 2001).

The most recent biosecurity incursions, centered in the south-west suburbs of Auckland city, have been ongoing since late 2001 (Ross 2001, 2003). While the first two incursions were localized and apparently eradicated at the time, the New Zealand Ministry of Agriculture and Forestry (MAF) considered that *U. lugens* had the potential, if it established, to spread widely and cause major damage to New Zealand's *Eucalyptus* timber plantations. MAF's response was to commission a team to identify the sex pheromone. The development of a synthetic sex pheromone attractant for male *U. lugens* moths was considered to be a high priority to detect any further incursions and to undertake delimitation surveys of infestations (Suckling et al. 2005).

While several authors have published on the biology of *U. lugens* (Campbell 1962; Morgan and Cobbinah 1977; Farr 2002), there has been no research on female calling behavior, pheromone identity, pheromone production, or longevity of the adults. Campbell (1962) noted that "Both emergence and copulation take place during the early hours of darkness."

In Australia, *U. lugens* exists in univoltine and bivoltine forms, and these are said to differ in the arrangement of their deposited eggs. Morgan and Cobbinah (1977) suggested that any biomorph differences were temperature induced, rather than representing separate taxa. Farr (2002) observed the ability of "the univoltine form to change to a bivoltine cycle following two consecutive warm winters" in the southern jarrah forest of Western Australia, but this was apparently not correlated with a change in egg deposition pattern. The population in Auckland is bivoltine (Suckling et al. 2005).

Our objectives in this study were to: (1) characterize the sex pheromone of *U. lugens* so as to provide a monitoring tool for cost-effective detection of populations; (2) measure any quantitative and/or qualitative differences in sex pheromone between a univoltine population of *U. lugens* from Hobart, Tasmania, Australia, and a bivoltine population from Auckland, New Zealand; and (3) investigate female calling behavior and temporal pheromone production in the bivoltine population.

Methods and Materials

Insects Egg batches of *U. lugens*, adhering to *Eucalyptus* leaves, were collected from *E. nicholi* trees in Walter Massey Park, Mangere East, Auckland, New Zealand, and air-freighted in doubly contained, sealed packaging to HortResearch, Lincoln for emergence in containment facilities [approximately 22°C and approximately 60% relative humidity (RH)]. Larvae were reared in 680-ml plastic containers (Huhtamaki Henderson Ltd., Auckland, New Zealand) and supplied with fresh leaves of *Eucalyptus viminalis*, as required. Groups of about 10 fifth instars were transferred to additional plastic containers to avoid cannibalism. Pupae were placed individually in 50-cm plastic Petri dishes for emergence. In addition, two shipments of female pupae from a Tasmanian population of *U. lugens* were imported and held in quarantine facilities at Landcare Research, Lincoln. The Tasmanian larvae originated from *E. viminalis* trees at Government Hills, Risdon, Hobart and were reared on *E. viminalis*, initially, and then *E. nitens*, in a similar fashion to the Auckland population.

Females from the Auckland and Tasmanian populations had their pheromone glands excised and extracted for qualitative and quantitative analyses by gas chromatography (GC) and gas chromatography–mass spectrometry

(GC-MS). Males from the Auckland population were used for coupled gas chromatography electroantennographic detection (GC-EAD) studies.

Chemicals Authentic samples of (10*Z*,12*E*)-hexadecadienal (*Z*10,*E*12–16:Ald) (91% isomerically pure), (10*E*,12*Z*)-hexadecadien-1-ol (*E*10,*Z*12–16:OH) (86% isomerically pure; 1%, 3%, and 9% of the *Z*,*E*-, *Z*,*Z*-, and *E*,*E*- isomers, respectively), and (10*E*,12*E*)-hexadecadien-1-ol (*E*10,*E*12–16:OH) (>98% isomerically pure) were obtained from D. R. Hall, Natural Resources Institute, University of Greenwich, Chatham Maritime, Kent, UK. Larger amounts of *E*10,*Z*12–16:OH (>95% isomerically pure), (*Z*)-11-hexadecen-1-yl acetate (*Z*11–16:Ac), and (*Z*)-9-tetradecen-1-yl acetate (*Z*9–14:Ac) (both >98% isomerically pure), for trapping trials, as well as (*E*)-11-hexadecen-1-yl acetate (*E*11–16:Ac) and (*Z*)-10-pentadecen-1-yl acetate (*Z*10–15:Ac) (>98% isomerically pure), were purchased from Pherobank, Wageningen, The Netherlands. (10*Z,*12*E*)-Hexadecadien-1-ol (*Z*10,*E*12–16:OH), (10*Z,*12*E*)-hexadecadien-1-yl acetate (*Z*10,*E*12–16:Ac), (10*E,*12*Z*)-hexadecadien-1-yl acetate (*E*10,*Z*12–16:Ac), and (10*E,*12*E*)-hexadecadien-1-yl acetate (*E*10,*E*12–16:Ac) were all synthesized at HortResearch, Palmerston North. Dimethyldisulfide (DMDS) was obtained from Merck, Darmstadt, Germany and octadecan-1-ol and 4-methyl-1,2,4-triazoline-3,5-dione (MTAD) from Sigma-Aldrich, Sydney, Australia.

Syntheses of (10E,12Z)-Hexadecadien-1-yl Acetate and (10E,12E)-Hexadecadien-1-yl Acetate Vinyl acetate (5.35 g, 62.2 mmol, Sigma-Aldrich, Sydney, Australia) and 1.0 g of lipase PS-C "Amano" II (Amano Enzyme Inc., Nagoya, Japan) were added to a solution of an authentic sample of either *E*10,*Z*12–16:OH or *E*10,*E*12–16:OH (0.92 g, 3.86 mmol) contained in dry, freshly distilled pentane (100 ml, from P_2O_5) under dry nitrogen. The slurry was shaken for 18 hr at room temperature, and the disappearance of either *E*10,*Z*12–16:OH or *E*10,*E*12–16:OH was monitored by thin layer chromatography on silica gel plates [Merck 1.05554; eluted with hexane/ethyl acetate 10:1; visualized under UV (234 nm) and developed with acidified ethanolic vanillin and heat]. The reaction mixture was filtered through a short pad of celite (10 mm) and rinsed with additional freshly distilled pentane (2×30 ml). Finally, the resulting colorless solution was concentrated under reduced pressure to give either *E*10,*Z*12–16:Ac or *E*10,*E*12–16:Ac as a pale, yellow oil. The identity and purity of the compounds was confirmed by GC-MS.

Syntheses of (10Z,12E)-Hexadecadien-1-ol and (10Z,12E)-Hexadecadien-1-yl Acetate The authentic sample of *Z*10,*E*12–16:Ald was converted to *Z*10,*E*12–16:OH by reduction with $NaBH_4$ (2 mg, 0.053 mmol) in ethanol (1 ml). Approximately 0.75 mg of the *Z*10,*E*12–16:OH were then converted to *Z*10,*E*12–16:Ac using the lipase PS-C "Amano" II method, as described previously for *E*10,*Z*12–16:Ac and *E*10,*E*12–16:Ac. The identity and purity of the compounds was confirmed by GC-MS.

Pheromone Gland Extracts All pheromone gland extracts were harvested from calling females held under a 12L/12D reverse-phase photoperiod in a room maintained at 22°C and 60% RH. Female moths were kept in 40-ml humidified polycarbonate plastic boxes and provided with 10% sugar solution to feed on *ad libitum*. Excised pheromone glands were placed into liquid nitrogen-cooled 0.5-ml V-vials (Wheaton, Millville, NJ, USA), containing 20–30 μl of *n*-hexane. After glands had been excised, the vial and its contents were brought to room temperature and the glands extracted for 5 min. The extract was transferred to a 1.1-ml conical glass vial and concentrated under a stream of argon to ca 10 μl, and stored at −20°C until analysis. To quantify pheromone titer per female, pheromone glands were extracted in 50-μl of *n*-hexane containing 50 ng of (*Z*)-10-pentadecen-1-yl acetate as internal standard.

To determine double-bond positions of the EAD-active compounds, two pheromone gland extracts were derivatized and analyzed by GC-MS. For the DMDS derivatizations of a 13-female gland extract and *Z*11–16:Ac and *E*11–16:Ac standards, we followed the procedure described by Buser et al. (1983). For details, see Gibb et al. (2006). For the MTAD derivatizations of a six-female gland extract and *E*10,*Z*12–16:Ac and *E*10,*Z*12–16:OH standards, we followed the procedure of McElfresh and Millar (1999).

Female Calling Behavior Experiments and Pheromone Titer Analysis over Time In an experiment to determine the most appropriate time for extracting pheromone glands, the calling behavior of female *U. lugens* (five replicate groups of 10 females, all from the Auckland Population) was observed and recorded every hour on the hour over a 12-h period, from the beginning to the end of each of the first four scotophases following female emergence. Female mortality at the end of each activity period was also noted. In a subsequent, related experiment, we extracted the pheromone glands of groups of 10 freshly emerged females for quantitative analysis at the beginning of their first scotophase (T0) and thereafter at 2, 4, 7, 24, and 48 hr (T2, T4, T7, T24, and T48). There were three replicate groups for each sample of 10 females.

Comparison of Auckland, New Zealand, and Tasmania U. lugens Gland Extracts The pheromone titers of the Auckland bivoltine and Tasmanian univoltine populations were compared by excising and extracting glands of 14 actively calling female moths (N=3) from each population

during the sixth hour of the first scotophase following emergence.

Gas Chromatography Electroantennographic Detection Pheromone gland extracts of *U. lugens* females were analyzed by GC-EAD using a Varian 3800 gas chromatograph, coupled to an EAD Recording Unit (Syntech Research and Equipment, Hilversum, The Netherlands). Extracts were run on ZB-5 (30 m×0.25 mm i.d.×0.5 μm film; Phenomenex, Torrance, CA, USA) and ZB-Wax (30 m×0.25 mm i.d.× 0.5 μm film; Phenomenex) columns, with a 1:1 split between the flame ionization detector and the EAD. Nitrogen was the carrier gas (1 ml min^{-1}), and the injections were splitless. Injector and detector temperatures were set at 250°C and 300°C, respectively, and the GC oven program was 80°C for 1 min, increasing at 20°C min^{-1} to 230°C, and held for 30 min. For details of the preparation of moth antennae and our setup of associated equipment for GC-EAD analyses, see Gibb et al. (2006). Kováts retention indices (KIs; Kováts 1965; Marques et al. 2000) were calculated for the EAD-active compounds.

Quantitative Analysis of Pheromone Glands For quantitative analysis of pheromone gland extracts, we used a Varian 3800 gas chromatograph fitted with a DB-5 column (30 m× 0.25 mm i.d.×0.5 μm film; Agilent Technologies, Palo Alto, CA, USA). Helium was the carrier gas (1 ml min^{-1}), and injections were splitless. Injector and detector temperatures were set at 220°C and 300°C, respectively, and the GC oven program was 100°C for 1 min, 10°C min^{-1} to 210°C, held for 16 min, then increased at 20°C min^{-1} to 240°C, and held for 20 min.

Gas Chromatography–Mass Spectrometry Gland extracts of insects from the Auckland and Tasmanian populations were analyzed by GC-MS using a Varian 3800 GC coupled to a Varian 2200 MS. Helium was used as carrier gas (1 ml min^{-1}) and injections were splitless. Injector, transfer line, and ion trap temperatures were 220°C, 250°C, and 200°C, respectively. The machine was fitted with a VF-5ms column (30 m×0.25 mm i.d.×0.25 μm film; Varian Inc., Walnut Creek, CA, USA), and the following temperature program was used: 100°C for 1 min, 10°C min^{-1} to 210°C, and held for 30 min. The DMDS- and MTAD-derivatized pheromone gland extracts, and standards were also analyzed using the VF-5ms column (injector temperature, 250°C), using a temperature program of: 80°C to 240°C at 10°C min^{-1} (DMDS) or 100°C to 300°C at 15°C min^{-1} (MTAD).

Trapping Bioassays Field trials were conducted in Queensland, Australia in 2002 (trial A) and in Auckland from 2003 to 2007 (trials B-D). Green delta traps (Clare et al. 2000) were attached to individual eucalypt trees at a height of approximately 2.5 m, with at least 5-m spacing between traps and at least 20-m spacing between replicates. Trap positions within each replicate were re-randomized between readings, and sticky inserts were replaced at each check for capture of male *U. lugens*. Test compounds, based on ratios from GC analyses of *U. lugens* gland extracts, were loaded onto red rubber septa (Thomas Scientific Inc., Philadelphia, PA, USA). All bioassays included a blank control.

In trial A (April to June 2002), the attraction of *U. lugens* males to various combinations of the three candidate EAD-active compounds was tested at a rural site near Goomeri (26°11′ S; 152°07′ E), 3 hr north of Brisbane (one replicate), where there had been previous outbreaks of *U. lugens*, and at Brisbane Forest Park (three replicates). An additional compound, Z9–14:Ac, was also included in trial A because a review of the literature (Arn et al. 1992) had revealed that Z9–14:Ac occurred frequently as a sex pheromone component in the superfamily Noctuoidea. Traps were checked three times at intervals of 4–8 d. Treatments comprised the following ratios (in microgram loadings) of *E*10,*Z*12–16:OH, *E*10,*Z*12–16:Ac, *Z*11–16:Ac, and *Z*9–14:Ac: (1) 26:70:3:3, (2) 0:100:0:0, (3) 100:0:0:0, (4) 25:75:0:0, (5) 24:72:3:0, (6) 24:72:0:3, (7) 0:95:0:5, (8) 0:95:5:0, (9) 0:92:4:4, (10) 88:0:12:0, (11) 88:0:0:12, and (12) 80:0:10:10.

Trial B (March 18 to May 10, 2003) examined the attraction of *U. lugens* males to various combinations of the two major candidate EAD-active compounds with and without *Z*11–16:Ac, a minor gland component. Replicates of the trial were placed in 10 locations in the greater Auckland area that were considered likely to be sites of infestation by *U. lugens*. Traps were checked weekly. Treatments were (1) 0:300:0, (2) 30:270:0, (3) 75:225:0, (4) 150:150:0, (5) 225:75:0, (6) 270:30:0, (7) 300:0:0, (8) 30:270:9, (9) 75:225:9, (10) 150:150:9, (11) 225:75:9, and (12) 270:30:9 μg loadings of *E*10,*Z*12–16:OH, *E*10,*Z*12–16:Ac, and *Z*11–16:Ac (n=3).

Trial C (March 14 to May 9 2004) was a dose–response trial based on results derived from earlier trials and a test of two minor components that had been identified in the gland extract. Treatments were (1) 40:60:0:0, (2) 400:600:0:0, (3) 1200:1800:0:0, (4) 250:750:30:0, (5) 225:75:0, and (6) 400:600:30:50 μg loadings of *E*10,*Z*12–16:OH, *E*10,*Z*12–16:Ac, *Z*11–16:Ac and octadecan-1-ol (18:OH). The last component had been found in the gland extract but due, to its relatively lower volatility, was considered more likely to be a pheromone precursor than an actual component but was included in the trial to test attraction. Ten replicates were used and traps were checked weekly.

Trial D (6 Nov to 13 Dec 2007) investigated the effect of the second minor component, *E*10,*E*12–16:Ac, on catch. Treatments were (1) 250:750:0:0, (2) 250:750:50:30, (3)

250:750:0:30 μg loadings of *E*10,*Z*12–16:OH, *E*10,*Z*12–16:Ac, and *Z*11–16:Ac. Ten replicates were used and traps were checked weekly.

Statistical Analyses Only treatments that trapped moths were included in the statistical analyses. For each trial, the sum of moths captured per trap was analyzed for treatment effects using one-way analysis of variance (ANOVA; SAS Institute Inc. 1998). Residual plots were used to check the validity of the ANOVA assumptions, with $\log_{10}(x+1)$ transformations of the summed captures, when necessary, to remove variance heterogeneity. Means were compared by Fisher's protected least significant differences test (P=0.05; SAS Institute Inc. 1998).

Results and Discussion

Identification of Pheromone Components Analyses of *U. lugens* gland extracts by GC-EAD revealed the presence of two major EAD-active compounds (compounds **1** and **2**) and one minor EAD-active compound (compound **3**) that consistently elicited responses from male antennae (Fig. 1). Two other compounds (compounds **4** and **5**) were also present in gland extracts, but EAD responses to these compounds were small and inconsistent.

The retention times of compound **1** on ZB-5 and ZB-wax columns were consistent with that of a conjugated C_{16} dienol-1-ol and were the same as those of the *E*10*Z*12–16:OH standard. The analysis of the MTAD-derivatized pheromone gland extract by GC-MS revealed an adduct with a molecular ion at *m/z* (relative intensity) 351 (3) and diagnostic ions at *m/z* 308 (18) ($[C_{16}H_{26}N_3O_3]^+$), and 208 (46), ($[C_{10}H_{14}N_3O_2]^+$), indicative of a C_{16} dien-1-ol with conjugation at the 10,12 position. Moreover, comparison of the mass spectrum [*m/z* (rel. int.) 238 (1, M^+), 220 (5), 149 (3), 135 (5), 121 (16), 109 (15), 95 (38), 81 (68), 67 (100), 55 (24), 41 (32)] and KI of compound **1** with those of all four possible 10,12-hexadecadien-1-ol stereoisomers confirmed compound **1** unambiguously as *E*10*Z*12–16:OH (KI of 1,924 on the VF-5ms column; KIs of the *Z*,*E*, *Z*,*Z*, and *E*, *E* isomers were 1,917, 1,936, and 1,940, respectively).

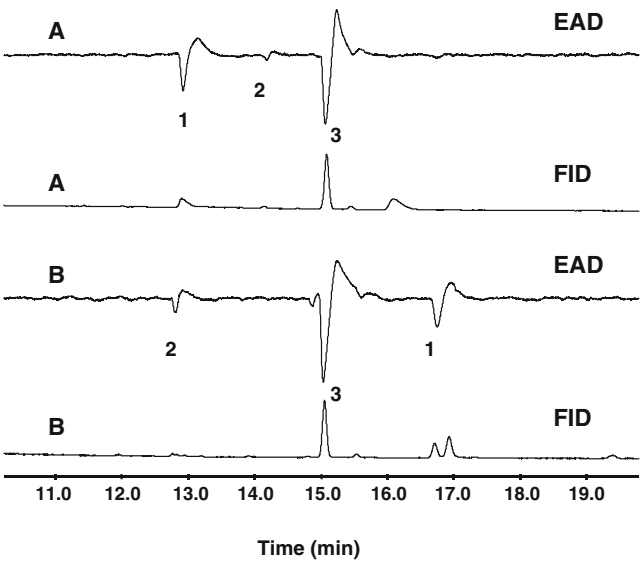

Fig. 1 Coupled gas chromatography electroantennogram analysis of pheromone gland extract of female *Uraba lugens*, using a conspecific male antenna, on ZB-5 (**a**) and ZB-Wax (**b**) capillary columns. *1* (*E*10, *Z*12)-hexadecadien-1-ol; *2* (*Z*)-11-hexadecenyl acetate; and *3* (*E*10, *Z*12)-hexadecadienyl acetate. *FID* Flame ionization detector response, *EAD* electroantennogram detector response

Similarly, the retention times of compound **2** on ZB-5 and ZB-wax columns suggested a conjugated C_{16} dien-1-yl acetate and were the same as those of authentic *E*10*Z*12–16:Ac. Analysis of the MTAD-derivatized pheromone gland extract revealed an adduct with a molecular ion at *m/z* (rel. int.) 393 (2) and diagnostic ions at *m/z* (rel. int.) 350 (32), $[C_{18}H_{28}N_3O_4]^+$, and 208 (100), $[C_{10}H_{14}N_3O_2]^+$, indicative of a C_{16} dien-1-yl acetate with conjugation at the 10,12 position. Moreover, comparison of the mass spectrum [*m/z* (rel. int.) 280 (1, M^+), 220 (5), 177 (3), 163 (4), 149 (8), 135 (19), 121 (33), 107 (18), 95 (35), 81 (70), 67 (100), 61 (3), 55 (17), 43 (48)] and retention time of compound **2** in pheromone gland extracts, with all four possible C_{16} dien-1-yl acetate stereoisomers for the 10,12 position, confirmed compound **2** unambiguously as *E*10*Z*12–16:Ac (KI=2,046 on the VF-5ms column; *Z*,*E*, *Z*,*Z*, and *E*,*E* stereoisomers of 10,12 hexadecadien-1-yl acetate had KIs of 2,037, 2,058, and 2,064, respectively). A small amount (approxiamtely 5% compared with the *E*,*Z*-isomer) of *E*10*E*12–16:Ac (compound **3**) was also present in gland extracts, based on its KI and mass spectrum.

Compound **4** had KIs of 2,001 and 2,364, respectively, on the ZB-5 and ZB-Wax columns, the same as those of *Z*11–16:Ac standard. In addition, the mass spectrum of compound **4** [*m/z* (rel. int.) 222 (5), 166 (3), 137 (8), 123 (11), 109 (33), 95 (56), 81 (100), 67 (96), 61 (6), and 55 (42)] and its retention time on the VF-5ms column was the same as that of authentic *Z*11–16:Ac. Finally, compound **4** was unequivocally confirmed to be *Z*11–16:Ac, rather than *E*11–16:Ac, by comparing the retention times of the DMDS adducts of the two standards, with the adduct formed in the DMDS derivatized gland extract. Both hexadecen-1-yl acetate-DMDS adducts had a molecular ion at *m/z* (rel. int.) 376 (7) and diagnostic ions at *m/z* (rel. int.) 117 (20, $[C_5H_{10}SCH_3]^+$) and 259 (100, $[O_2C_{13}H_{24}SCH_3]^+$), corresponding to the addition of DMDS to a double bond at position 11.

The spectrum of compound **5** suggested an aliphatic primary alcohol (Pretsch et al. 2000) and was confirmed as

octadecan-1-ol by comparing its KI (2,088) on the VF-5 ms column and mass spectrum [*m/z* (rel. int.) 270 (1, M^+), 138 (3), 125 (8), 111 (39), 97 (88), 83 (89), 69 (91), 55 (100), and 41 (46)] with that of the authentic standard.

Qualitative and Quantitative Comparison of Auckland and Tasmania U. lugens Gland Extracts Gland extracts from the Auckland and Tasmanian populations contained four main compounds (Fig. 2), with two components dominant. Neither the quantities nor ratios of the four compounds varied significantly between these populations.

Female U. lugens Calling Behavior and Sex Pheromone Analysis Female *U. lugens* moths emerged during late photophase and early dusk. Characteristic lepidopteran calling behavior by females was first observed 2 hr into the scotophase and continued until dawn, with a peak at 7 hr into the scotophase (Fig. 3). A similar calling pattern was observed for 24-, 48-, and 72-hr-old females, although the proportion of females calling decreased significantly after the third scotophase.

Consistent with the calling behavior, pheromone titer reached a peak 7 hr into the first scotophase (Fig. 4) and

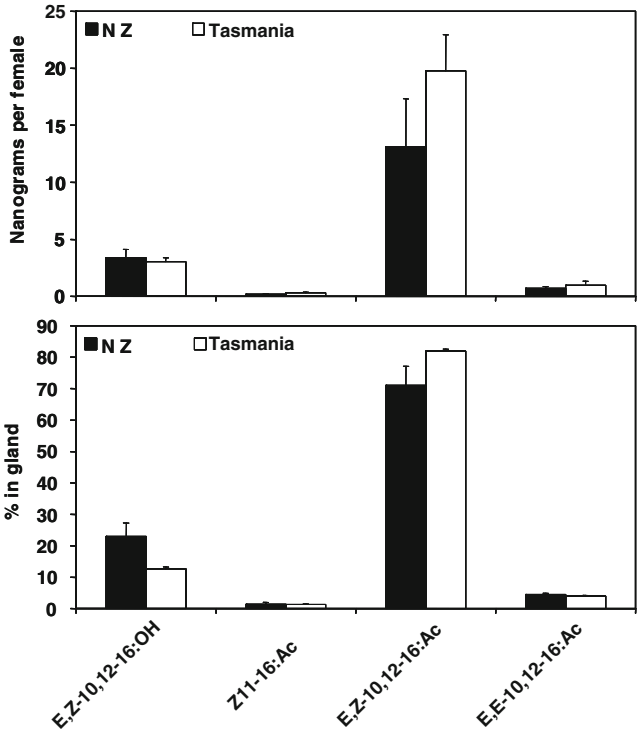

Fig. 2 Pheromone gland extract of *Uraba lugens* from Auckland (New Zealand) and Tasmania showing titers of (*E*10,*Z*12)-hexadecadien-1-ol (E,Z-10,12–16:OH), (*Z*)-11-hexadecenyl acetate (Z11–16:Ac), (*E*10,*Z*12)-hexadecadienyl acetate (E,Z10,12–16:Ac), and (*E*10,*E*12)-hexadecadienyl acetate (E,E-10,12–16:Ac). *Bars* indicate one standard error

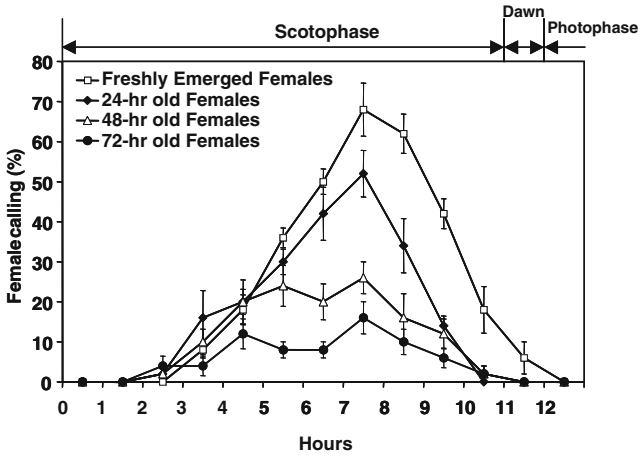

Fig. 3 Percentage of *Uraba lugens* females observed calling over a 12-h period from the first to the fourth scotophases. Age of females given is at start of the scotophase. *Bars* indicate one standard error

was at a comparable level during the scotophase on the next day and the day after that. Except at the beginning of the first scotophase (0 hr), when the amount was barely detectable, there was no significant difference in the amount of 18:OH in the gland at any of the time periods (i.e., from 2–48 hr) analyzed (Fig. 4). By the fourth scotophase, mortality of females was 35% and, after 96 hr, was 80% (Fig. 4).

Trapping Bioassays Trap tests in Australia were comparatively unsuccessful, catching a total of only 14 *U. lugens* to synthetic blends (Fig. 5a), which precluded meaningful statistical analysis. However, a second species (*Nola* spp.), visually distinct from *U. lugens* by the lack of a dark band across the wings, was caught in traps. We were unable to identify this species beyond the genus level. The catches of this insect to the blends tested are included in Fig. 5b for future reference.

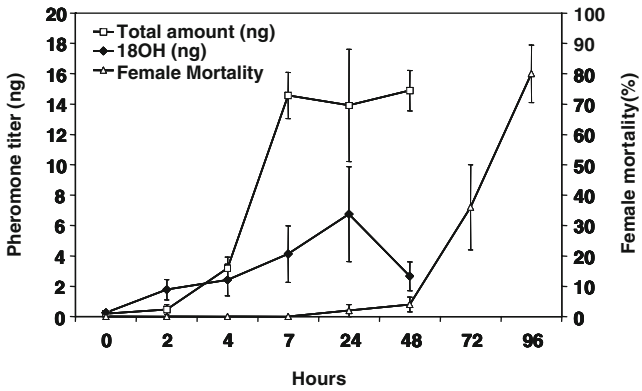

Fig. 4 Titer of pheromone (total amount of four compounds in Fig. 1) and octadecanol (18:OH) in the gland of *Uraba lugens* females and mortality over time. *0* Beginning of first scotophase. *Bars* indicate one standard error

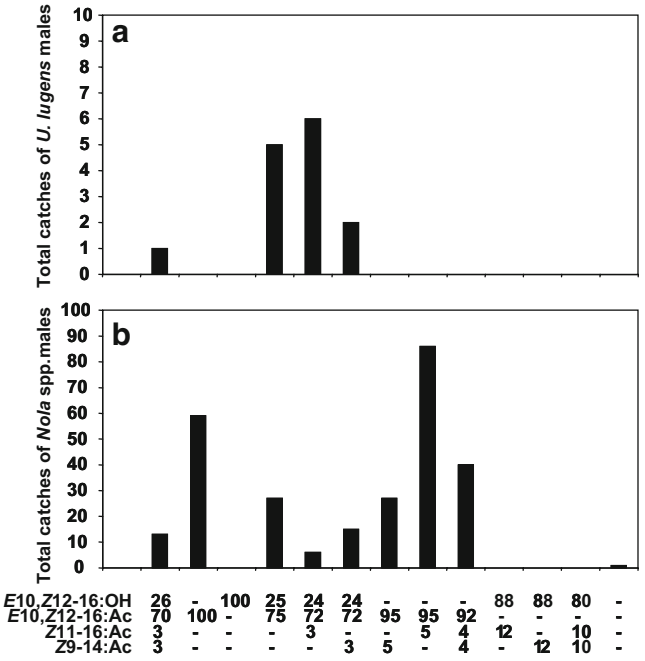

Fig. 5 Trap catch of *Uraba lugens* males (**a**) and *Nola* spp. males (**b**) in pheromone traps, baited with 100-μg total loadings of various combinations of candidate pheromone components, at a rural site near Goomeri, north of Brisbane (trial A). E,Z-10,12–16:OH=(*E*10,*Z*12)-hexadecadien-1-ol; Z11–16:Ac=(*Z*)-11-hexadecenyl acetate; E,Z10,12–16:Ac=(*E*10,*Z*12)-hexadecadienyl acetate; and Z9–14:Ac=(*Z*)-9-tetradecenyl acetate

Trapping assays in New Zealand confirmed that the binary combination of *E*10*Z*12–16:Ac and *E*10*Z*12–16:OH was necessary and sufficient for trap catch of male *U. lugens*. Treatment effects were significant on log-transformed counts ($F_{12,91}$=2.21, P<0.05) in trial B (Fig. 6) and on untransformed counts in trial C ($F_{5,48}$=14.21, P<0.001; Fig. 7a) and trial D ($F_{3,36}$=33.59, P<0.001; Fig. 7b). None of the tested compounds identified from female *U. lugens* pheromone glands was attractive by itself. Furthermore, the addition of the minor gland components *Z*11–16:Ac, *E*10*E*12–16:Ac, and octadecan-1-ol to the binary mix of *E*10*Z*12–16:Ac and *E*10*Z*12–16:OH did not result in increased trap catches relative to the binary mix alone. Except at very low ratios (1:9, Fig. 6) of *E*10*Z*12–16:Ac: *E*10*Z*12–16:OH, trap catches did not differ significantly over the ratios of these two compounds tested. Trap catch at a constant ratio of 2:3 (*E*10*Z*12–16:Ac: *E*10*Z*12–16:OH) increased from the lowest dosage tested (100 μg total of the two components) to the intermediate dosage (1,000 μg) but did not increase further to the highest dosage (3,000 μg) (Fig. 7a).

Unless an invasive moth is a major pest in another country, it is unlikely that its pheromone has been identified previously. Moreover, it is difficult to anticipate problematic invasive moths, since it is difficult to predict risk of a species arriving and its potential impact in a new

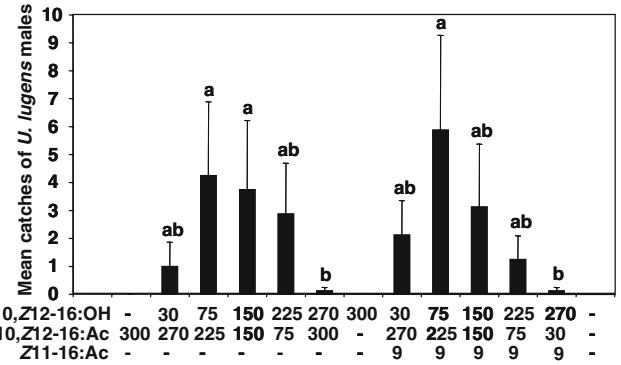

Fig. 6 Trap catch of *Uraba lugens* males in Auckland using lures containing a range of ratios of one, two, or three components at 300 μg (total components) (trial B). *Differences in letters* indicate differences between treatments at the P<0.05 level. E,Z-10,12–16:OH=(*E*10,*Z*12)-hexadecadien-1-ol; Z11–16:Ac=(*Z*)-11-hexadecenyl acetate; E,Z10,12–16:Ac=(*E*10,*Z*12)-hexadecadienyl acetate. *Bars* indicate one standard error

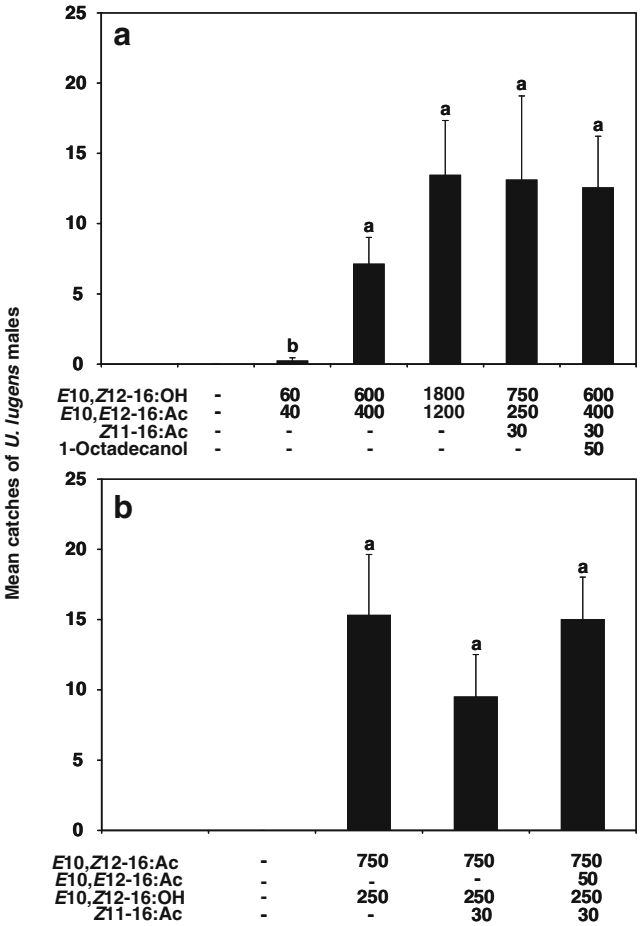

Fig. 7 Trap catch of *Uraba lugens* males in Auckland to **a** different doses (μg) and blends (trial C), and **b** addition of minor gland components to the two component blend (trial D). E,Z-10,12–16:OH=(*E*10,*Z*12)-hexadecadien-1-ol; Z11–16:Ac=(*Z*)-11-hexadecenyl acetate; E,Z10,12–16:Ac=(*E*10,*Z*12)-hexadecadienyl acetate. *Differences in letters* indicate differences between treatments at the P<0.05 level. *Bars* indicate one standard error

ecosystem. Therefore, pheromone identifications of such moth pests are likely to occur only after the insect has arrived and established. The gum leaf skeletonizer is not considered a major pest in its native Australia. A parasitoid complex (Brimblecombe 1962; Austin and Allen 1989; Allen 1990; Farr 2002), microbial diseases (Strelein 1988; Farr 2002), and periods of protracted drought help keep population levels relatively low, although occasional outbreaks of populations do occur (Brimblecombe 1962; Farr et al. 2004).

We commenced our study on the gum skeletonizer after it had been found in New Zealand, but to the knowledge at the time, it had yet to establish a permanent population. We were able to supply attractive lures to the official quarantine delimitation program in New Zealand (Suckling et al. 2005) within 3 mo of commencing the study, in part due to the initial field trial we conducted in Australia. Lures containing a 1-mg dose of a 75:25:3 mix of *E*10,*Z*12–16:Ac, *E*10, *Z*12–16:OH and *Z*11–16:Ac have since been used widely to collect phenological data, conduct delimitation surveys in the Auckland area (Suckling et al. 2005), conduct a survey in Tasmania in 2005 to determine the distribution of native Australian populations, and also to develop a model to determine the potential spread of this invasive pest (Kriticos et al. 2007). The surveys in New Zealand have shown that the insect has spread quite rapidly. An interactive map of the distribution of *U. lugens* in New Zealand, based on pheromone trapping data, is available (http://gis.scionresearch.com/maful/viewer.htm).

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References

ALLEN, G. R. 1990. *Uraba lugens* Walker (Lepidoptera: Noctuidae): larval survival and parasitoid biology in the field in South Australia. *J. Aust. Ent. Soc.* 29:301–312.

ALLEN, G. R., and KELLER, M. A. 1991. *Uraba lugens* (Lepidoptera: Nolidae) and its parasitoids (Hymenoptera: Braconidae): temperature, host size, and development. *Env. Entomol.* 20:458–469.

ARN, H., TOTH, M., and PRIESNER, E. 1992. List of sex pheromones of Lepidoptera and related attractants. 2nd edn.International Organization for Biological Control, Monfavet.

AUSTIN, A. D., and ALLEN, G. R. 1989. Parasitoids of *Uraba lugens* Walker (Lepidoptera: Noctuidae) in South Australia, with description of two new species of Braconidae. *Trans. Royal Soc. S. Aust* 113:169–184.

BERNT, L. 2006. Gumleaf skeletoniser—potential for biological control. *Biosecurity* 69:13.

BERRY, J. A., and MANSFIELD, S. 2006. Hyperparasitoids of the gum leaf skeletoniser, *Uraba lugens* Walker (Lepidoptera: Nolidae), with implications for the selection of a biological control agent for *Uraba lugens* in New Zealand. *Aust. J. Entomol.* 45:215–218.

BRIMBLECOMBE, A. R. 1962. Outbreaks of the eucalypt leaf skeletoniser. *Queensland J. Agr. Sc.* 19:209–217.

BUSER, H., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

CAMPBELL, K. G. 1962. The biology of *Roeselia lugens* (Walk.), the gum-leaf skeletonizer moth, with particular reference to the *Eucalyptus camaldulensis* Dehn. (River Red Gum) forests of the Murray Valley region. *Proceedings of the Linnean Society of New South Wales, vol.* 87, 316–338.

CLARE, G., SUCKLING, D. M., BRADLEY, S. J., WALKER, J. T. S., SHAW, P. W., DALY, J. M., MCLAREN, G. F., and WEARING, C. H. 2000. Pheromone trap colour determines catch of non-target insects. *N.Z. Plant Protect.* 53:216–220.

COMMON, I. F. B. 1990. Moths of Australia. Melbourne University Press, Carlton, Victoria, Australia.

DERRAIK, J. 2006. Erucism in New Zealand: exposure to gum leaf skeletoniser (*Uraba lugens*) caterpillars in the differential diagnosis of contact dermatitis in the Auckland region. *N.Z. Med J.* 119:U2142.

FARR, J. D. 2002. Biology of the gumleaf skeletoniser, *Uraba lugens* Walker (Lepidoptera: Noctuidae), in the southern jarrah forest of Western Australia. *Aust. J. Entomol.* 41:60–69.

FARR, J. D., SWAIN, D., and METCALF, F. 2004. Spatial analysis of an outbreak of *Uraba lugens* (Lepidoptera: Noctuidae) in the south-west of Western Australia: does logging, vegetation type or fire influence outbreaks? *Aust. For.* 67:101–113.

GIBB, A. R., SUCKLING, D. M., MORRIS, B. D., DAWSON, T. E., BUNN, B., COMESKEY, D., and DYMOCK, J. J. 2006. *Z*7-Tricosene and monounsaturated ketones as sex pheromone components of the Australian guava moth *Coscinoptycha improbana*: Identification, field trapping, and phenology. *J. Chem. Ecol.* 32:221–237.

HARRIS, J. A., NEUMANN, R. G., and WARD, B. 1977. An outbreak of the gum leaf skeletoniser, *Uraba lugens* Walker, in river red gum forest near Barmah. Forestry Commission, Victoria.

HOARE, R. J. B. 2001. Adventive species of Lepidoptera recorded for the first time in New Zealand since 1988. *N.Z. Entomol.* 24:23–47.

KOVÁTS, E. 1965. Gas chromatographic characterization of organic substances in the retention index system, pp. 229–247, in J. C. Giddings, and R. A. Keller (eds.). Advances in Chromatography, vol. 1. Edward Arnold, London.

KRITICOS, D. J., POTTER, K. J. B., ALEXANDER, N. S., GIBB, A. R., and SUCKLING, D. M. 2007. Using a pheromone lure survey to establish the native and potential distribution of an invasive Lepidopteran, *Uraba lugens*. *J. Applied Ecol.* 44:853–863.

MANSFIELD, S. M., KRITICOS, D. J., POTTER, K. P., and WATSON, M. 2005. Parasitism of gum leaf skeletoniser populations in New Zealand. *N. Z. Plant Prot.* 58:191–196.

MARQUES, F. A., MCELFRESH, J. S., and MILLAR, J. G. 2000. Kováts retention indexes of monounsaturated C_{12}, C_{14}, and C_{16} alcohols, acetates and aldehydes commonly found in lepidopteran pheromone blends. *J. Braz. Chem. Soc.* 11:592–599.

MCELFRESH, J. S., and MILLAR, J. G. 1999. Sex pheromone of the common sheep moth, *Hemileuca eglanterina*, from the San Gabriel Mountains of California. *J. Chem. Ecol.* 25:687–709.

MORGAN, F. D., and COBBINAH, J. R. 1977. Oviposition and establishment of *Uraba lugens* (Walker), the gum leaf skeletoniser. *Aust. For.* 40:44–55.

POTTER, K. J. B., and STEPHENS, A. E. A. 2005. Suitability of valued eucalypt species for the larval development of the gum leaf skeletoniser, *Uraba lugens*. *N.Z. Plant Prot.* 58:184–190.

PRETSCH, E., BÜHLMANN, P., and AFFOLTER, C. 2000. Structure Determination of Organic Compounds—Tables of Spectral Data. Springer, Berlin.

ROSS, M. 2001. Skeletoniser found in Auckland cemetery. *Biosecurity* 30:8.

ROSS, M. 2003. Gum leaf skeletoniser found in Onehunga. *Biosecurity* 41:6.

SAS Institute Inc. 1998. SAS OnlineDoc(TM), Version 7-0. SAS Institute, Cary, NC.

STRELEIN, G. J. 1988. Gum leaf skeletoniser moth, *Uraba lugens*, in the forests of Western Australia. *Aust. For.* 51:197–204.

SUCKLING, D. M., GIBB, A. R., DENTENER, P. R., SELDON, D. S., CLARE, G. K., JAMIESON, L., BAIRD, D., KRITICOS, D. J., and EL-SAYED, A. M. 2005. *Uraba lugens* (Lepidoptera: Nolidae) in New Zealand: pheromone trapping for delimitation and phenology. *J. Econ. Entomol.* 98:1187–1192.

potheses, and act as standards for comparison. Models of mating disruption and mass trapping may need to consider parameters such as (a) odor dispersion (e.g., plume structure, emission rates, meteorology, and densities of chemical dispensers), (b) behavior (e.g., orientation mechanisms, sensitivity to components, mating, and dispersal), and (c) population ecology (e.g., duration, percentage mating or trapped, densities of males and females).

One parameter that could be used in models described above is the active space, defined by Elkinton and Cardé (1984) as the volume of air inside in which the odor concentration is above the threshold that elicits a behavioral reaction in the receiving organism (Sower et al. 1971; Nakamura and Kawasaki 1977; Baker and Roelofs 1981). This concept is based on various Gaussian equations that calculate the odor concentrations in still and moving air (Sutton 1953; Bossert and Wilson 1963; Fares et al. 1980). The active space has been investigated in several studies by observing wing-fanning of male moths in cages at various points within a pheromone plume (Elkinton and Cardé 1984; Elkinton et al. 1984). The fine structure of pheromone plumes on a small time scale is extremely complicated but thought to comprise filaments of higher concentrations due to turbulent eddies of air (Mankin et al. 1980; Elkinton and Cardé 1984; Baker et al. 1998).

While plumes and active spaces are three-dimensional in nature, they can be simplified further to two dimensions for practical purposes. This is because mate- and host-seeking insects disperse and orient over hundreds of meters within a relatively thin layer of air a few meters thick, as shown in many studies (e.g., Meyerdirk and Moreno 1984; Meyerdirk and Oldfield 1985; Chandler 1985; Meyer and Colvin 1985; Stone 1986; Byers et al. 1989; Stewart and Gaylor 1991; Isaacs and Byrne 1998; Pearsall and Myers 2001; Weber et al. 2005). Thinking in a two-dimensional way (Fig. 1a), the active space can be thought of as an instantaneous snapshot or as a time-averaged area (Sutton 1953; Bossert and Wilson 1963; Elkinton and Cardé 1984; Elkinton et al. 1984; Byers 1987). Elkinton and Cardé (1984) reviewed reports that suggested that the plume's active space is affected by wind speed, turbulence, temperature, topography, vegetation, and time scale. Thus, under field conditions, the active space might be at least as chaotic as suggested in Fig. 1a.

Another concept connected to the active space is the capture probability (CP). There is likely a specific CP at any position in the active space that depends on the semiochemical concentration and orientation ability of the insect species (McClendon et al. 1976; Wall and Perry 1987; Branco et al. 2006). It is well known from theoretical and experimental studies above and others that concentrations of odor decline with distance from the source. In addition, it is known that at lower release rates, insects are less attracted. This is probably due to a combination of individual variation in response threshold, less receptor firing frequency, and lower plume filament flux frequency at these low concentrations (Baker et al. 1998). Therefore, we expect that insects that enter the active space of a plume at a position far from the source would have a lower probability of finding the source than an insect that enters much nearer the source. For example, McClendon et al. (1976) presented a capture probability response surface based on catch of marked boll weevils, *Anthonomus grandis* Boheman, released at various distances from a synthetic pheromone source. The probabilities ranged from 5% at about 150 m from the source to about 45% near the source. Branco et al. (2006) proposed a declining logistic function to predict capture probability with distance for scale insects that respond to sex pheromone.

The CP does not depend solely on pheromone concentration since even if the concentration is the same at two points downwind, one further than the other from the source, the insect entering farther from the source will likely have a lower CP due to more time needed to fly to the source and more possibilities of becoming disoriented. Thus, the CP depends on where the insect enters the active space (Fig. 1b). If the semiochemical release rate is increased, it is apparent that the active space increases in area as well as the varied CP values, all of which would be impractical to measure. The semiochemical release rate can be increased to a point that adaptation or confusion occurs with little catch (Bartell and Roelofs 1973; Baker and Roelofs 1981; Kuenen and Baker 1981; Baker et al. 1989; Rumbo and Vickers 1997; Judd et al. 2005). In this case, the active space should move a distance downwind from

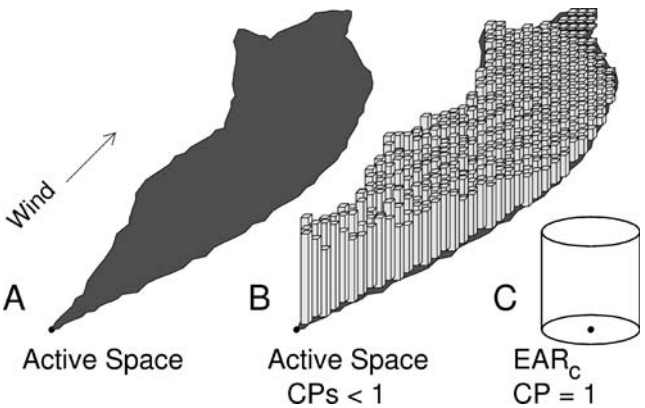

Fig. 1 **a** Representation of a pheromone plume as an active space in which semiochemical concentration is above a behavioral threshold eliciting orientation; **b** the same plume represented with CP<1 indicating the probability of an insect reaching the pheromone source upon initial observation at a particular position; **c** the capture finding probability surface compressed into an effective attraction radius with the same effect on catch by reducing the diameter but maximizing the CP at a probability of 1

the source, although it may be of similar size, while CP values would be drastically reduced.

The *effective attraction radius* (EAR) was proposed by Byers et al. (1989) as the radius of a passive sphere that would intercept the same number of insects as does a specific attractive trap baited with semiochemicals. A similar concept used in models of mate finding, mass trapping, and host-tree finding (Byers 1991, 1993a, b, 1996a, 1999) is here termed the EAR_c (c for circle) to distinguish it from the spherical EAR. As discussed below, the EAR_c concept is more useful in developing practical control with mating disruption and mass trapping than the better-known active space concept. The two ideas are related. In the EAR_c used in models, the CP response volume within the active space is compressed into a cylindrical volume with CP equal to 1 so that insects entering will find the source and be trapped (Fig. 1c). The cylinder can be further compressed to two dimensions so that the EAR_c is a circular area. The time-averaged active space and its various CPs are extremely difficult to determine in nature but are hypothesized to attract an equivalent number of insects under the same population conditions as would a corresponding EAR_c. Fortunately, the spherical EAR is easily determined by a time-averaged catch ratio of active and unbaited traps in the field as well as the interception area of the unbaited trap. Once this EAR is established, it is advantageous to transform it to an EAR_c for use in encounter rate models of mass trapping and mating disruption (Byers 1993a, b, 1999; El-Sayed et al. 2006; Byers 2007, and unpublished). Of particular importance is that measurement of the EAR at different population densities should give the same value because it is derived from a ratio of active and passive catches (Byers et al. 1989).

No algorithms have been developed to transform the spherical EAR to a circular EAR_c. In addition, the assumption that the EAR_c can simulate encounter rates (catches) that are equivalent to that for plumes with more complex CP-active space has not been investigated. In contrast to molecular diffusion that is random, animals disperse in a correlated random walk (CRW) of forward direction because the direction of each step is correlated to some degree with their previous step direction (Turchin 1998; Byers 2001). My objective was to design graphical simulations of insects that move in a CRW while sometimes entering elongated active space plumes that have an inverse linear CP relationship with distance in order to calculate catch at various constant insect densities and flight speeds. This was to demonstrate merely that changes in CP and/or active space dimensions in this model would result in corresponding changes in catch. Then, for a particular catch obtained in the CP-active space model, I wanted to vary the EAR_c circle of CP=1 to iteratively solve for the same catch. This would demonstrate there is an EAR_c that is equivalent to any complex interaction of active space and varying CP, of which the CP-active space is virtually impossible to obtain experimentally. In this case, the EAR_c would provide a simple but effective alternative to a model that uses the active space and its spatially complex probabilities of attraction. The goal is then to determine the EAR in the field and convert it to an EAR_c for use in models of mating disruption and mass trapping in order to develop cost-effective control programs with these methods (Miller et al. 2006a, b; El-Sayed et al. 2006; Byers 2007).

Methods and Materials

Simulation of Insect Catch with Capture Probabilities in the Active Space of Plumes Insects were simulated in a two-dimensional area with *x*-axis (xa) and *y*-axis (ya) that can be adjusted but was held at 1,000×1,000 m in which a pheromone source was placed at (xa/2, ya/2) and an active space plume extended as an angular sector of 15° with a radial length of 100 m (Byers 1996b). Insects were simulated at a constant density in the area outside of the plume (usually $0.001/m^2$ or 997 insects for the plume above). Any insect that left the simulation area was replaced by another that was taking a half step from the perimeter at random into the area. Also, any insects that reached the source (i.e., caught) were counted and replaced outside the active space at random. Insects flew in the area according to a CRW and entered active space plumes as in earlier models (Byers 1996a, b, 1999, 2001). Each insect was given an initial direction and position outside the active space at random. Thereafter, each male followed a CRW made of a series of steps with a distance covered per second (speed), each step calculated as a polar vector from the former position. The vector length was the average distance traveled per second (usually 1 m/s speed), and the direction was the former direction plus a turning angle chosen at random from a normal distribution, usually an SD° (angular standard deviation) of 5° centered on the former direction (Byers 2001).

Insects that entered the plume active space of length L were assumed to reach the source with a probability that depended on their initial entry distance, d, from the source. Thus, to obtain CP as a function of distance from the source, a linear function beginning with P=0.05 at L= 100 m and rising to a CP=1 at the source was used:

$$\mathrm{CP} = \frac{L - d + P \cdot d}{L} \tag{1}$$

However, other values of L, $d \le L$, and $0 \le P \le 1$ can be used. This equation generally accounts for the expectation, based on general knowledge of dosage–response relationships and mark–recapture studies (e.g., Zolubas and Byers

1995), that catch is higher when entering the active space nearer the source than when entering farther downwind. However, the exact nature of the relationship is known poorly due to little information about variation in odor concentration, behavioral responses, and orientation time while approaching the source. In the model, an insect reaches the source (is caught) if a random number (0 to 1) is ≤CP at the point where the insect enters the active space (Eq. 1). If not caught, then the insect moves in the opposite direction away from the plume so only one test is made at that point in time. This algorithm merely approximates the effect of the plume CPs because only the edges of the plume are encountered.

The model was programmed in QuickBASIC 4.5 (Microsoft, Redmond, WA, USA) for use in simulations as well as Java 1.4.2_10 or later (Sun Microsystems, Santa Clara, CA, USA) for general demonstration on the Internet with a web browser and Java runtime installed (http://www.chemical-ecology.net/java2/act-ear.htm).

Simulation of Equivalent Insect Catch by the Effective Attraction Radius The same model parameters and algorithms as above were used except there was no plume but rather a circle representing the EAR_c placed at (xa/2, ya/2). Insects that entered the EAR_c were captured with a probability of 1. Since insects could be allowed steps that jump over the circular EAR_c, the algorithm for encountering the circle was that shown in Byers (1991, his Fig. 3). The goal was to find an EAR_c in simulations with a catch that was approximately the same as for the assumed active space and CP relationship with the same CRW model parameters. This goal was facilitated by the functional response type I equation of Holling (1959). His equation defines the number of prey found (y) by a predator as:

$$y = a \cdot T_s \cdot x \tag{2}$$

where T_s is the total foraging time, x is the prey density, and a is the instantaneous attack rate. The attack rate is equal to the area covered by the predator each time unit and is, thus, the size of the predator or prey ($2\times EAR_c$) times the speed of prey or predator (depending on which is moving). This equation assumes one trap (i.e., predator) does not appreciably deplete insect densities and that predators/prey do not revisit previous areas.

In regard to an EAR_c of a pheromone plume (or trap), substitution of the relevant variables into Eq. 2 and solution for the radius (EAR_c) gives the equation:

$$EAR_c = \frac{C \cdot xa \cdot ya}{T \cdot S \cdot 2 \cdot N} \tag{3}$$

where T is the test duration (same as T_s above), S is the insect speed, C is the number caught, and N is the number of insects in the area (xa·ya) or prey density (x). In the simulations, N is known but would not be measured easily in nature. Thus, the catch obtained in the CP-active space model (mean of ten simulations) was used in Eq. 3 to predict an equivalent EAR_c. Simulations were then performed to bracket the predicted EAR_c by incrementing in 3-m steps the simulated EAR_c from 1 to twice the predicted (each increment had ten simulations). Linear regression was used to solve for the EAR_c that caught the equivalent catch as that in the CP-active space response area.

To gain insight about effects of various model parameters used in both the CP-active space and EAR_c in models, simulation results were compared to predicted results of Eq. 3. Both EAR_c (1, 2, 5, 10, and 20 m) and number of insects (100, 200, 500, 1,000, and 2,000) were varied in simulations of ten replicates each for all combinations of these parameters. The standard deviation of turning angle distribution (SD°) was varied similarly (0, 2, 5, 10, 15, 20, 25, and 30°) in simulations to test for effects on catch.

Relationship Between EAR of Sphere and EAR_c of Circle Used in Models The original EAR (Byers et al. 1989) was the radius of a sphere as calculated by the equation:

$$EAR = \sqrt{\frac{A_c \cdot 2 \cdot r \cdot h}{P_c \cdot \pi}} \tag{4}$$

where A_c is the catch on the active trap (semiochemical), P_c is the catch on the passive (unbaited) trap, and r is the radius and h is the height of the passive trap cylinder. For hanging flat panel traps of width × height, $2{\cdot}r{\cdot}h$ in Eq. 4 can be substituted with ($0.637{\cdot}width{\cdot}height$) for the average interceptive trap area, TA (Byers et al. 1989). In simulation models, the EAR is better described in a new way:

$$EAR_c = \frac{A_c \cdot TA/2}{P_c \cdot F_L} \tag{5}$$

where F_L is the thickness of the air layer in which insects search primarily while flying. Regressions were done by enlarging r from 0.1 to 2 m with various h, A_c, and P_c in F_L from 1 to 10 m thickness in order to determine the relationships between Eqs. 4 and 5. The goal was to try and convert EAR of Eq. 4, measured conveniently with two trap catches, to corresponding EAR_c of Eq. 5, which is most useful in simulation models.

The scientific literature was searched (BIOSIS Previews) for articles on flight heights of insects caught by traps to estimate the effective flight layer of search, F_L, as well as catches on attractant and passive sticky traps to estimate EAR from Eq. 4 or EAR_c from Eq. 5. The F_L was estimated from published catches at various heights by calculating the mean height of catch and standard deviation (SD) by using standard statistical formulas (McCall 1970). In some

reports, actual catch was not given but rather the total catch proportion at each trapping height. It was assumed that at least 100 insects were caught among all trap heights. Effects of this assumption were tested on simulated data of either 20, 100, or 2,000 catches at five trap levels (1, 2, 3, 4, and 5) assuming catch proportions of 0.1, 0.3, 0.3, 0.2, 0.1, respectively. The F_L was then calculated from $SD \cdot \sqrt{2 \cdot \pi}$. This gives a probability area equal to the height of the normal distribution ($1/(SD \cdot \sqrt{2 \cdot \pi})$; McCall 1970) times the layer's thickness that would equal the area under the normal curve (Byers, unpublished).

Results

Simulation of Insect Catch with Capture Probabilities in the Active Space of Plumes The simulation of the 15°-sector× 100-m long CP-active space response plume in the square kilometer arena caught an average of 121.4±6.9 (±95% confidence limits (C.L.) N=10) insects when a constant density of 1,000 in the area each took 3,600 steps of 1 m each and turned with an SD° of 5°. By assuming the same simulation conditions and using Eq. 3, an equivalent number of insects should be caught by a predicted EAR_c of 16.86 m. An increase of the plume length (X) from 50 to 200 m increased the catch (Y) as expected as a linear function (Y= 5.5+1.145X, R^2=1.0, N=7). Other CP-response surfaces in the active space altered the simulated catch depending on the values. For example, assuming an exponential decline in probability with distance from the source (Mason et al. 1990; Zolubas and Byers 1995), CP = $P/\exp(d)^{5/L}$ with P=1, gave a mean catch of 43.6±6.08 with the parameters above. This predicts an EAR_c of 6.06 m by using Eq. 3. Simulations also showed that the CRW parameter of the SD° of angular turns affected catch, as is presented below.

Simulation of Equivalent Insect Catch by the Effective Attraction Radius An increase in the number of insects in the arena (density) caused a linear increase in catch (Table 1) in accordance with that predicted from solving catch (C) in Eq. 3. Also, an increase in the EAR_c caused a linear increase in catch (Table 1) that was close to that predicted. Other simulations in which the SD° of turning angles was increased from straight at 0° to sinusoidal at 30° gave results that were surprising (Table 2). At an SD° of 0°, the catch was 60.3±5.9, which was less than 72 predicted from Eq. 3, while as the SD° was increased to 5°, the catch increased to 71.3±6.4 and was indistinguishable from predicted. Further increases in SD° to 10° (65.7±4.1), 15° (56±4.8), 20° (42.2±4.2), 25° (33.5±2.6), and 30° (27.9± 4.4) caused the catch to decline well below predicted. The general reasons for this will be discussed below.

The EAR_c was increased incrementally in simulations from 1 m to about twice the predicted EAR_c in order to find the catch that was equivalent to the catch by the CP-active space model. The results fit a linear function, Y=7.209X+ 0.26 (R^2=1.0, N=11, Fig. 2). By using a catch of 121.4 for Y above in the CP-active space plume, an equivalent EAR_c is found by solving for X=(Y−0.26)/7.209=16.80 m, which is close to 16.86 m predicted from Eq. 3. Another random seed gave an equivalent EAR_c of 16.85 m. This shows in principle that any CP-active space response surface can be transformed into an equivalent EAR_c. While the CP-active space response surface is exceedingly difficult to measure, the EAR can be found experimentally in the field (see below).

Relationship between EAR of Sphere and EAR_c of Circle Used in Models There is a positive linear relationship between radius of the passive trap and the EAR of the sphere, while the EAR_c increases as a function of the square of the trap radius (Fig. 3). The exact relationships depend on the ratio of catch (active/passive), the interception area of the passive trap, and in the case of the EAR_c, the effective flight layer thickness. When the EAR_c (Y) is plotted against EAR (X), the EAR_c increases as the square of the EAR (Y=aX^2, Fig. 4). This relationship does not depend on the catch ratio or the dimensions of the passive trap; however, the proportion of the passive trap height that intercepts the flight layer (F_L) does influence the power curves. For example, if the F_L=10 m then Y=0.157X^2,

Table 1 Effect of EAR_c and number of insects on catch when each insect moved 3,600 steps of 1 m (1 m/s) with an SD° of turning angles of 5° in an area of 1,000×1,000 m (catches are means±95 C.L. of ten simulations)

	Effective attraction radius (EAR_c; m)				
Number of insects per square kilometer	1	2	5	10	20
100	0.6±0.5 (0.72)[a]	1.5±0.8 (1.44)	3.5±1.3 (3.6)	7.9±2.1 (7.2)	16.4±2.4 (14.4)
200	1.4±1.1 (1.44)	3.3±1.5 (2.88)	9.1±2.5 (7.2)	14.1±2.4 (14.4)	28.4±3.1 (28.8)
500	4.7±1.0 (3.6)	8.0±2.5 (7.2)	17.3±2.8 (18)	37.3±3.4 (36)	70.7±4.8 (72)
1,000	8.5±1.6 (7.2)	12.5±1.6 (14.4)	33.9±4.9 (36)	67.9±6.6 (72)	139.2±8.8 (144)
2,000	14.4±3.1 (14.4)	28.8±3.0 (28.8)	68±7.9 (72)	139.4±5.1 (144)	290.6±13.2 (288)

[a] Values in parentheses are predicted catch (C) by solving Eq. 3.

Table 2 Effect of SD° of turning angles on catch by an EAR_c of 10 m when each insect moved 3,600 steps in an area of 1,000×1,000 m with a constant 1,000 insects (catches are means±95 C.L. of ten simulations)

	Average speed of insect (m/s)			
SD° of turn angles	0.1	0.5	1	2
0	6.0±0.6 (7.2)[a]	27.9±3.5 (36)	60.3±5.9 (72)	115.4±7.2 (144)
2	5.8±1.5 (7.2)	33±4.7 (36)	66.5±6.5 (72)	123.2±8.3 (144)
5	6.3±1.1 (7.2)	35.5±3.8 (36)	71.3±6.4 (72)	140.6±10.3 (144)
10	3.8±1.2 (7.2)	31.1±3.7 (36)	65.7±4.1 (72)	136.6±6.8 (144)
15	3.0±1.1 (7.2)	20.8±3.4 (36)	56±4.8 (72)	131±7.0 (144)
20	1.4±0.8 (7.2)	16.9±2.7 (36)	42.2±4.2 (72)	113.6±6.0 (144)
25	1.7±0.9 (7.2)	12.0±3.5 (36)	33.5±2.6 (72)	96±8.0 (144)
30	1.1±0.6 (7.2)	9.1±1.4 (36)	27.9±4.4 (72)	80±5.6 (144)

[a] Values in parentheses are predicted catch (C) by solving Eq. 3.

while if F_L=3 m, then $Y=0.524X^2$. Plotting of the regression coefficient a above against F_L gives an inverse power relationship $a=1.571/F_L$. This a used in $Y=aX^2$ above can be used with an effective flight layer thickness to convert any spherical EAR to the corresponding model EAR_c with the equation:

$$EAR_c = \frac{1.571 \cdot EAR^2}{F_L} \quad (6)$$

It turns out that 1.571 is $\pi/2$.

Over 100 articles were found on insect flight heights of which some were suitable to estimate the flight layer of search, F_L (Table 3). Most insects, while searching for mates and/or food, fly within a few meters above the ground (Table 3). Insects that are of agricultural importance fly within an even thinner layer just above the canopy. In some studies, only proportions of catch at each trap height were reported, so N=100 or 1,000 insects were assumed when calculating mean height of catch and SD. However, there was little possible error from this assumption since calculation with 20, 100, or 2,000 catch on specific proportions (see "Methods and Materials") gave the same mean height of catch (2.9 m), while the SD also was similar at 1.165, 1.142, or 1.136, respectively.

A few studies were found on catches of attractant and passive sticky traps of various insects and used to calculate EAR from Eq. 4, which was then used with an estimated F_L to determine an EAR_c by Eq. 6 (Table 4). The EAR_c is directly proportional to the ratio of catch, so the radius of its circle catches in direct proportion to its size in simulations as expected.

Discussion

The active space concept is readily understood as the plume area where male moths detect female moth pheromone (Sower et al. 1971; Nakamura and Kawasaki 1977; Baker and Roelofs 1981; Elkinton and Cardé 1984). It is time-

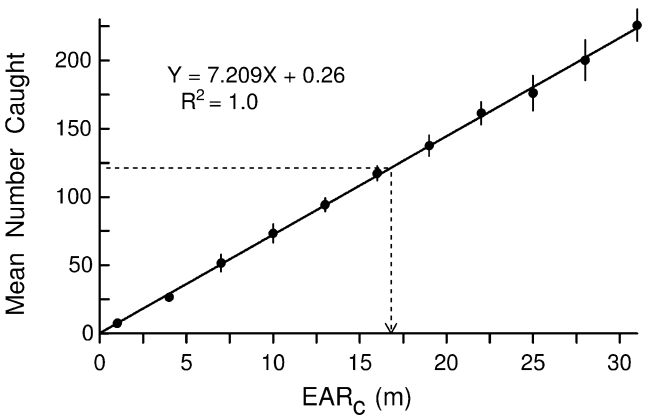

Fig. 2 Mean number of insects caught in an area of 1,000×1,000 m with a constant density of 1,000 insects, each taking 3,600 steps of 1 m with a 5° SD of turning angle distribution as a function of EAR_c in meters (*bars* represent 95% C.L., ten simulations each point)

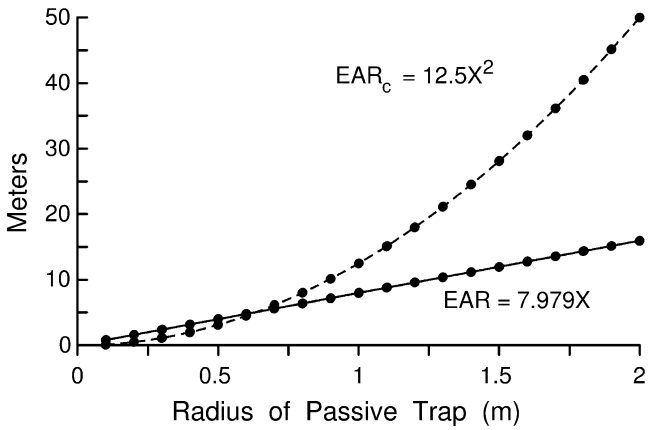

Fig. 3 EAR from Eq. 4 and EAR_c from Eq. 5 as a function of the passive cylinder trap radius when the pheromone trap caught 50 times more than the passive trap (height of passive trap was twice the radius and F_L=8 m)

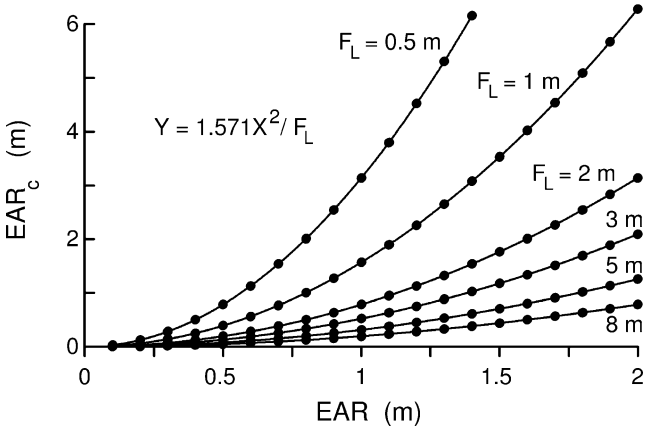

Fig. 4 EAR$_c$ as a function of EAR in various flight layers

consuming and tedious to measure the general dimensions of the active space for a particular pheromone release rate and moth species by observing male wing-fanning in cages at many distances downwind from the source (Elkinton and Cardé 1984; Elkinton et al. 1984). Knowledge of the active space would be useful in mass-trapping programs if moths entering the space have a constant probability of being caught by a trap. Unfortunately, a constant probability is unlikely and not consistent with our knowledge of moth orientation behavior in wind tunnels or in the field. It is likely that males entering the active space far from the pheromone source have lower CP than males entering the active space nearer the source (McClendon et al. 1976; Branco et al. 2006). However, the difficulties of determining the many CPs in the active space are enormous, and as such, no studies have attempted precise measurements. Still, knowledge of the CP-active space dimensions could be useful in modeling of mate- and host-finding as well as mating disruption and mass trapping with synthetic pheromone dispensers (Byers 2007).

The simulations demonstrated that males encountering CP-active space plumes of a specific dimension reach the pheromone source at a rate that depends on the model parameters chosen. The equivalent attraction rate of a CP-active space plume of any arbitrary complexity was obtained by adjusting the circular EAR$_c$ in size until this caught an equivalent number during the same period. In modeling insect encounters with semiochemical lures, the use of highly complex CP-active space plumes is less desirable than the simple-to-measure-and-use EAR$_c$.

The EAR, as used originally (Byers et al. 1989) in Eq. 4, is calculated from the passive trap interception area as seen from any particular direction multiplied by the ratio of catch on the pheromone trap (A_c) and passive trap (P_c). This interception area is solved for a circular area of radius EAR (also the radius of a sphere of this size). The EAR (or EAR$_c$) is useful to compare the relative strengths of semiochemical attractants of various blends and release rates among insect species and studies. Catches alone are not sufficient for such comparisons because catch depends on trap dimensions as well as on population density, both of which vary between locations and times. In contrast to simple catch comparisons, the EAR is not affected significantly by flight density variations, as Eq. 4 uses a ratio of attractive to passive catches that, in principle, would not vary with flight density. It is relatively simple to obtain an average catch on passive sticky traps as well as one on attractive pheromone traps. The size of the passive trap then determines the EAR as long as there is some catch on both trap types (division by zero is undefined).

An insect species with a larger EAR than a second species generally means that individuals of the first species are attracted from a longer distance, assuming the CP gradients in the two active spaces are about the same. However, if the CP-active spaces are different, it is possible that the second species could, on average, be attracted from further away. Because we have little or no knowledge of the CP-active space, it is assumed that the larger the EAR, the longer the average distance of attraction. In the simulations, insects did not reach the inner areas of the plume, so the response surface actually applied only on the periphery, while in nature, CP could be observed anywhere in the plume. If simulated insects were allowed to continue into the plume, they would have a new chance of being caught according to the CP at each step, which would incorrectly cause nearly all to be caught. The EAR with CP=1 does not exist in nature, as does a plume, but merely acts as a convenient method of comparison of attractive bait strengths or as a convenient substitute for plumes (as EAR$_c$) in encounter rate models. The attractive trap must catch more than the passive trap, the passive trap should catch some insects, and higher catches on many passive traps will improve the accuracy of the EAR measurement. The attractive trap should be placed far enough from the passive trap as to hardly influence the latter's catch, but both kinds of traps should be within the same area of flight density. The EAR is usually up to only a few meters and, thus, considerably smaller than the attraction range (maximum orientation distance), the sampling range (maximum distance over which an insect can reach a trap in a given time; Wall and Perry 1987), or the average distance of attraction. A related concept is the effective sampling area (trap catch divided by the population density; Turchin and Odendaal 1996), which, for European pine sawflies *Neodiprion sertifer* Geoffroy, released away from a pheromone trap, was estimated to have a radius of 125 m (Östrand and Anderbrant 2003), much larger than any expected EAR.

The EAR calculation is convenient to measure in the field with sticky traps that sample a small portion of the flight layer (usually within meters of the ground for most agricultural insects). The trouble in modeling with the EAR as measured

Table 3 Mean height of catch ±SD (m) and the effective F_L of various insects calculated from catches at trap heights reported in the literature

Species	Trapping method	Range of trap heights	Number of trap heights	Total catch[a]	Mean height of catch ±SD	F_L (m)
Coleoptera: Scolytidae						
Hylurgops palliatus (Gryllenhal)[b]	St-screen	0.7–11.5	10	86	4.98±2.63	6.59
Ips typographus (L.)[b]	St-screen	0.7–11.5	10	103	4.63±2.75	6.89
T. piniperda (L.)[b]	St-screen	0.7–11.5	10	10	5.98±3.00	7.52
Trypodendron domensticum (L.)[b]	St-screen	0.7–11.5	10	17	2.82±1.67	4.19
Cryphalus abietus (Ratz.)[b]	St-screen	0.7–11.5	10	242	3.44±2.73	6.84
Pityogenes bidentatus (Herbst)[b]	St-screen	0.7–11.5	10	23	3.10±1.62	4.06
Pityogenes chalcographus (L.)[b]	St-screen	0.7–11.5	10	84	6.89±2.90	7.27
Pityogenes quadridens (Hartig)[b]	St-screen	0.7–11.5	10	50	4.08±2.80	7.02
Ips calligraphus (Germar)[c]	X-pane	1–5	3	(100)	2.62±1.31	3.28
Ips grandicollis (Eichh.)[c]	X-pane	1–5	3	(100)	3.43±1.29	3.22
Hylastes tenius Eichh.[c]	X-pane	1–5	3	(100)	2.43±0.91	2.27
Xyleborus affinis (Eichh.)[c]	X-pane	1–5	3	(100)	1.33±0.94	2.35
Xyleborinus saxesini (Ratz.)[c]	X-pane	1–5	3	(100)	2.25±1.44	3.61
Ambrosiodmus lecontei Hopk.[c]	X-pane	1–5	3	(100)	2.38±1.69	4.24
Xylosandrus compactus (Eichh.)[c]	X-pane	1–5	3	(100)	1.78±1.31	3.28
Coleoptera: Curculionidae						
Balanogastris kolae (Desbr.)[d]	B-St-card	0.4–2.2	4	701	0.68±0.44	1.11
Anthonomus grandis Boheman[e]	Y-St-X-pane-P	0–9.1	6	22,310	1.65±1.96	4.91
Hylobius pales (Herbst.)[c]	X-pane	1–5	3	(100)	2.24±1.66	4.17
Coleoptera: Platypodidae						
Platypus compositus Say[c]	X-pane	1–5	3	(100)	1.67±1.07	2.69
Coleoptera: Scarabaeidae						
Phyllophaga crinita Burmeister[f]	St-card-UV	0.15–2.28	15	3,244	0.68±0.59	1.49
Hoplia equina LeConte[g]	X-pane-P	0.2–1	3	852	0.35±0.25	0.62
Coleoptera: Chrysomelidae						
Diabrotica virgifera LeConte[h]	Y- St-box	3.05–7.62	4	4,528	4.12±1.44	3.60
Trirhabda virgata LeConte[i]	W- St-card	1–4	4	377	1.75±1.00	2.51
Phyllotreta cruciferae (Goeze)[j]	Y- St-card	0.5–7	8	173	2.68±1.82	4.57
Coleoptera: Elateridae						
Ctenicera appropinquans (Randall)[k]	Y–X-pane	0.8–14.3	10	115	5.40±3.83	9.59
Ctenicera pulchra (LeConte)[k]	Y–X-pane	0.8–14.3	10	56	5.14±3.89	9.74
Ctenicera tarsalis (Melsheimer)[k]	Y–X-pane	0.8–14.3	10	74	3.88±3.07	7.70
Melanotus similes (Kirby)[k]	Y–X-pane	0.8–14.3	10	97	2.50±1.77	4.43
Sericus brunneus (L.)[k]	Y–X-pane	0.8–14.3	10	118	6.56±3.72	9.31
Lepidoptera: Geometridae						
Idaea squamipunctata Warren[l]	R-light	1–30	3	529	18.02±11.13	27.90
Hypomecis costaria Guenée[l]	R-light	1–30	3	252	2.17±3.88	9.72
Ornithospila avicularia Guenée[l]	R-light	1–30	3	85	8.98±8.93	22.38
Godonela avitusaria Walker[l]	R-light	1–30	3	63	9.38±10.86	27.23
Hypomecis tetragonata Walker[l]	R-light	1–30	3	37	10.54±8.94	22.42
Lepidoptera: Nymphalidae						
Heliconius numata Cramer males[m]	B-fly-net	–	–	90	2.30±0.95	2.38
Heliconius numata Cramer females[m]	B-fly-net	–	–	34	1.09±0.64	1.61
Hemiptera: Cicadellidae						
Circulifer tenellus (Baker) males[n]	Y-St-card	0.11–2.81	7	2,905	0.17±0.27	0.67
Circulifer tenellus (Baker) females[n]	Y-St-card	0.11–2.81	7	657	0.23±0.35	0.89
Scaphytopius magdalensis (Provancher)[o]	Y-St-card	0.12–1.82	8	(100)	0.30±0.19	0.48
Hemiptera: Aleyrodidae						
Parabemisia myricae (Kuwana)[p]	Y-St-card	0.76–6.1	8	5,445	1.93±1.42	3.55
Bemisia tabaci Gennadius males[q]	W-St-card	0.16–7.36	4	258	1.09±1.82	4.55
Bemisia tabaci Gennadius females[q]	W-St-card	0.16–7.36	4	265	1.23±1.89	4.73

Table 3 (continued)

Species	Trapping method	Range of trap heights	Number of trap heights	Total catch[a]	Mean height of catch ±SD	F_L (m)
Hemiptera: Miridae						
Lygus lineolaris (P. de Beauvois) males[r]	W-St-card	0.6–3.05	3	1,780	0.93±0.66	1.66
Lygus lineolaris (P. de Beauvois) females[r]	W-St-card	0.6–3.05	3	1,396	0.99±0.71	1.79
Thysanoptera: Thripidae						
Frankliniella occidentalis (Pergande)[s]	Y-St-card	0.25–2.5	5	(1,000)	1.14±0.70	1.76
Diptera: Agromyzidae						
Liriomyza trifolii (Burgess) fall[t]	Y-St-card	0.3–1.5	5	(100)	0.55±0.39	0.99
Liriomyza trifolii (Burgess) spring[t]	Y-St-card	0.3–1.5	5	(100)	0.50±0.27	0.69

St-screen Sticky screen; *X-pane* cross window panes; *B-St-card* brown sticky card; *Y-St-X-pane-P* yellow sticky cross panes with pheromone bait; *St-card-UV* sticky card with UV light; *X-pane-P* cross window panes with pheromone bait; *Y-St-box* yellow sticky box; *W-St-card* white sticky card; *Y-St-card* yellow sticky yellow card; *Y-X-pane* yellow cross panes; *R-light* Rothamsted light trap; *B-fly-net* butterfly net pole

[a] Trap catch reported as proportions so catch in parentheses was assumed in order to calculate mean height of catch and variation.

[b] Byers et al. 1989

[c] Atkinson et al. 1988

[d] Ivbijaro and Daramola 1977

[e] Rummel et al. 1977

[f] Stone 1986

[g] Weber et al. 2005

[h] VanWoerkom et al. 1983

[i] Messina 1982

[j] Lamb 1983

[k] Boiteau et al. 2000

[l] Intachat and Holloway 2000

[m] Joron 2005

[n] Meyerdirk and Oldfield 1985

[o] Meyer and Colvin 1985

[p] Meyerdirk and Moreno 1984

[q] Isaacs and Byrne 1998

[r] Stewart and Gaylor 1991

[s] Pearsall and Myers 2001

[t] Chandler 1985.

in the field is that its radius is a sphere and extends vertically and horizontally in three dimensions and often would not fill the flight layer or could theoretically protrude above the flight layer. Byers (2007) used a circular EAR in simulation models of mating disruption and mass trapping and implied that field-measured EAR could be used to predict catch rates. However, in the present work, I found that the spherical EAR must be transformed into the circular EAR_c for appropriate prediction due to the effect of the effective flight layer, F_L, which varies among species. It is simpler to model insect search in two dimensions because they fly over wide areas in essentially a two-dimensional layer of a few meters thickness. However, if the passive trap is a tall cylinder that extends vertically through the flight layer, then the EAR_c of Eq. 5 can be measured in nature. In this case, the interception is proportional to the radius of the cylinder in simulations with CP=1 (Fig. 1c) or as a circle of EAR_c.

Byers et al. (1989) measured EAR_c in the field with several 12-m columns of sticky cylinder traps baited or not with attractants for insects. In this study, the flight layer was sampled throughout by the sticky traps whose catch was multiplied by 4 to account for gaps in coverage. Therefore, the EAR_c of the aggregation pheromone baits of the bark beetle *Ips typographus* (L.) is simply the ratio of catches times the passive trap radius (0.7 m, Table 4). These ten baits were released at one point in another study (Schlyter et al. 1987) that gave an EAR of 1.9 m, which, assuming an F_L of 8 m, gives an identical EAR_c of 0.7 m (Eq. 6). Other F_L estimates would alter the calculated EAR_c. For example, the estimate of F_L for *I. typographus* from flight height data (Table 3) was 6.9 m, giving an EAR_c of 0.82 m. Equation 6 can be used to convert the EAR to EAR_c if a flight layer is estimated from observations or trap catches at various heights. By using data from Byers et al. (1989) for two species of bark beetle caught on baits of host tree monoterpenes or aggregation pheromone components, the two types of EAR calculations are compared (Table 4). In these cases, the EAR_c was smaller than the spherical EAR because of the size of the

Table 4 EAR and EAR_c of monoterpene or aggregation pheromone component release rates attractive to bark beetles, *Tomicus piniperda* and *Ips typographus*, respectively, based on passive and active catches on cylindrical sticky trap screens and effective flight layer estimate (from Table 3)

Test	Insect catch		Passive trap interception area (m^2)	EAR sphere (m)	Flight Layer estimate (m)	EAR_c (m)
	Passive	Active				
Tomicus piniperda						
Scots pine log (1982)[a]	52	623	0.06	0.59	7.53	0.048
Log + 30 males + 30 females[a]	52	774	0.06	0.65	7.53	0.059
(+)-3-Carene[a]	7	48	0.06	0.44	7.53	0.027
(+)-α-Pinene[a]	7	60	0.06	0.50	7.53	0.034
(−)-α-Pinene[a]	7	79	0.06	0.57	7.53	0.045
Terpinolene[a]	7	104	0.06	0.65	7.53	0.059
Scots pine log (1983)[a]	7	256	0.06	1.02	7.53	0.146
April 15, Monoterpenes[b]	2.33	27	0.09	0.58	7.53	0.069
April 21, Monoterpenes[b]	1	19	0.09	0.74	7.53	0.114
Ips typographus						
High release pheromone[c]	6	753	0.06	1.90	6.9	0.546
Medium release pheromone[d]	7	80	0.06	0.57	6.9	0.050
May 17, Pheromone[e]	5.67	194	0.09	0.99	6.9	0.223
May 19, Pheromone[e]	1.33	18	0.09	0.62	6.9	0.088
May 20, Pheromone[e]	16.67	269	0.09	0.67	6.9	0.105
May 21, Pheromone[e]	7.5	44	0.09	0.41	6.9	0.038
May 22, Pheromone[e]	8.5	215	0.09	0.85	6.9	0.165

[a] Released about 30 mg each compound per day; Scots pine, *Pinus sylvestris* L.; Byers et al. 1985.
[b] Released about 10–20 mg/day of (+)-α-pinene, (−)-α-pinene, and (+)-3-carene, and 3–5 mg/day of terpinolene; Byers et al. 1989.
[c] High release of 57 mg 2-methyl-3-buten-2-ol (MB) per day and 1 mg (1S,4S,5S)-cis-verbenol (cV) per day; Schlyter et al. 1987.
[d] Medium release: 5.8 mg MB per day and 1 mg cV per day; Schlyter et al. 1987.
[e] Byers et al. 1989, medium release MB and cV.

EAR and estimated F_L. However, the EAR_c can be larger than the EAR (Fig. 4), where both the F_L and the size of the EAR as a function of EAR^2 affect the EAR_c.

The SD° of turning angles usually does not affect encounter rates of insects with mates, host plants, or pheromone traps because the distances of insect search are far greater than distances to these objects at natural densities (Byers 1991, 2007). However, the SD° does influence the encounter rate of insects that must travel relatively far in proportion to their allotted search distance to encounter an object at low density (Table 2). Surprisingly, a straight path (SD°=0°) caused insects to less often encounter an object than slightly more sinuous tracks (SD°=5°), while highly sinuous tracks (e.g., SD°=30°) caused significantly fewer encounters. Short travel distances did not cause many encounters when insects began anywhere at random. This was because many insects could simply not reach the single object no matter how they traveled, as their search distance was less than their initial distance from the object. At larger search distances, insects could intercept the object by going straight but would have only one chance that was proportional to the object's diameter and initial distance because usually the insect could not return once it "missed" the object (only if meeting area's boundary). However, with a slightly more sinuous path, the insect had the same probability of intercepting the object, but there was an additional small probability that it could miss and then return to find the object due to the ability to turn (SD°>0°). At still higher SD°, many turns would tend to prevent many insects placed relatively far from the object from reaching the object, even though a few placed nearer the object could turn back and have one or more chances of interception. This means that predators and parasitoids that land on a leaf and search for insect hosts would benefit if they evolved a small but sufficient SD° of about 5° to cause more frequent host encounters than if they used a straight path or a highly sinuous path.

Estimation of the mean flight height and SD is done easily from either proportions or numbers caught on trap heights, although rarely are they calculated. Appropriate calculation of the SD assumes that the distribution is approximately normal and that catch does not continue to increase with height. The number of trap levels increases the accuracy and confidence of the calculated values. The type of traps used to monitor flight height should not matter as long as they are the same and catch a correct proportion of flying insects at the respective heights. However, many previous studies (Table 3) have not used passive traps but rather attractive traps (yellow color, ultraviolet lights, and sex/aggregation pheromone) that might alter the natural flight patterns. For example, Byers et al. (1989) found that sticky traps baited with aggregation pheromone or host plant monoterpenes altered the pattern of catch with height for two

species of bark beetle compared to passive sticky traps. The mean flight height for *I. typographus* on passive traps was 4.63±2.75 (±SD) and F_L=6.89, while for the pine shoot beetle, *Tomicus piniperda* (L.), mean height was 5.98±3.00 and F_L=7.52. However, when aggregation pheromone was released at each height, *I. typographus* had a mean flight height of only 1.50±1.63 and F_L=4.08 (N=740), while *T. piniperda* attraction to host tree monoterpenes altered its mean flight height to 2.90±2.77 and F_L=6.95 (N=48). An estimation of the EAR from published results is usually not possible because (a) few studies have used unattractive sticky traps to intercept flying insects in comparison with attractive traps, or (b) the blank traps did not catch insects. For example, most moth pheromone studies (Pherobase: El-Sayed 2007) have used traps where moths must enter to be caught (e.g., Delta traps), and so few were caught on blank traps (causing the EAR to be undefined).

The EAR_c can be used to predict mating disruption and mass trapping with competitive attraction and camouflage by modeling male moth search, female calling, moth and dispenser densities, and EAR_c of females and dispensers (Byers 2007, unpublished). However, many of these parameters are difficult to quantify in nature, such as moth density and male search distance. Other parameters, although usually not measured, can be estimated more easily such as EAR of females and dispensers by using catch ratios on sticky traps and converting to EAR_c with F_L estimated from sticky trap catches at fixed heights. The estimated EAR_c of 0.82 m discussed above for a standard *I. typographus* trap can be used in the model of Byers (2007) with no competition (female EAR=0) to determine the catch of these bark beetles. For example, if five of these traps are placed in a 200×200 m Norway spruce forest with 360 beetles (Byers et al. 1989) flying at 2 m/s for up to 6 km, then 99% are expected to be caught. This assumes there are no pheromone sources of competitive attraction such as might occur shortly after flight initiation in the spring. Models can predict mass trapping and mating disruption outcomes correctly only when the relevant parameters are known with good precision. Otherwise, models are still useful to gain better understanding of the effects of the parameters on decisions regarding control and the likely efficacy of deploying pheromone dispensers of various numbers and EAR_c.

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References

ATKINSON, T. H., FOLTZ, J. L., and CONNOR, M. D. 1988. Flight patterns of phloem- and wood-boring Coleoptera (Scolytidae, Platypodidae, Curculionidae, Buprestidae, Cerambycidae) in a north Florida slash pine plantation. *Environ. Entomol.* 17:259–265.

BAKER, T. C., and ROELOFS, W. L. 1981. Initiation and termination of oriental fruit moth male response to pheromone concentrations in the field. *Environ. Entomol.* 10:211–218.

BAKER, T. C., HANSSON, B. S., LÖFSTEDT, C., and LÖFQVIST, J. 1989. Adaptation of male moth antennal neurons in a pheromone plume is associated with cessation of pheromone-mediated flight. *Chem. Senses* 14:439–448.

BAKER, T. C., FADAMIRO, H., and COSSÉ, A. A. 1998. Fine-grained resolution of closely spaced odor strands by flying male moths. *Nature* 393:530.

BARTELL, R. J. 1982. Mechanisms of communication disruption by pheromones in control of Lepidoptera: a review. *Physiol. Entomol.* 7:353–364.

BARTELL, R. J., and ROELOFS, W. L. 1973. Inhibition of sexual response in males of the moth *Argyrotaenia velutinana* by brief exposures to synthetic pheromone or its geometric isomer. *J. Insect Physiol.* 19:655–661.

BOITEAU, G., BOUSQUET, Y., and OSBORN, W. 2000. Vertical and temporal distribution of Carabidae and Elateridae in flight above an agricultural landscape. *Environ. Entomol.* 29:1157–1163.

BOSSERT, W. H., and WILSON, E. O. 1963. The analysis of olfactory communication among animals. *J. Theor. Biol.* 5:443–469.

BRANCO, M., JACTEL, H., FRANCO, J. C., and MENDEL, Z. 2006. Modelling response of insect trap captures to pheromone dose. *Ecol. Model.* 197:247–257.

BYERS, J. A. 1987. Interactions of pheromone component odor plumes of western pine beetle. *J. Chem. Ecol.* 13:2143–2157.

BYERS, J. A. 1991. Simulation of mate-finding behaviour of pine shoot beetles, *Tomicus piniperda*. *Anim. Behav.* 41:649–660.

BYERS, J. A. 1993a. Simulation and equation models of insect population control by pheromone-baited traps. *J. Chem. Ecol.* 19:1939–1956.

BYERS, J. A. 1993b. Orientation of bark beetles *Pityogenes chalcographus* and *Ips typographus* to pheromone-baited puddle traps placed in grids: A new trap for control of scolytids. *J. Chem. Ecol.* 19:2297–2316.

BYERS, J. A. 1996a. An encounter rate model for bark beetle populations searching at random for susceptible host trees. *Ecol. Model.* 91:57–66.

BYERS, J. A. 1996b. Temporal clumping of bark beetle arrival at pheromone traps: Modeling anemotaxis in chaotic plumes. *J. Chem. Ecol.* 22:2133–2155.

BYERS, J. A. 1999. Effects of attraction radius and flight paths on catch of scolytid beetles dispersing outward through rings of pheromone traps. *J. Chem. Ecol.* 25:985–1005.

BYERS, J. A. 2001. Correlated random walk equations of animal dispersal resolved by simulation. *Ecology* 82:1680–1690.

BYERS, J. A. 2007. Simulation of mating disruption and mass trapping with competitive attraction and camouflage. *Environ. Entomol.* 36:1328–1338.

BYERS, J. A., ANDERBRANT, O., and LÖFQVIST, J. 1989. Effective attraction radius: A method for comparing species attractants and determining densities of flying insects. *J. Chem. Ecol.* 15:749–765.

BYERS, J. A., LANNE, B. S., LÖFQVIST, J., SCHLYTER, F., and BERGSTRÖM, G. 1985. Olfactory recognition of host-tree susceptibility by pine shoot beetles. *Naturwissenschaften* 72:324–326.

CARDÉ, R. T. 1990. Principles of mating disruption, pp. 47–71, in R. L. Ridgway, and R. M. Silverstein (eds.). Behavior-Modifying Chemicals for Pest Management: Applications of Pheromones and other Attractants. Marcel Dekker, New York.

CARDÉ, R. T., and MINKS, A. K. 1995. Control of moth pests by mating disruption: successes and constraints. *Annu. Rev. Entomol.* 40:559–585.

CHANDLER, L. D. 1985. Flight activity of *Liriomyza trifolii* (Diptera: Agromyzidae) in relationship to placement of yellow traps in bell pepper. *J. Econ. Entomol.* 78:825–828.

EL-SAYED, A. M. 2007. The Pherobase: database of insect pheromones and semiochemicals. (http://www.pherobase.com).

EL-SAYED, A. M., SUCKLING, D. M., WEARING, C. H., and BYERS, J. A. 2006. Potential of mass trapping for long-term pest management and eradication of invasive species. *J. Econ. Entomol.* 99:1550–1564.

ELKINTON, J. S., and CARDÉ, R. T. 1984. Odor dispersion, pp. 73–91, in W. J. Bell, and R. T. Cardé (eds.). Chemical Ecology of Insects. Sinauer Associates, Sunderland.

ELKINTON, J. S., CARDÉ, R. T., and MASON, C. J. 1984. Evaluation of time-averaged dispersion models for estimating pheromone concentration in a deciduous forest. *J. Chem. Ecol.* 10:1081–1108.

FARES, Y., SHARPE, P. J. H., and MAGNUSON, C. E. 1980. Pheromone dispersion in forests. *J. Theor. Biol.* 84:335–359.

HOLLING, C. S. 1959. Some characteristics of simple types of predation and parasitism. *Can. Entomol.* 91:385–398.

INTACHAT, J., and HOLLOWAY, J. D. 2000. Is there stratification in diversity or preferred flight height of geometroid moths in Malaysian lowland tropical forest? *Biodiv. Conserv.* 9:1417–1439.

ISAACS, R., and BYRNE, D. N. 1998. Aerial distribution, flight behaviour and eggload: their inter-relationship during dispersal by the sweetpotato whitefly. *J. Anim. Ecol.* 67:741–750.

IVBIJARO, M. F., and DARAMOLA, A. M. 1977. Flight activity of the adult kola weevil, *Balanogastris kolae* (Coleoptera: Curculionidae) in relation to infestation. *Entomol. Exp. Appl.* 22:203–207.

JORON, M. 2005. Polymorphic mimicry, microhabitat use, and sex-specific behaviour. *J. Evol. Biol.* 18:547–556.

JUDD, G. J. R., GARDINER, M. G. T., DELURY, N. C., and KARG, G. 2005. Reduced antennal sensitivity, behavioural response, and attraction of male codling moths, *Cydia pomonella*, to their pheromone (*E*,*E*)-8,10-dodecadien-1-ol following various pre-exposure regimes. *Entomol. Exp. Appl.* 114:65–78.

KUENEN, L. P. S., and BAKER, T. C. 1981. Habituation versus sensory adaptation as the cause of reduced attraction following pulsed and constant sex pheromone pre-exposure in *Trichoplusia ni*. *J. Insect Physiol.* 27:721–726.

LAMB, R. J. 1983. Phenology of flea beetle (Coleoptera: Chrysomelidae) flight in relation to their invasion of canola fields in Manitoba. *Can. Entomol.* 115:1493–1502.

MANKIN, R. W., VICK, K. W., MAYER, M. S., COFFELT, J. A., and CALLAHAN, P. S. 1980. Models for dispersal of vapors in open and confined spaces: Applications to sex pheromone trapping in a warehouse. *J. Chem. Ecol.* 6:929–950.

MASON, L. J., JANSSON, R. K., and HEATH, R. R. 1990. Sampling range of male sweetpotato weevils (*Cylas formicarius elegantulus*) (Summers) (Coleoptera: Curculionidae) to pheromone traps: Influence of pheromone dosage and lure age. *J. Chem. Ecol.* 16:2493–2502.

MC CALL, R. B. 1970. Fundamental Statistics for Psychology. Harcourt, Brace and World, New York.

MC CLENDON, R. W., MITCHELL, E. B., JONES, J. W., MC KINION, J. M., and HARDEE, D. D. 1976. Computer simulation of pheromone trapping systems as applied to boll weevil population suppression: a theoretical example. *Environ. Entomol.* 5:799–806.

MESSINA, F. J. 1982. Timing of dispersal and ovarian development in goldenrod leaf beetles *Trirhabda virgata* and *T. borealis*. *Ann. Entomol. Soc. Am.* 74:78–83.

MEYER, J. R., and COLVIN, S. A. 1985. Diel periodicity and trap bias in sticky trap sampling of sharpnosed leafhopper populations. *J. Entomol. Sci.* 20:237–243.

MEYERDIRK, D. E., and MORENO, D. S. 1984. Flight behavior and color-trap preference of *Parabemisis myricae* (Kuwana) (Homoptera: Aleyrodidae) in a citrus orchard. *Environ. Entomol.* 13:167–170.

MEYERDIRK, D. E., and OLDFIELD, G. N. 1985. Evaluation of trap color and height placement for monitoring *Circulifer tenellus* (Baker)(Homoptera: Cicadellidae). *Can. Entomol.* 117:505–511.

MILLER, J. R., GUT, L. J., DE LAME, F. M., and STELINSKI, L. L. 2006a. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 1): Theory. *J. Chem. Ecol.* 32:2089–2114.

MILLER, J. R., GUT, L. J., DE LAME, F. M., and STELINSKI, L. L. 2006b. Differentiation of competitive vs. non-competitive mechanisms mediating disruption of moth sexual communication by point sources of sex pheromone (Part 2): Case Studies. *J. Chem. Ecol.* 32:2115–2143.

NAKAMURA, K., and KAWASAKI, K. 1977. The active space of the *Spodoptera litura* (F.) sex pheromone and the pheromone component determining this space. *Appl. Entomol. Zool.* 12:162–177.

ÖSTRAND, F., and ANDERBRANT, O. 2003. From where are insects recruited? A new model to interpret catches of attractive traps. *Agricul. Forest Entomol.* 5:163–171.

PEARSALL, I. A., and MYERS, J. H. 2001. Spatial and temporal patterns of dispersal of western flower thrips (Thysanoptera: Thripidae) in nectarine orchards in British Columbia. *J. Econ. Entomol.* 94:831–843.

RUMBO, E. R., and VICKERS, R. A. 1997. Prolonged adaptation as possible mating disruption mechanism in oriental fruit moth, *Cydia* (=*Grapholita*) *molesta*. *J. Chem. Ecol.* 23:445–457.

RUMMEL, D. R., JORDAN, L. B., WHITE, J. R., and WADE, L. J. 1977. Seasonal variation in the height of boll weevil flight. *Environ. Entomol.* 6:674–678.

SCHLYTER, F., BYERS, J. A., and LÖFQVIST, J. 1987. Attraction to pheromone sources of different quantity, quality, and spacing: Density-regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol.* 13:1503–1523.

SHOREY, H. H. 1977. Manipulation of insect pests of agricultural crops, pp. 353–367, in H. H. Shorey, and J. J. McKelvey Jr. (eds.). Chemical Control of Insect Behaviour: Theory and Application. Wiley, New York.

SOWER, L. L., GASTON, L. K., and SHOREY, H. H. 1971. Sex pheromones of noctuid moths. XXVI. Female release rate, male response threshold, and communication distance for *Trichoplusia ni*. *Ann. Ent. Soc. Am.* 64:1448–1456.

STEWART, S. D., and GAYLOR, M. J. 1991. Age, sex, and reproductive status of the tarnished plant bug (Heteroptera: Miridae) colonizing mustard. *Environ. Entomol.* 20:1387–1392.

STONE, J. D. 1986. Time and height of flight of adults of white grubs (Coleoptera: Scarabaeidae) in the southwestern United States. *Environ. Entomol.* 15:194–197.

SUTTON, O. G. 1953. Micrometeorology. McGraw-Hill, New York.

TURCHIN, P. 1998. Quantitative Analysis of Movement. Sinauer Associates, Sunderland.

TURCHIN, P., and ODENDAAL, F. J. 1996. Measuring the effective sampling area of a pheromone trap for monitoring population density of southern pine beetle (Coleoptera: Scolytidae). *Environ. Entomol.* 25:582–588.

VANWOERKOM, G. J., TURPIN, F. T., and BARRETT, J. R. Jr. 1983. Wind effect on western corn rootworm (Coleoptera: Chrysomelidae) flight behavior. *Environ. Entomol.* 12:196–200.

WALL, C., and PERRY, J. N. 1987. Range of action of moth sex-attractant sources. *Entomol. Exp. Appl.* 44:5–14.

WEBER, D. C., ROBBINS, P. S., and AVERILL, A. L. 2005. *Hoplia equina* (Coleoptera: Scarabaeidae) and nontarget capture using 2-tetradecanone-baited traps. *Environ. Entomol.* 34:158–163.

WORNER, S. P. 1991. Use of models in applied entomology: the need for perspective. *Environ. Entomol.* 20:768–773.

ZOLUBAS, P., and BYERS, J. A. 1995. Recapture of dispersing bark beetle, *Ips typographus* L. (Col., Scolytidae) in pheromone-baited traps: regression models. *J. Appl. Entomol.* 119:285–289.

In a recent study, we found that juvenile aphids reared in social isolation on artificial diet release less EβF than those reared in a colony or in isolation but exposed to colony odors (Verheggen et al., unpublished). We suggested that aphid volatiles may stimulate other aphids to produce EβF. In this study, we tested this by stimulating release of EβF from aphids and measuring whether additional EβF is released by individuals that perceive these volatiles. Such a contagious phenomenon could be adaptive if there are benefits to disseminating the alarm farther than would be achieved by the release of EβF by an individual.

Methods and Materials

Insects and Plants Pea aphids were reared on broad beans, *Vicia faba*, in an environmentally controlled greenhouse (L16:D8, RH 35±5%, 25±2°C) for several months prior to the experiments. Plants were grown in 9×9 cm plastic pots, filled with a peat-based, general-purpose potting soil (Metro Mix 200 Series, SunGrow Agriculture Distribution Inc., Bellevue, WA, USA).

Push–pull Headspace Collection System The push–pull headspace collection system consisted of two cylindrical chambers (12 cm diam.×30 cm) made of glass and Teflon®. Chambers were connected to one another with Teflon® tubing. To maintain ambient humidity and normal atmospheric pressure within the chambers, activated-carbon-filtered air was pumped into the system at the same rate that air was removed via air-entrainment filters, in a manner consistent with push–pull headspace collection setups described elsewhere (e.g., Tholl et al. 2006).

To generate natural EβF emissions, we crushed 50 third instar aphids inside the collection chambers with a glass pestle, which along with the aphids was left inside the chamber after crushing. To quantify EβF produced by the crushed (lead) and undisturbed (downstream) aphids, an adsorbent filter, containing 40 mg of SuperQ® (Alltech, Deerfield, IL, USA), was connected to each chamber. Clean air was pushed into the system at a rate of 1.5 L min^{-1} and sampled air was pulled through the filters from both the lead and downstream chambers at a rate of 0.75 L min^{-1} per chamber. Five experiments were conducted; each of 1 h with nine replicates (Table 1). The first experiment (crushed–empty) was a positive control designed to document the distribution of EβF, released upwind, in the two chambers. The second experiment (empty–infested) measured the amount of EβF released by a colony of 50 *A. pisum* under the laboratory conditions. The third (empty–non-infested) and fifth (crushed–non-infested) experiments were, respectively, controls devoted to the evaluation of the potential amount of EβF that could be released from an uninfested broad bean plant unexposed or exposed to EβF. The fourth experiment (crushed–infested) was conducted to show whether downstream aphids emit additional alarm signal when exposed to an alarm signal from upstream conspecifics.

Volatile Analysis Filters were eluted with 150 μl of dichloromethane and nonyl acetate (320 ng) was added to each sample as an internal standard. Extracts were analyzed by gas chromatography with flame ionization detection with a Hewlett–Packard 6890 series gas chromatograph equipped with a splitless injector (260°C). The column (Equity-1, Supelco, Bellefonte, PA, USA, 30 m×0.25 mm i.d.) was held at 40°C for 1 min before being heated to 260°C at 15°C min^{-1}. Quantification of compounds was carried out by comparing individual peak areas to that of the internal standard. Identification of EβF was made by comparison of its retention time with that of synthetic EβF (Bedoukian Research, Inc., Danbury, CT, USA) and confirmed by gas chromatography-mass spectrometry.

Table 1 Mean (*E*)-β-farnesene (EβF) emission amounts released by *Acyrthosiphon pisum* and detected in lead and downstream chambers, as well as average lead/downstream EβF ratios (±SE), in different experiments

Number	Lead chamber	Downstream chamber	Average EβF amounts (ng±SE)[d]		Average downstream/lead EβF ratios (±SE)[d]
			Lead chamber	Downstream chamber	
1	Crushed aphids[a]	Empty	1,295.74±261.43	1,130.25±148.87	1.056±0.190
2	Empty	Infested plant[b]	–	–	–
3	Empty	Non-infested plant[c]	–	–	–
4	Crushed aphids	Infested plant	1,585.06±288.37	957.69±153.83	0.769±0.094
5	Crushed aphids	Non-infested plant	1,384.22±275.00	1,048.26±133.65	0.859±0.113

[a] 50 crushed third Instar larvae *A. pisum*
[b] Single 20-cm-high *Vicia faba* infested with 50 third Instar *A. pisum*
[c] Single 20-cm-high non-infested *V. faba*
[d] Nine replicates were performed for each experiment

Results and Discussion

EβF was the only volatile released by *A. pisum* detected in our experiments, which is consistent with previous findings (Francis et al. 2005). In experiment one (crushed–empty), third instar *A. pisum* released an average (sum of upstream and downstream collections) of 48.5 ng of EβF per individual. The higher EβF levels observed in our study, compared to those found by Mondor et al. (2000) and Schwartzberg et al. (2008), may be explained by differences in eliciting EβF release (crushed vs. probed or natural attack). These EβF amounts are larger than what we might expect to see in a natural condition. However, within a colony, signaling and receiving aphids are much closer to each other than in our experiment and, therefore, the higher release rate in our experiment may compensate for this. Moreover, a higher release rate should be advantageous for testing our hypothesis.

The ratio of downstream aphid to lead aphid emission should be 1.0 if no additional EβF were produced from aphids in the downstream chamber. A ratio greater than 1.0 should, therefore, reflect emission of EβF from aphid/host plant complexes subjected to the alarm signal. No EβF was emitted from downstream plant and plant/aphid complexes in experiments with empty lead chambers (Table 1, experiment 2 [empty–infested] and 3 [empty–non-infested]). These data confirm that undisturbed aphids under the conditions of this experiment do not produce a detectable alarm signal. EβF was detected in experiments 1 (crushed–empty), 4 (crushed–infested), and 5 (crushed–non-infested). However, there was no difference in EβF ratios among these experiments (ANOVA, $F_{2,24}$=1.12, P=0.342). Although not significant, the small reduction in ratio in experiment 1 compared to experiments 4 and 5, may have been due to absorption of some EβF by the downstream plants used in the latter experiments. Interestingly, in experiment 4, the downstream aphids appeared to perceive EβF as 14% of these aphids dropped from their host plant.

These results indicate that the EβF alarm signal is not contagious. This conclusion is consistent with further observations that the amount of EβF released by a single aphid under attack is similar to the average amount of alarm pheromone released per consumed aphid in a colony (Schwartzberg et al., unpublished).

An understanding of how alarm pheromone is emitted in a natural setting may be important when studying the effects of alarm signaling among aphids, since a single alarm signal can influence aphid ecology through both inter- and intra-specific signaling. The way that such signals convey information in an aphid colony may be important for the effectiveness of a signaling alarm, as well as in reducing the costs of signal production in an environment in which signal eavesdropping by predators can add a fitness cost to signal production.

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References

Beale, M. H., Birkett, M. A., Bruce, T. J. A., Chamberlain, K., Field, L. M., Huttly, A. K., Martin, J. L., Parker, R., Phillips, A. L., Pickett, J. A., Prosser, I. M., Shewry, P. R., Smart, L. E., Wadhams, L. J., Woodcock, C. M., and Zhang, Y. 2006. Aphid alarm pheromone produced by transgenic plants affect aphid and parasitoid behavior. *Proc. Natl. Acad. Sci. USA* 103:10509–10513.

Braendle, C., and Weisser, W. W. 2001. Variation in escape behavior of red and green clones of the pea aphid. *J. Insect Behav.* 14:497–509.

Francis, F., Vandermoten, S., Verheggen, F., Lognay, G., and Haubruge, E. 2005. Is the (*E*)-β-farnesene only volatile terpenoid in aphids? *J. Appl. Entomol.* 129:6–11.

Mondor, E. B., Baird, D. S., and Slessor, K. N. 2000. Ontogeny of alarm pheromone secretion in pea aphid, *Acyrthosiphon pisum*. *J. Chem. Ecol.* 26:2875–2882.

Pickett, J. A., and Glinwood, R. T. 2007. Chemical Ecology. In H. van Emden, and R. Harrington (eds.). Aphids as Crop Pests, pp. 235–260. CAB International, Wallingford.

Schwartzberg, E. G., Kunert, G., Stephan, C., David, A., Röse, U. S. R., Gershenzon, J., Boland, W., and Weisser, W. W. 2008. Real-time analysis of alarm pheromone emission by the pea aphid (*Acyrthosiphon pisum*) under predation. *J. Chem. Ecol.* 34:76–81.

Tholl, D., Boland, W., Hansel, A., Loreto, F., Röse, U. S. R., and Schnitzler, J. P. 2006. Practical approaches to plant volatile analysis. *Plant J.* 45:540–560.

Verheggen, F., Ryne, C., Olsson, P. O. C., Arnaud, L., Lognay, G., Hogberg, H. E., Persson, D., Haubruge, E., and Lofstedt, C. 2007a. Electrophysiological and behavioral activity of secondary metabolites in the confused flour beetle, *Tribolium confusum*. *J. Chem. Ecol.* 33:525–539.

Verheggen, F. J., Fagel, Q., Heuskin, S., Lognay, G., Francis, F., and Haubruge, E. 2007b. Electrophysiological and behavioral responses of the multicolored asian Lady Beetle, *Harmonia axyridis* Pallas, to sesquiterpene semiochemicals. *J. Chem. Ecol.* 33:2148–2155.

Verheggen, F. J., Arnaud, L., Bartram, S., Gohy, M., and Haubruge, E. 2008. Aphid and plant secondary metabolites induce oviposition in an aphidophagous hoverfly. *J. Chem. Ecol.* 34:301–307.

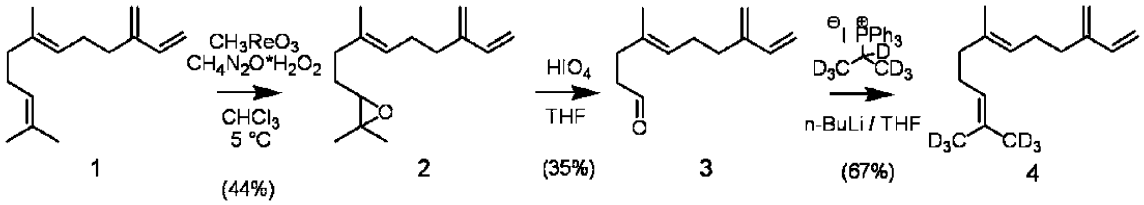

Fig. 1 Synthesis of [12,12,12,13,13,13]-$^2H_6$-(*E*)-β-farnesene

(DEBF), labeled on the geminal dimethyl group, to investigate signal reinforcement in the pea aphid.

Methods and Materials

Synthesis of Deuterated EBF Following Cravotto et al. (2004) (Fig. 1), epoxidation of (*E*)-β-farnesene (**1**) (408.70 mg, 2 mmol), employing methyltrioxorhenium (VII) and urea-hydrogen peroxide adduct in $CHCl_3$ at 5°C, gave a mixture of mono- and di-oxiranes. The 10,11-epoxy-(*E*)-β-farnesene (**2**) (180.3 mg, 44%, R_f 0.48) was separated on Florisil 100–200 (petroleum ether 40–60/ether 40:1 *v*/*v*). As specified by Fielder and Rowan (1994) (Fig. 1), the epoxide **2** was cleaved with periodic acid to give (*E*)-4-methyl-8-methylenedeca-4,9-dienal (**3**) (49.1 mg, 35%, R_f 0.27), which was purified on silica 60 (petroleum ether 40–60/ether 40:1 *v*/*v*). Wittig reaction of **3** with d_7-isopropyltriphenylphosphonium iodide produced the d_6-(*E*)-β-farnesene (**4**) ([12,12,12,13,13,13]-$^2H_6$-(*E*)-β-farnesene; DEBF) (221.60 µg, 67%, R_f 0.60). Purification of **4** was carried out by flash chromatography on silica gel (silica 60, Merck, Darmstadt, Germany) with pentane. The chemical purity of DEBF after chromatographic purification was 97% as determined by gas chromatography-mass spectrometry (GC-MS).

NMR Data of Deuterated EBF ^{1}H NMR (500 MHz, $CDCl_3$): δ (ppm)=1.53 (s, 3H), 1.89–1.94 (m, 2H), 1.97–2.03 (m, 2H), 2.08–2.19 (m, 4H), 4.92 (d, *J*=1.3 Hz, 1H), 4.94 (d, *J*=1.3 Hz, 1H), 4.98 (d, *J*=10.8 Hz, 1H), 5.03 (t, *J*=6.8 Hz, 1H), 5.09 (tq, *J*=6.8, 1.0 Hz, 1H), 5.17 (d, *J*=17.7 Hz, 1H), 6.31 (dd, *J*=17.7, 10.8 Hz, 1H).

Aphid Lines We used a red clone (BP) of the pea aphid, *A. pisum*, maintained on *Vicia faba* plants in controlled conditions at 20°C, 16L/8D photoperiod, and approximately 75% RH. For experiments, aphid lines descended from a single founder were established (Kunert et al. 2005).

Experiment 1: DEBF and Aphid Behavior Three groups of 15 apterous adults of the same line were transferred to three plants and covered with cellophane bags (18.8×39 cm, *N*=12 lines). A piece of filter paper, to which DEBF, EBF (both 1 µg in 3 µl hexane), or hexane (3 µl) was applied, was placed inside the bags. The solutions were applied three times per day over 5 d. After the first application of each solution, the number of pea aphids walking was counted for 5 min. At the end of the experiment, mothers were counted and removed, and nymphs left for four more days on plants. Nymphs were then frozen and scored as winged or apterous morphs.

Experiment 2: DEBF and EBF Release For volatile collection, two groups of 15 third/fourth instars, of the same line, were transferred to two plants (*N*=13 lines) and placed in glass chambers (modified 1-L beaker). Teflon plates were placed around the base of the plant, keeping the soil out of

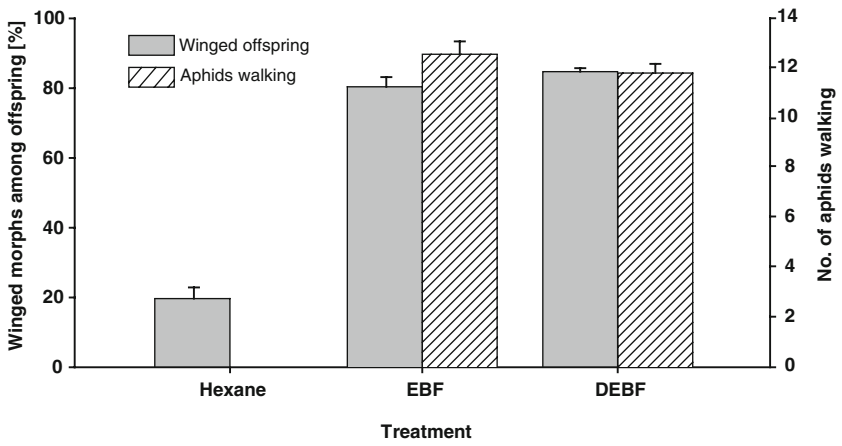

Fig. 2 Percentage of winged offspring produced by adult pea aphids and number of pea aphid mothers walking after exposure to hexane, (*E*)-β-farnesene (*EBF*) or deuterated (*E*)-β-farnesene (*DEBF*). The bars show the mean value±SE

the collection system. Two openings (Ø=1 cm) were present on top of the chamber: one provided air (2 L min^{-1}) filtered through active charcoal, and the other held a filter paper to which either DEBF (1 μg in 3 μl hexane) or hexane (3 μl) was applied. The chambers had an additional opening 3 cm from the rim holding a Super-Q filter (80/100 mesh; Alltech, Deerfield, IL, USA) connected to an air pump (1 L min^{-1}). With this set-up, six plants (three lines) could be tested simultaneously.

Volatile Analysis Super Q filters were eluted with 140 μl of dichloromethane and analyzed by GC-MS on a DB-5MS (J&W) column. For analysis, the column oven was kept at 60°C for 2 min., increased to 180°C at 5°C min^{-1}, and then increased at 60°C L min^{-1} until 300°C. Mass spectra from each peak were compared to those in the NIST and Wiley libraries for tentative peak identification. An internal standard of (*E*)-β-caryophyllene (400 ng in 30 μl of CH_2Cl_2) was added to all samples as an internal standard.

Statistical Analyses The number of aphids walking was analyzed by analysis of variance (ANOVA). Wing induction was analyzed by a generalized linear model (glm) with a quasi-binomial error structure. The aphid lines and number of nymphs produced in each replicate were included in the model, which was then simplified by removing non-significant variables or interactions, and accepted after an ANOVA ($P>0.05$; Crawley 2007). The survival of the mothers was analyzed with a glm using a Poisson error structure and simplified as described above. Data were analyzed with R software 2.6.0 (2007) and are presented as mean±SE.

Results and Discussion

In experiment 1, there was no significant difference among treatments in the mean number of adult pea aphids that survived (hexane=13.75±0.52; EBF=17.83±0.44; DEBF=13.58±0.47; $t=1.317$; $P=0.197$; $N=36$). In both EBF and DEBF treatments, the proportion of winged morphs among aphid offspring was higher than in the hexane treatment (Fig. 2; $t=15.075$; $P<0.001$, $N=36$). The number of aphids that responded to treatment by walking did not differ between EBF and DEBF (Fig. 2, $t=-1.542$, $P=0.152$, $N=36$); no walking responses were observed in the hexane treatment (Fig. 2, $t=27.211$, $P<0.001$, $N=36$).

In experiment 2, aphids started dispersing after DEBF application, but there was no measurable emission of endogenous alarm pheromone, suggesting that only attacked aphids emit EBF. Given the amounts of DEBF applied and re-collected, we estimated that the minimum amount of EBF detected by our experimental system was about 30 ng, an amount equivalent to that typically released by two to three third/fourth instars (Schwartzberg et al. 2008). Therefore, because we used colonies of 30 aphids, no more than 10% of the individuals tested, if any, could have responded by emitting their own EBF.

While signal amplification would have the advantage of alerting more aphids in the colony, it also has disadvantages. For example, it is thought that aphids may use the frequency of alarm pheromone perception as a measure of danger. Experiments with predators that induce alarm pheromone release and synthetic EBF, respectively, have shown that the proportion of winged offspring is related to the number of aphids consumed and the frequency of application of EBF (Kunert et al. 2005). Signal amplification would preclude aphids from employing the frequency of alarm pheromone release as a measure of the severity of an attack. Additionally, some natural enemies use EBF to detect their prey (Acar et al. 2001; Beale et al. 2006), and amplification of the signal would increase the danger of predator attraction.

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References

ACAR, E. B., MEDINA, J. C., LEE, M. L., and BOOTH, G. M. 2001. Olfactory behavior of convergent lady beetles (Coleoptera: Coccinellidae) to alarm pheromone of green peach aphid (Hemiptera: Aphididae). *Can. Entomol.* 133:3389–397.

BEALE, M. H., BIRKETT, M. A., BRUCE, T. J. A., CHAMBERLAIN, K., FIELD, L. M., HUTTLY, A. K., MARTIN, J. L., PARKER, R., PHILLIPS, A. L., PICKETT, J. A., PROSSER, I. M., SHEWRY, P. R., SMART, L. E., WADHAMS, L. J., WOODCOCK, C. M., and ZHANG, Y. H. 2006. Aphid alarm pheromone produced by transgenic plants affects aphid and parasitoid behavior. *Proc. Natl. Acad. Sci. USA* 103:10509–10513.

CRAVOTTO, G., BALLIANO, G., ROBALDO, B., OLIARO-BOSSO, S., CHIMICHI, S., and BOCCALINI, M. 2004. Farnesyloxycoumarins, a new class of squalene-hopene cyclase inhibitors. *Bioorg. Med. Chem. Lett.* 14:1931–1934.

CRAWLEY, M. J. 2007. The R Book. p. 950. Wiley, Chichester, England.

FIELDER, S., and ROWAN, D. D. 1994. The synthesis of d(6)-alpha-farnesene. *J. Labelled Comp. Rad.* 34:1075–1085.

FRANCIS, F., VANDERMOTEN, S., VERHEGGEN, F., LOGNAY, G., and HAUBRUGE, E. 2005. Is the (E)-beta-farnesene only volatile terpenoid in aphids? *J. Appl. Entomol.* 129:16–11.

KUNERT, G., OTTO, S., ROSE, U. S. R., GERSHENZON, J., and WEISSER, W. W. 2005. Alarm pheromone mediates production of winged dispersal morphs in aphids. *Ecol. Lett.* 8:596–603.

NAULT, L. R., EDWARDS, L. J., and STYER, W. E. 1973. Aphid alarm pheromones: secretion and reception. *Environ. Entomol.* 2:101–105.

PICKETT, J. A., and GLINWOOD, R. 2007. Chemical ecology, pp. 255–260, in H.F. Van Emden, and R.H. Harrington (eds.). Aphids as Crop PestsCABI, Wallingford, Oxfordshire.

SCHWARTZBERG, E. G., KUNERT, G., STEPHAN, C., DAVID, A., RÖSE, U. S. R., GERSHENZON, J., BOLAND, W., and WEISSER, W. W. 2008. Real-time analysis of alarm pheromone emission by the pea aphid (*Acyrthosiphon pisum*) under predation. *J. Chem. Ecol.* 34:76–81.

At a distance, aphids are able to use visual cues to recognize their hosts (David and Hardie 1988; Gish and Inbar 2006), with gustatory cues playing a role in acceptance or rejection of a plant after landing (Powell et al. 1999). Olfaction plays a prominent role in both the pre- and post-alighting stages of host selection and has been widely demonstrated in a variety of aphid species (Pickett et al. 1992; Powell and Hardie 2001). Aphids possess a sophisticated ability to recognize a wide variety of plant-volatile compounds by using olfactory receptor neurons housed in sensilla located on the antennae (Visser et al. 1996). Two types of olfactory sensilla exist, the primary and secondary rhinaria (Bromley et al. 1979, 1980). Distal and proximal primary rhinaria are found on the sixth and fifth antennal segments, respectively, and are used in the detection of plant volatiles (Hardie et al. 1994b; Park and Hardie 2002). As well as housing olfactory receptor neurons used to recognize host volatiles, the primary rhinaria also house olfactory receptor neurons used in the detection of non-host volatiles that may act as deterrents (Nottingham et al. 1991; Isaacs et al. 1993). Olfactory-mediated host location in aphids is thus a combination of host recognition and non-host avoidance.

An improved understanding of aphid olfaction could facilitate the development of novel crop protection strategies that rely on semiochemicals to reduce aphid populations in the field (Bruce et al. 2003, 2005a; Ninkovic et al. 2003). Semiochemicals used in host location could be used to help predict outbreaks by incorporation into traps used for monitoring populations. Semiochemicals can also be used within "push–pull" or stimulo-deterrent diversionary strategies (SDDS) (Miller and Cowles 1990; Cook et al. 2007). This involves protecting a main crop with repellents or masking compounds (the 'push' away from crops) and using a trap crop to lure insects away from the main crop (the 'pull'). Attractive semiochemicals could potentially be incorporated into the 'pull' element, and manipulation of the host odor to deter pest insects could constitute the 'push'.

There is already evidence that winged virginoparous *A. fabae* use olfaction to locate their summer host plant, *V. faba* (Nottingham et al. 1991; Pickett et al. 1992), but the volatile compounds used in host location have not yet been identified. The work carried out here sought to identify the semiochemicals released by *V. faba* that *A. fabae* uses to identify its host.

Methods and Materials

Insect Rearing The Kennedy and Booth clone of *Aphis fabae* (Kennedy and Booth 1950) was reared on tick beans (*Vicia faba* var. minor) in a Perspex rearing cabinet under controlled conditions (22±1°C, L:D, 16:8 hr). Winged aphids were induced by allowing the colony to become crowded. Prior to behavioral bioassays, aphids were starved for 24 hr.

Chemicals Authentic chemical standards used in electro-antennogram and laboratory behavioral studies were (*E*)-2-hexenal, benzaldehyde, 6-methyl-5-hepten-2-one, decanal, 1-hexanol (all 99% purity, Sigma Aldrich, St. Louis, MO, USA), (1*R*,5*S*)-myrtenal (98% purity, Sigma Aldrich), undecanal (97% purity, Sigma Aldrich), (*R*)-linalool and (*S*)-linalool (98% purity, Sigma Aldrich), octanal, methyl salicylate, (*Z*)-3-hexen-1-ol, and (*Z*)-3-hexen-1-yl acetate (all 98% purity, Avocado Research Chemicals, Lancs., UK), (*Z*)-3-hexen-1-yl acetate (99% purity, Avocado Research Chemicals), and (*E*)-caryophyllene (85% purity, Pfaltz & Bauer inc., Stamford, CT, USA). (*E*)-β-Farnesene (98% purity) was synthesized from (*E*)-farnesyl chloride (Kang et al. 1987). (*S*)-(−)-Germacrene D and (*R*)-(+)-germacrene D (both 98% purity) were obtained by incubation of farnesyl pyrophosphate with purified, expressed (R)-(+) or (S)-(−)-germacrene-D synthase and subsequent hexane extraction and purification through a short column of silica gel (BDH, 40–63 μm)/$MgSO_4$ (10:1) (Prosser et al. 2004). (*E*,*E*)-4,8,12-Trimethyl-1,3,7,11-tridecatetraene was synthesized from (*E*,*E*)-farnesol by oxidation to its aldehyde followed by Wittig methylenation (Leopold 1990).

Air Entrainment of Plants All equipment was washed with Teepol detergent (Herts County Supplies, Herts, UK), rinsed with acetone and distilled water, and baked overnight at 160°C. Porapak Q tubes were eluted with redistilled diethyl ether and heated at 132°C for 2 hr to remove contaminants. *Vicia faba* (var. Sutton dwarf) plants were grown individually in 7.5-cm pots in a glasshouse maintained at 20°C. Three-week-old plants at the vegetative growth stage were enclosed individually in glass vessels (190 mm high × 100 mm wide), open at the bottom and closed at the top except for an inlet and outlet port. The bottom was closed without pressure around the plant stem by using two semicircular aluminum plates with a hole in the center to accommodate the stem. Charcoal filtered air was pumped in at 400 ml min^{-1} and drawn out at 300 ml min^{-1} through a Porapak Q adsorbent tube (Alltech Associates, Lancashire, UK) in a 5-mm diam glass tube (Alltech Associates, Carnforth, Lancashire, UK; 50 mg Porapak Q in 5-mm diam glass tube). The difference in flow rates created a slight positive pressure to ensure that unfiltered air did not enter the system, thus removing the need for an airtight seal around the stem. All connections were made with PTFE tubing (Alltech Associates, Lancashire, UK) with brass ferules and fittings (North London Valve, London, UK) and sealed with PTFE tape (Gibbs & Dandy, Luton,

UK). Plants were entrained for 7 d, and the Porapak Q filter was eluted with 0.5 ml of redistilled diethyl ether, providing a solution that contained the isolated volatile compounds. Four plants were entrained. Gas chromatography (GC) analysis of each entrainment sample revealed similar GC traces, and these samples were combined to give a bulk air entrainment sample.

Gas Chromatography Analysis Volatiles were analyzed on a Hewlett-Packard 6890 GC equipped with a cold on-column injector, a flame ionization detector (FID), a non-polar HP-1 bonded-phase fused silica capillary column (50 m × 0.32 mm i.d., film thickness 0.52 μm) and a polar DB-WAX column (30 m × 0.32 mm i.d., film thickness 0.82 μm). The oven temperature was maintained at 30°C for 1 min, and programmed at 5°C min^{-1} to 150°C and held for 0.1 min, then 10°C min^{-1} to 230°C. The carrier gas was hydrogen. Stereochemistry of linalool and germacrene D was determined by using an HP5890 GC (Agilent Technologies, UK) equipped with a cool on-column injector and a FID, fitted with a *β*-cyclodextrin chiral capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness). The GC oven was maintained at 40°C for 1 min and then raised by 5°C min^{-1} to 150°C where it was held for 30 min. The carrier gas was hydrogen. A 1-μl aliquot solution that contained equal quantities of both enantiomers of the compounds in redistilled hexane was injected onto the chiral GC to establish that successful separation of enantiomers took place. This was followed by co-injections of the air entrainment sample, first with an authentic standard of one enantiomer and then with the second enantiomer. Peak enhancement with either enantiomer confirmed the presence of that enantiomer in the air entrainment sample.

Coupled Gas Chromatography–Electroantennography (GC-EAG) Electroantennogram (EAG) recordings were made with Ag-AgCl glass microelectrodes filled with Ringer solution (7.55 g l^{-1} sodium chloride, 0.64 g l^{-1} potassium chloride, 0.22 g l^{-1} calcium chloride, 1.73 g l^{-1} magnesium chloride, 0.86 g l^{-1} sodium bicarbonate, 0.61 g l^{-1} sodium orthophosphate). The head of an *A. fabae* winged virginopara was separated from the body with a scalpel, and the tips of the antennae were removed to ensure good contact with the electrode. The head was placed into the tip of the indifferent electrode, and the tips of the antennae were positioned in the end of the recording electrode. The coupled GC–EAG system, in which the effluent from the GC column was simultaneously directed to the antennal preparation and the GC detector, has been described previously (Wadhams 1990). Separation of the volatiles was achieved on a Hewlett-Packard 6890 gas chromatograph equipped with a cold on-column injector and a FID. The column used was 50 m × 0.32-mm i.d. HP-1. The oven temperature was maintained at 30°C for 2 min and then programmed at 5°C min^{-1} to 100°C and then at 10°C min^{-1} to 250°C. The carrier gas was hydrogen. Electroantennogram signals were passed through a high-impedance amplifier (UN-06; Syntech, the Netherlands), and simultaneous recordings of the EAG and FID responses were obtained with specialized software (EAD version 2.3; Syntech, the Netherlands). Six coupled runs were completed. Only FID peaks that corresponded to an EAG peak in three or more replicates were considered electrophysiologically active.

Coupled Gas Chromatography–Mass Spectrometry (GC-MS) Tentative identification of electrophysiologically active FID peaks was achieved by GC-MS. A 1-μl aliquot of the air entrainment sample was injected onto a capillary GC column (50 m × 0.32 mm i.d. HP1) directly coupled to a mass spectrometer (Thermo-Finnigan, MAT95, Bremen, Germany). Ionization was achieved by electron impact at 70 eV, 250°C. The oven temperature was maintained at 30°C for 5 min and then programmed at 5°C min^{-1} to 250°C. Tentative identifications of electrophysiologically active FID peaks were made by comparison of spectra with those of authentic samples in a database (NIST 2005). Tentative identifications by GC-MS were confirmed by co-injection of the air entrainment sample with authentic standards on both HP-1 and DB-WAX columns, with peak enhancement indicating co-elution. Quantification of identified compounds was achieved by injecting authentic standards at three known concentrations onto the HP 1 GC column and recording the peak area. Each standard was injected three times, and a calibration curve was plotted for each compound, and used to determine quantities of each compound in the air entrainment sample.

Electrophysiological Responses of Aphids to Identified Compounds Electrophysiological activity of identified compounds was confirmed by EAG. The stimulus delivery system, which employed a piece of filter paper in a disposable Pasteur pipette cartridge, has been described previously (Wadhams 1982). Authentic standards of each test compound were applied to a strip of filter paper at a dose of 10 μg in 10 μl redistilled hexane, and responses were compared to a solvent control. For compounds where there was no significant response to 10 μg, a dose of 100 μg in 10-μl redistilled hexane also was tested. The stimulus (2 sec duration) was delivered into a purified air stream (1 l min^{-1}) that flowed continuously through a glass tube (i.d. 8 mm) and over the preparation. Electrophysiological responses were recorded by using specialized software (EAD version 2.3; Syntech, the Netherlands). A standard stimulus of (1*R*,5*S*)-myrtenal, previously demonstrated to be electrophysiologically active for *A. fabae*

(Hardie et al. 1994a), was tested at the start and end of each replicate to ensure that insect preparations were functional throughout the experiment. The hexane control was tested at the start and end of each experiment, and an average was taken. Responses to test compounds were compared to the average of the two hexane control stimuli for each replicate. All comparisons were made by using a paired *t*-test (Genstat).

Olfactometer Bioassays A Perspex four-arm olfactometer (Pettersson 1970) was used to determine behavioral responses of *A. fabae* winged virginoparae to intact *V. faba* (var. Sutton dwarf) plants, an air entrainment sample of *V. faba* (var. Sutton dwarf) plants, and a synthetic volatile blend that comprised all identified electrophysiologically active compounds at the same concentrations and ratio as in the air entrainment sample. Prior to each experiment, all glassware was washed with Teepol detergent, rinsed with acetone and distilled water, and baked in an oven overnight at 160°C. Perspex components were washed with Teepol solution, rinsed with 80% ethanol solution and distilled water, and left to air dry. The olfactometer was fitted with a filter-paper base to provide traction for the walking insect, and was illuminated from above by diffuse uniform lighting from two 18W/35 white fluorescent light bulbs screened with greaseproof paper. It was surrounded by black paper to remove any external visual stimuli. For bioassays with plant material, a 2-l glass vessel that contained a single 3-wk-old plant was connected to the treatment inlet of the olfactometer by PTFE tubing. Three identical glass vessels that contained 110-mm filter paper saturated with distilled water were connected to the three control inlets. When the air entrainment sample and synthetic blend were used as test stimuli, the glass vessels were replaced with glass arms. Ten microliters of the air entrainment sample/synthetic blend were placed onto a piece of filter paper, 30 sec was allowed for the solvent to evaporate, and then it was placed in the treated arm. In these experiments, the three control arms contained a piece of filter paper with 10 μl of redistilled diethyl ether or redistilled hexane (for tests with the air entrainment sample and synthetic blend, respectively).

A single aphid was introduced through a hole in the top of the olfactometer with a fine paintbrush. Air was drawn through the central hole at a rate of 400 ml min^{-1} and subsequently exhausted from the room. Each aphid was given 2 min to acclimatize in the olfactometer after which the experiment was run for 16 min. The olfactometer was rotated 90 degrees every 2 min to control for any directional bias in the room. It was divided into four regions that correspond to each of the four glass vessels/arms, and time spent in each region was recorded with Olfa software (F. Nazzi, Udine, Italy). If an aphid remained motionless continuously for 2 min, it was considered inactive, and the replicate was repeated by using a new aphid (Pettersson 1970). Twelve replicates were carried out for each odor source tested. Time spent in the treated region was converted to a percentage of total time and transformed by using a logit transformation. The transformed data were normally distributed and compared to a test mean of −1.099 (logit transformation of 25%) by using a one-sample *t*-test (Genstat v. 10).

A choice test between 10 μl synthetic blend and 10 μl air entrainment sample was carried out. These two treatments were assigned randomly to different glass arms in each replicate. The other two arms contained filter paper with 10 μl solvent and were used as controls. Time spent in each region was recorded for 16 min. Twenty-four replicates were carried out. To determine if more time was spent in either of the treated regions, the difference between the time spent in each was calculated. The data were normally distributed and compared to a test mean of zero by using a one-sample *t*-test.

Results

Identification of Electrophysiologically Active Compounds in Air Entrainment Sample Coupled GC-EAG revealed 16 electrophysiologically active compounds in the air entrainment sample (Fig. 1). By using coupled GC-MS and GC peak enhancement on two GC columns of different polarity, 15 of these compounds were identified as (*Z*)-3-hexen-1-ol, 1-hexanol, (*E*)-2-hexenal, benzaldehyde, 6-methyl-5-hepten-2-one, octanal, (*Z*)-3-hexen-1-yl acetate, (*R*)-linalool, methyl salicylate, decanal, undecanal, (*E*)-caryophyllene, (*E*)-β-farnesene, (*S*)-(−)-germacrene D, and (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene (TMTT). The quantities and ratio of all identified compounds are listed in Table 1. One compound, with a retention index of 1377 on an HP-1 column, could not be identified.

Electrophysiological Responses of Aphids to Identified Compounds All compounds elicited statistically significant electrophysiological responses when tested at 10 μg, with the exception of (Z)-3-hexen-1-ol, 6-methyl-5-hepten-2-one and benzaldehyde. These three compounds, however, did yield a statistically significant electrophysiological response when tested at 100 μg (Table 2).

Behavioral Responses to Plant Material, Air Entrainment Sample and Synthetic Blend In the olfactometer bioassays, the aphids spent significantly more time in the treated region of the olfactometer than the controls when a *V. faba* plant was used as an odor source ($P<0.001$), and also when a 10-μl aliquot of the *V. faba* air entrainment sample was

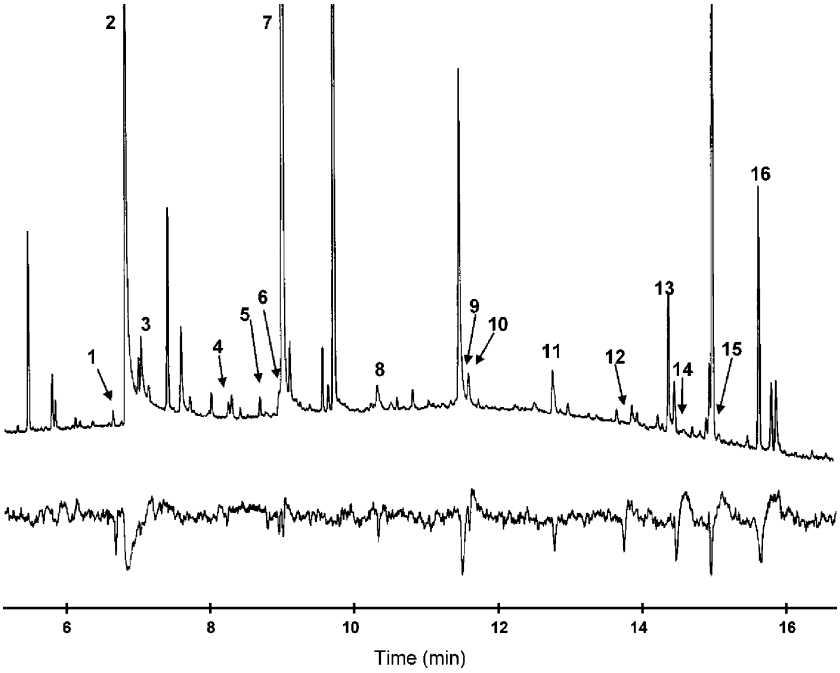

Fig. 1 Amalgamation of coupled GC-EAG traces showing electrophysiologically active peaks. 1 = (*E*)-2-hexenal, 2 = (*Z*)-3-hexen-1-ol, 3 = 1-hexanol, 4 = benzaldehyde, 5 = 6-methyl-5-hepten-2-one, 6 = octanal, 7 = (*Z*)-3-hexen-1-yl acetate, 8 = (*R*)-linalool, 9 = methyl salicylate, 10 = decanal, 11 = undecanal, 12 = unknown, 13 = (*E*)-caryophyllene, 14 = (*E*)-β-farnesene, 15 = (*S*)-(−)-germacrene D, 16 = (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene

used (P=0.048). A similar response was observed when the 15-component synthetic blend was used as an odor source, with the aphids spending more time in the treated region of the olfactometer than the controls (P=0.018) (Fig. 2).

When the synthetic blend and air entrainment samples were tested in a choice test, the total time spent in the two olfactometer treatment arms was greater than the total time spent in the two control arms (P=0.012). The aphids did not show any preference for either of the two treatments (P=0.844) (Fig. 3), thus demonstrating that the synthetic blend had activity similar to the natural sample.

Discussion

Aphis fabae has been found previously to move toward the odor of its host plant, *V. faba* (var. Sutton dwarf), in olfactometer studies (Nottingham et al. 1991), but the volatile compounds responsible were not identified. In this study, a synthetic kairomone blend that comprised all identified volatile compounds in the headspace of *V. faba* (var. Sutton dwarf) plants that were electrophysiologically active at physiologically relevant levels to *A. fabae* was tested. This blend elicited a similar behavioral response to

Table 1 Names, quantities and ratio of compounds

Compound no.	Compound name	Quantity in 10 µl of *Vicia faba* air entrainment sample (ng)	Ratio
1	(*E*)-2-hexenal	0.36	9
2	1-hexanol	14.2	355
3	(*Z*)-3-hexen-1-ol	85.8	2145
4	benzaldehyde	0.04	1
5	6-methyl-5-hepten-2-one	0.58	14.5
6	octanal	0.34	8.5
7	(*Z*)-3-hexen-1-yl acetate	42	1050
8	(*R*)-linalool	1.02	25.5
9	methyl salicylate	0.7	17.5
10	decanal	0.76	19
11	undecanal	0.52	13
12	unknown	–	–
13	(*E*)-caryophyllene	4.6	115
14	(*E*)-β-farnesene	0.44	11
15	(*S*)-(-)-germacrene D	0.36	9
16	(*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	4.96	124

Table 2 Corrected EAG responses of winged virginoparae *Aphis fabae* to compounds identified in headspace of *Vicia faba*

Compound name	Corrected EAG response (±SE) [-μV] at 10 μg dose	Corrected EAG response (±SE) [-μV] at 100 μg dose
(*E*)-2-hexenal	56 (±3)**	Not tested
1-hexanol	48 (±1)***	Not tested
(*Z*)-3-hexen-1-ol	7 (±1)	50 (±2)**
Benzaldehyde	60 (±2)	36 (±2)**
6-methyl-5-hepten-2-one	45 (±3)	59 (±3)*
Octanal	85 (±2)***	Not tested
(*Z*)-3-hexen-1-yl acetate	22 (±2)*	Not tested
(*R*)-linalool	43 (±2)**	Not tested
Methyl salicylate	36 (±2)**	Not tested
Decanal	82 (±4)**	Not tested
Undecanal	60 (±3)***	Not tested
(*E*)-caryophyllene	110 (±2)***	Not tested
(*E*)-β-farnesene	36 (±2)*	Not tested
(*S*)-(-)-germacrene D	111 (±4)**	Not tested
(*E*,*E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene	75 (±4)**	Not tested

Only those compounds which did not elicit a significant EAG response at a dose of 10 μg were tested at a dose of 100 μg. EAG responses were corrected by subtracting the response to the hexane solvent control. *Significantly different from control ($P<0.05$); **Significantly different from control ($P<0.01$); *** Significantly different from control ($P<0.001$).

the odor of a *V. faba* (var. Sutton dwarf) plant and also to the air entrainment sample, suggesting that the volatile compounds used in host location have successfully been identified. This was confirmed by the fact that aphids did not show any preference for the air entrainment sample over the synthetic blend when offered the choice. One compound could not be identified and so could not be included in the synthetic blend. However, since the aphids showed no preference for the air entrainment sample over the synthetic blend, it is unlikely that this unidentified compound is essential in host location.

Several of the compounds reported here have been identified previously in the headspace of undamaged *V. faba* plants, namely (*E*)-2-hexenal, (*Z*)-3-hexen-1-ol, benzaldehyde, (*Z*)-3-hexen-1-yl acetate, linalool, (*E*)-caryophyllene, and 4,8,12-trimethyl-1,3,7,11-tridecatetraene (Blight et al. 1984; Griffiths et al. 1999; Colazza et al. 2004). Methyl salicylate has not been found previously in the headspace of undamaged *V. faba* plants but is known to be released by bean upon damage by chewing insects (Blight and Pickett, unpublished data) and by other plants in the Fabaceae family (Dicke et al. 1999; Mithofer et al. 2005;

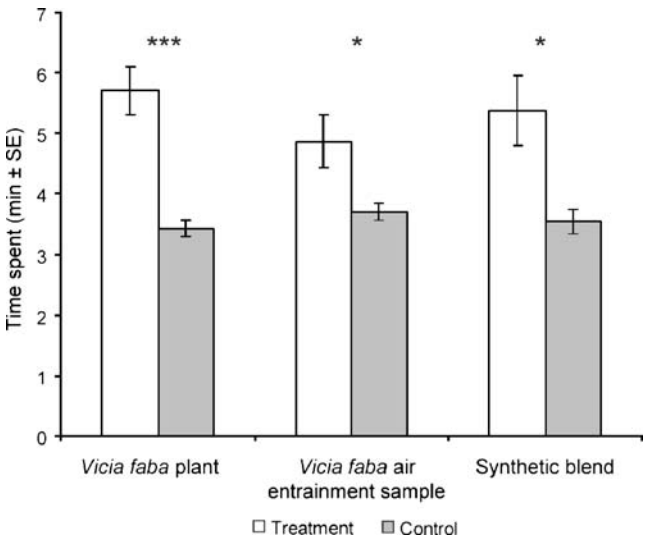

Fig. 2 Behavioral responses of winged virginoparae *Aphis fabae* to different odor sources in four-arm olfactometer ($N=12$). *Single asterisk* Treatment significantly different from control ($P<0.05$) *Triple asterisk* Treatment significantly different from control ($P<0.001$). Time spent in treated arm was converted to percentage, logit transformed, and then compared to a test mean of −1.099 (logit transformation of 25%) by using a one-sample *t*-test

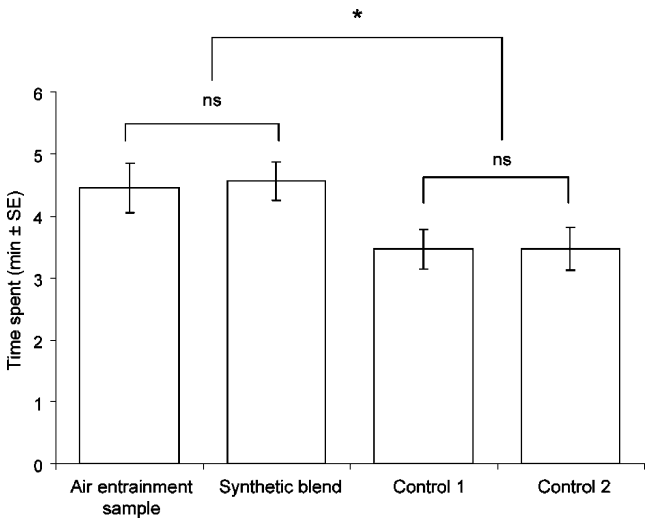

Fig. 3 Behavioral response of winged virginoparae *Aphis fabae* to *Vicia faba* air entrainment sample and synthetic blend when both odor sources present together in the same olfactometer ($N=24$). *Single asterisk* Significantly different ($P<0.05$). The difference between time spent in each treated arm was calculated and compared to a test mean of zero by using a one-sample *t*-test

Ishiwari et al. 2007). Its presence in a relatively small quantity (0.7 ng in 10 μl of air entrainment sample tested) suggests that this compound may be produced constitutively but only in small amounts that may not have been detected in previous work. Hardie et al. (1994a) observed that *A. fabae* made oriented movement away from methyl salicylate in a linear-track olfactometer at release rates at and above 50 μg hr^{-1}. The relatively small quantity of methyl salicylate in the air entrainment sample is likely to be below the threshold for which a repellent effect normally would be observed; hence, the aphids responded to the air entrainment sample and synthetic blend despite the presence of this compound. (*E*)-β-Farnesene is used by aphids as an alarm pheromone, released by aphids under attack from predators or parasitoids and causing nearby aphids to disperse (Nault et al. 1973; Pickett et al. 1992; Bruce et al. 2005a). It is also a widely occurring plant volatile (Knudsen et al. 1993), and aphids distinguish between plant- and aphid-originating (*E*)-β-farnesene by the presence of certain other plant volatiles that inhibit the behavioral response to the alarm pheromone (Dawson et al. 1984). *Aphis fabae* uses linalool as an alarm pheromone inhibitor (Bruce, unpublished data), which explains why the aphids orientate toward the air entrainment sample and synthetic blend despite the presence of (*E*)-β-farnesene.

The work carried out here has resulted in considerable progress in identifying which volatiles *A. fabae* could use in host location. However, a behavioral response to a blend of 15 volatile compounds does not necessarily mean that all 15 compounds are necessary to elicit that behavioral response. There are many examples in the literature that demonstrate host location can be mediated by only a small fraction of the electrophysiologically active volatiles identified in the headspace of an insect's host plant, the other compounds being redundant (Blight et al. 1997; Nojima et al. 2003; Birkett et al. 2004; Tasin et al. 2007). Other examples show that host location can be mediated by a single compound (Guerin et al. 1983; Hern and Dorn 2004), although such cases usually involve taxonomically specific volatile compounds that are not found in unrelated plant species (Bruce et al. 2005b). All of the electrophysiologically active volatile compounds identified in the headspace of *V. faba* are found in a diverse range of taxonomic plant groups and not specific to this species (Knudsen et al. 1993; Pare and Tumlinson 1997; Buttery et al. 2000; Blackmer et al. 2004; Williams et al. 2005), making it unlikely that a single volatile compound is used in host location. Visser (1986) suggested that host recognition by using ubiquitous rather than host-specific volatile compounds must be achieved by recognizing host-specific ratios of these compounds. Since none of the compounds identified here are specific to *V. faba*, it is likely that host location is meditated by a blend of several in a particular ratio.

Aphis fabae has a broad host range, and by using ratios of commonly occurring plant volatiles, it has the ability to recognize a large number of host plants. If an insect with a broad host range used host-specific volatile compounds, it would require specialized olfactory receptor neurons for volatile compounds associated with each host. By recognizing species-specific ratios of commonly occurring plant volatiles, the ability to recognize a larger range of plants with a much smaller number of receptor neurons is possible. The use of commonly occurring plant volatiles to recognize a large range of plant species could also facilitate learning behavior and preference for whichever host is more abundant in the habitat. *A. fabae* exists as a number of 'host races', each race specialized on a particular host (Douglas 1997). Host recognition by using ratios of commonly occurring plant volatiles could partly explain the abundance of these host races, as a change in integration in the central nervous system, rather than the need to evolve new receptor neurons when a new host race forms, could possibly facilitate more rapid evolution. The determination of how *A. fabae* responds to blends of volatile compounds in different ratios, and how this is used in host location will form the basis of future work.

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References

Birkett, M. A., Bruce, T. J. A., Martin, J. L., Smart, L. E., Oakley, J., and Wadhams, L. J. 2004. Responses of female orange wheat blossom midge, *Sitodiplosis mosellana*, to wheat panicle volatiles. *J. Chem. Ecol.* 30:1319–1328.

Blackmer, J. L., Rodriguez-Saona, C., Byers, J. A., Shope, K. L., and Smith, J. P. 2004. Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. *J. Chem. Ecol.* 30:1547–1564.

Blight, M. M., Pickett, J. A., Smith, M. C., and Wadhams, L. J. 1984. An aggregation pheromone of *Sitona lineatus*—identification and initial field studies. *Naturwissenschaften* 71:480–480.

Blight, M. M., Lemetayer, M., Delegue, M. H. P., Pickett, J. A., Marionpoll, F., and Wadhams, L. J. 1997. Identification of floral volatiles involved in recognition of oilseed rape flowers, *Brassica napus* by honeybees, *Apis mellifera*. *J. Chem. Ecol.* 23:1715–1727.

Bromley, A. K., Dunn, J. A., and Anderson, M. 1979. Ultrastructure of the antennal sensilla of aphids.1. coeloconic and placoid sensilla. *Cell Tissue Res.* 203:427–442.

Bromley, A. K., Dunn, J. A., and Anderson, M. 1980. Ultrastructure of the antennal sensilla of aphids.2. Trichoid, chordotonal and campaniform sensilla. *Cell Tissue Res.* 205:493–511.

Bruce, T. J. A., Martin, J. L., Pickett, J. A., Pye, B. J., Smart, L. E., and Wadhams, L. J. 2003. cis-Jasmone treatment induces resistance in wheat plants against the grain aphid, *Sitobion avenae* (Fabricius) (Homoptera: Aphididae). *Pest Manag. Sci.* 59:1031–1036.

BRUCE, T. J. A., BIRKETT, M. A., BLANDE, J., HOOPER, A. M., MARTIN, J. L., KHAMBAY, B., PROSSER, I., SMART, L. E., and WADHAMS, L. J. 2005a. Response of economically important aphids to components of Hemizygia petiolata essential oil. *Pest Manag. Sci.* 61:1115–1121.

BRUCE, T. J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2005b. Insect host location: a volatile situation. *Trends Plant Sci.* 10(6):269–274.

BUTTERY, R. G., LIGHT, D. M., NAM, Y., MERRILL, G. B., and ROITMAN, J. N. 2000. Volatile components of green walnut husks. *J. Agric. Food Chem.* 48:2858–2861.

CAMMELL, M. E. 1981. The black bean aphid, *Aphis fabae*. *Biologist* 28(5):247–258.

COLAZZA, S., MCELFRESH, J. S., and MILLAR, J. G. 2004. Identification of volatile synomones, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalis*. *J. Chem. Ecol.* 30:945–964.

COOK, S. M., KHAN, Z. R., and PICKETT, J. A. 2007. The use of push–pull strategies in integrated pest management. *Annu. Rev. Entomol.* 52:375–400.

DAVID, C. T., and HARDIE, J. 1988. The visual responses of free-flying summer and autumn forms of the black bean aphid, *Aphis fabae*, in an automated flight chamber. *Physiol. Entomol.* 13:277–284.

DAWSON, G. W., GRIFFITHS, D. C., PICKETT, J. A., SMITH, M. C., and WOODCOCK, C. M. 1984. Natural inhibition of the aphid alarm pheromone. *Entomol. Exp. Appl.* 36:197–199.

DICKE, M., GOLS, R., LUDEKING, D., and POSTHUMUS, M. A. 1999. Jasmonic acid and herbivory differentially induce carnivore-attracting plant volatiles in lima bean plants. *J. Chem. Ecol.* 25:1907–1922.

DOUGLAS, A. E. 1997. Provenance, experience and plant utilisation by the polyphagous aphid, *Aphis fabae*. *Entomol. Exp. Appl.* 83:161–170.

EL-AMRI, A. 1999. Identification of vectors and natural hosts of faba bean necrotic yellows virus in Morocco. *Al Awamia* 99:27–31.

FERNANDEZ-QUINTANILLA, C., FERERES, A., GODFREY, L., and NORRIS, R. F. 2002. Development and reproduction of *Myzus persicae* and *Aphis fabae* (Hom., Aphididae) on selected weed species surrounding sugar beet fields. *J. Appl. Entomol.* 126:198–202.

GISH, M., and INBAR, M. 2006. Host location by apterous aphids after escape dropping from the plant. *J. Insect Behav.* 19:143–153.

GRIFFITHS, D. W., ROBERTSON, G. W., SHEPHERD, T., and RAMSAY, G. 1999. Epicuticular waxes and volatiles from faba bean (*Vicia faba*) flowers. *Phytochemistry* 52:607–612.

GUERIN, P. M., STADLER, E., and BUSER, H. R. 1983. Identification of host plant attractants for the carrot fly, *Psila rosae*. *J. Chem. Ecol.* 9:843–861.

HARDIE, J., ISAACS, R., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1994a. Methyl salicylate and (−)-(1R,5S)-myrtenal are plant-derived repellents for black bean aphid, *Aphis fabae* Scop (Homoptera, Aphididae). *J. Chem. Ecol.* 20:2847–2855.

HARDIE, J., VISSER, J. H., and PIRON, P. G. M. 1994b. Perception of volatiles associated with sex and food by different adult forms of the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* 19:278–284.

HERN, A., and DORN, S. 2004. A female-specific attractant for the codling moth, *Cydia pomonella*, from apple fruit volatiles. *Naturwissenschaften* 91:77–80.

ISAACS, R., HARDIE, J., HICK, A. J., PYE, B. J., SMART, L. E., WADHAMS, L. J., and WOODCOCK, C. M. 1993. Behavioral responses of *Aphis fabae* to isothiocyanates in the laboratory and field. *Pestic. Sci.* 39:349–355.

ISHIWARI, H., SUZUKI, T., and MAEDA, T. 2007. Essential compounds in herbivore-induced plant volatiles that attract the predatory mite Neoseiulus womersleyi. *J. Chem. Ecol.* 33:1670–1681.

KANG, S. K., CHUNG, G. Y., and LEE, D. H. 1987. A convenient synthesis of (E)-beta-farnesene. *Bull. Kor. Chem. Soc.* 8:351–353.

KENNEDY, J. S., and BOOTH, C. O. 1950. Methods for mass rearing and investigating the host relations of *Aphis fabae* Scop. *Ann. Appl. Biol.* 37:451–470.

KNUDSEN, J. T., TOLLSTEN, L., and BERGSTROM, L. G. 1993. Floral scents—a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* 33:253–280.

LEES, A. D. 1966. The control of polymorphisms in aphids. *Adv. Insect Physiol.* 3:207–277.

LEOPOLD, E. J. 1990. Selective hydroboration of a 1,3,7-triene: homogeraniol. *Org. Synth.* 64:164–171.

MILLER, J. R., and COWLES, R. S. 1990. Stimulo-deterrent diversion—a concept and its possible application to onion maggot control. *J. Chem. Ecol.* 16:3197–3212.

MITHOFER, A., WANNER, G., and BOLAND, W. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137:1160–1168.

MÜLLER, C. B., WILLIAMS, I. S., and HARDIE, J. 2001. The role of nutrition, crowding and interspecific interactions in the development of winged aphids. *Ecol. Entomol.* 26:330–340.

NAULT, L. R., EDWARDS, L. J., and STYER, W. E. 1973. Aphid alarm pheromones: secretion and reception. *Environ. Entomol.* 2:101–105.

NEERAJ, V., DANISH, M., DOOMAR, S., and NAQVI, Q. A. 1999. Studies' on bean yellow mosaic virus infecting broad bean (*Vicia faba L.*) in Aligarh, India. *Ann. Plant Prot. Sci.* 7:51–54.

NINKOVIC, V., AHMED, E., GLINWOOD, R., and PETTERSSON, J. 2003. Effects of two types of semiochemical on population development of the bird cherry oat aphid *Rhopalosiphum padi* in a barley crop. *Agric. For. Entomol.* 5:27–33.

NIST 2005. NIST mass spectral search for the NIST/EPA/NIH mass spectral library version 2.0. Office of the Standard Reference Data Base, National Institute of Standards and Technology, Gaithersburg, Maryland.

NOJIMA, S., LINN, C., MORRIS, B., ZHANG, A. J., and ROELOFS, W. 2003. Identification of host fruit volatiles from hawthorn (Crataegus spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol.* 29:321–336.

NOTTINGHAM, S. F., HARDIE, J., DAWSON, G. W., HICK, A. J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1991. Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles. *J. Chem. Ecol.* 17:1231–1242.

PARE, P. W., and TUMLINSON, J. H. 1997. De novo biosynthesis of volatiles induced by insect herbivory in cotton plants. *Plant Physiol.* 114:1161–1167.

PARK, K. C., and HARDIE, J. 2002. Functional specialisation and polyphenism in aphid olfactory sensilla. *J. Insect Physiol.* 48:527–535.

PARKER, W. E., and BIDDLE, A. J. 1998. Assessing the damage caused by black bean aphid (*Aphis fabae*) on spring beans, pp. 1077–1082, in Brighton Crop Protection Conference: Pests & Diseases 1998. Brighton, UK.

PETTERSSON, J. 1970. An aphid sex attractant. *Entomol. Scand.* 1: 63–73.

PICKETT, J. A., WADHAMS, L. J., WOODCOCK, C. M., and HARDIE, J. 1992. The chemical ecology of aphids. *Annu. Rev. Entomol.* 37:67–90.

POWELL, G., and HARDIE, J. 2001. The chemical ecology of aphid host alternation: how do return migrants find the primary host plant? *Appl. Entomol. Zool.* 36:259–267.

POWELL, G., MANIAR, S. P., PICKETT, J. A., and HARDIE, J. 1999. Aphid responses to non-host epicuticular lipids. *Entomol. Exp. Appl.* 91:115–123.

PROSSER, I., ALTUG, I. G., PHILLIPS, A. L., KONIG, W. A., BOUWMEESTER, H. J., and BEALE, M. H. 2004. Enantiospecific (+)- and (−)-germacrene D synthases, cloned from goldenrod, reveal a functionally active variant of the universal isoprenoid-biosynthesis aspartate-rich motif. *Arch. Biochem. Biophys.* 432:136–144.

PRUTER, C., and ZEBITZ, C. P. W. 1991. Effects of *Aphis fabae* and *Uromyces viciae* favae on the growth of a susceptible and an aphid resistant cultivar of *Vicia faba*. *Ann. Appl. Biol.* 119:215–226.

SHANNAG, H. K., and ABABNEH, J. A. 2007. Influence of black bean aphid, *Aphis fabae* Scopoli. on growth rates of faba bean. *World J. Agric. Sci.* 3:344–349.

TASIN, M., BACKMAN, A. C., CORACINI, M., CASADO, D., IORIATTI, C., and WITZGALL, P. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. *Phytochemistry* 68:203–209.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

VISSER, J. H., PIRON, P. G. M., and HARDIE, J. 1996. The aphids' peripheral perception of plant volatiles. *Entomol. Exp. Appl.* 80:35–38.

WADHAMS, L. J. 1982. Coupled gas-chromatography–single cell recording—a new technique for use in the analysis of insect pheromones. *Z. Naturforsch. C* 37:947–952.

WADHAMS, L. J. 1990. The use of coupled gas chromatography: electrophysiological techniques in the identification of insect pheromones, pp. 289–298, in A. R. McCaffery, and I. D. Wilson (eds.). Chromatography and Isolation of Insect Hormones and PheromonesPlenum, New York.

WILLIAMS, L., RODRIGUEZ-SAONA, C., PARE, P. W., and CRAFTS-BRANDNER, S. J. 2005. The piercing-sucking herbivores *Lygus hesperus* and *Nezara viridula* induce volatile emissions in plants. *Arch. Insect Biochem.* 58:84–96.

2007). Like other bark beetle species belonging to the genus *Tomicus* Latreille, *T. destruens* has two dispersal phases per generation: mature adults seek recently cut or dying pines for reproduction, whereas newly emerged callow adults fly toward healthy trees and tunnel into vigorous shoots to become sexually mature (Chararas 1962). Maturation feeding continues for several months, making trees susceptible to stem attacks the following year. Host selection by bark beetles is governed mainly by volatile attractive signals (Wood 1982). In many cases, individuals of only one sex (pioneers) find suitable hosts, begin the attack, and then release a species-specific aggregation pheromone (Byers 1989). Individuals of the nonhost-searching sex join the pioneers, simply by orienting to the pheromone. Although a sexual attractant has been reported in *T. destruens* (Carle 1974, 1978; Carle et al. 1978), an effective aggregation pheromone has never been found, and adult aggregations appear to be mediated only by host volatiles (Guerrero et al. 1997). However, depending on their degree of maturation, adults appear to be attracted to volatiles emitted by either dying or healthy pines, and thus, flying adults need effective cues to find suitable breeding or feeding material (Guerrero et al. 1997). In some cases, efforts to trap *T. destruens* have been made with commercially available lures for *Tomicus piniperda*, a closely related species that lives mainly on Scots pine (*Pinus sylvestris*) and often considered synonymous with *T. destruens* until a recent systematic revision (Faccoli et al. 2005c; Faccoli 2006). The bait contains a synergistic blend of the host-volatile α-pinene and ethanol (Czokajlo and Teale 1999), with or without 3-carene and α-terpinolene (Byers et al. 1985; Schröeder and Eidmann 1987; Poland et al. 2004). α-Pinene is one of the main monoterpenes released by conifers, and ethanol is a product of the anaerobic fermentation of phloematic sugars in weakened and dying trees (Kimmemer and Kozlowski 1982). However, the number of *T. destruens* adults captured in traps baited only with α-pinene and ethanol is always very low (Sabbatini Peverieri et al. 2004; Vasconcelos et al. 2005) when compared with catches of other bark beetles. Thus, *T. destruens* adults probably follow more specific chemical cues than α-pinene and ethanol, as they are able to recognize the right host species, its physiological state (healthy or dying), as well as specific parts of the host tree (shoots or bark).

Few studies have focused on maturation feeding of *Tomicus* species (Långström 1980, 1983; Ye 1996; Ye and Lieutier 1997; Poland and Haack 2000). Långström et al. (2002) suggest that, if aggregation occurs during the shoot-feeding phase, it could be mediated by visual and/or chemical cues. Eidmann (1977) reported that callow adults are guided from brood logs toward pine shoots by visual stimuli, and Långström et al. (2002) noted that Yunnan pines (*Pinus yunnanensis*) with a high level of shoot infestation tended to have a canopy larger than trees with little damage. Borg-Karlson et al. (1999) found that β-pinene contents were higher in more severely rather than only slightly damaged Yunnan pines. Nevertheless, it was not clear if trees that released more (−)β-pinene were preferred by callow adults or if the observed differences among monoterpene profiles were the result of the attack. However, these studies were performed on *T. piniperda*, and there is no information on the chemical and/or physical stimuli that govern shoot selection by callow adults of *T. destruens*. The identification of semiochemicals attractive to both callow and mature adults of *T. destruens* should allow the development of effective control programs, based on traps baited with different lures in relation to season and degree of maturation of the insects. The aims of the present study were: (1) to identify the volatiles released by the shoots and bark of stone pine (*Pinus pinea*) that are behaviorally and electrophysiologically active on *T. destruens* and (2) to verify if the blends and concentrations of such volatiles effect callow and mature males and females differently, during the two host search phases (breeding and feeding) of the insect's biology.

Methods and Materials

Sample Collection and Handling Callow adults of *T. destruens* were obtained under laboratory conditions [21 ± 1°C, 70% R.H., 16:8 h (L:D) photoperiod] from naturally infested logs of stone pine (10 cm in diameter, 30 cm long). Emerging callow adults were removed daily from the cages and starved at room temperature for 24 h before the beginning of the trials. Mature adults were collected during winter 2006 in a stone pine forest growing in NE Italy (Caorle (VE), 45° 54′ N; 12° 36′ E) from attacked pine shoots on the litter. Both callow and mature adults were sexed by using acoustical and morphological features (Faccoli 2006). Host material used for chemical and behavioral studies, including logs and shoots of healthy pine, was collected from the same stand as the insects.

Odor Collection Headspace collections were made from fresh shoots and bark of stone pine over 24 h in a climatic chamber at 25 ± 2°C, 60 ± 10% R.H., 16:8 h (L:D) photoperiod, 1,000 lx during the light period. Five shoots (approximately 150 g) or one freshly cut log (approximately 800 g, length 20 cm, diameter 10 cm), with the cut ends sealed with paraffin, were placed into 2,000-ml glass jars (Tasin et al. 2005) and charcoal-filtered air was pumped through the system at 150 ml/min and over a Porapak Q cartridge that contained 50 mg of adsorbent (Sigma-Aldrich, Milan, Italy). Volatiles were desorbed by eluting the cartridge with 500 µl

of redistilled hexane. Additional extracts (N=5) of both shoots and bark were prepared for chemical quantification, and 0.5 μg of heptyl acetate (purity≥99%) were added as an internal standard (Bengtsson et al. 2001). Sample volumes were reduced to 50 μl at room temperature, sealed in glass vials, and stored at −18°C until used.

Chemical Analysis Five samples of both extracts were analyzed with a Hewlett-Packard 5890 gas chromatograph (GC), with a polar Innowax column (30 m×0.32 mm; J & W Scientific, Folsom, CA, USA) programmed from 60°C (hold 3 min) at 8°C min^{-1} to 220°C (hold 7 min) and interfaced with a Hewlett-Packard 5970B MS with electron impact ionization (70 eV). The identity of most compounds from the volatile collections was verified by comparison with synthetic compounds purchased from Sigma-Aldrich and Fluka Chemie (Buchs, Switzerland). Compounds that did not elicit antennal responses, and for which no standards were available, were tentatively identified by using the Wiley mass spectral database. Identified compounds were quantified by comparing their peak areas with that of the internal standard (Zhang et al. 2007).

Gas Chromatography and Electroantennographic Detection Two microliters of each extract were injected onto the same GC setup mentioned above, interfaced with an electroantennogram apparatus (Arn et al. 1975). The outlet of the GC column was split in a 1:1 ratio between the flame ionization detector (FID) and an antenna of *T. destruens*. A glass capillary indifferent electrode filled with Kaissling solution (Kaissling 1987) added with 5 g l^{-1} polyvinylpyrrolidone K90 (Fluka Chemie) was inserted into the severed head of the beetle, while the different electrode in a similar pipette was brought into contact with the distal end of the antennal club. Compounds that eluted from the capillary column were delivered to the antenna through a glass tube (12 cm × 8 mm) by a charcoal-filtered and humidified airstream. The antennal and FID signals were amplified and recorded simultaneously by Syntech software (Hilversum, The Netherlands). Samples from both stone pine shoot and bark extracts were tested on ten males and ten females of *T. destruens*, five callow and five mature individuals for each sex. A compound was considered electrophysiologically active when it elicited antennal responses at least three times greater than background noise (Zhang et al. 2001). The absolute EAD amplitudes were calculated.

Behavioral Responses A four-arm olfactometer (from Pettersson 1970, modified by Vet et al. 1983) was used to test the behavioral responses of walking callow and mature adults to various sources of volatiles. Incoming air passed through an activated carbon filter (Whatman Carbon-Cap 75), before passing into separate but identical lines leading to each corner of the olfactometer. The air passed through a flowmeter (0–2 l/min, Gapmeter Type GT), allowing the fine adjustment of the flow, into a 300-ml glass jar that contained the odor source. Air was then drawn directly to the corners of the exposure chamber and exited through a tube placed in the center of the olfactometer floor, leading to a Plexiglas vial containing a test insect. The clearest boundaries of the flows, with no intermingling, occurred at a rate of 800 ml/min. Fluorescent tubes were attached to all the sides of the olfactometer, providing homogeneous, diffuse illumination. Only one arm of the olfactometer contained a test odor (Arm 1); the other arms were empty. To prevent possible disturbing effects of movement around the apparatus (Vet et al. 1983), the olfactometer was kept in a box with walls covered by white sheets, and a digital camera, positioned centrally above the chamber, was linked to a recording video monitor. Digital records were analyzed later, as described below. With this system, we tested 50 g of either fresh shoots or bark, 10 ml of collected volatiles, two blends of synthetic compounds α-pinene and β-myrcene (blend A) and α-pinene, β-myrcene, and α-terpinolene (blend B), and a blank (empty olfactometer). The synthetic blends were chosen based on EAG results and the known attraction of the compounds to other species of bark beetles. The blends were prepared at α-pinene, β-myrcene, and α-terpinolene ratios of 79:21:0 (blend A) and 18:80:2 (blend B) based on the ratios found from shoots and bark, respectively. The two blends were then tested at five concentrations, ranging from 10^{-2} to 10^{2} of the amounts collected from 150 g of fresh shoots and 800 g of a cut branch (Table 1). For each source, ten adults were tested according to sex (male–female) and maturation (callow–mature), except for the synthetic blends where sex was not considered as a variable. For every five insects tested, the whole apparatus was washed and Arm 1 randomly reassigned. Experiments were carried out under constant laboratory conditions (21 ± 1°C, 70% R.H.), assuming that samples kept at the same temperature and having the same size and similar physiological conditions release a similar concentration of volatiles. Each insect and each sample tissue was used only once.

Analysis of Data The digital record of the activity of each insect over 10 min in the olfactometer was analyzed by the Micro Measure program (Wye College Programs). A video-mixing desk was used to lay a computer-generated map of the olfactometer on the video image. The borders of the odor fields were drawn on the computer image and labeled as zones. All the tracks of the insect were traced from the video image by a cursor. For each insect tested, and for each arm of the olfactometer, the program measured time spent walking (second), time spent stationary (second), and

total time spent (second) in each arm. Data were analyzed by multiway analysis of variance to find statistical differences in time spent by adults in Arm 1, in relation to their degree of maturation, sex, and tested odor source. Possible interactions among the three variables were also analyzed. Homogeneity of variance was tested by Cochran's test (test C) and normality by Kolmogor Smirnov's test (test D); when necessary, data were log-transformed ($X'=\log(x+1)$) or arcsine-transformed ($X'=\arcsin\sqrt{x}$) to obtain homogeneous data and normal variance. Whenever significant differences occurred, Tukey's honestly significant difference multiple comparison test was applied for separation of means (Zar 1999). Differences at the 0.05 level of confidence, adjusted by Bonferroni correction for mass comparison, were considered significant. For each compound, differences in the absolute EAD responses (millivolt) of males and females, either mature or callow, were evaluated by using Student's *t* test. Analyses were performed by STATISTICA® 3.1 for WINDOWS® software (Statistica®, Tulsa, OK, USA).

Results

Chemical Analysis Monoterpenes and sesquiterpenes were identified from both shoot and bark samples. (*Z*)-3-hexenol and 3 hydrocarbons were collected only from shoot samples, while among the terpenes, α-terpinolene, limonene oxide, (*Z*)-limonene oxide, and α-longipinene were found only in bark, whereas (*E*)-furanic-linalool oxide, (*Z*)-furanic-linalool oxide, and germacrene-D were found only in shoots (Table 1). Furthermore, shoots and bark also differed in the proportion of α-pinene and β-myrcene, with shoots having higher amounts of α-pinene (78.58:21.42, respectively) while bark had more β-myrcene (17.78:80; Table 1). Limonene represented more than 80% of the total blend of both shoots and bark (Table 1).

Gas Chromatography and Electroantennographic Detection GC–electroantennography (EAD) analyses of headspace collections detected six active compounds (Table 1), of which the monoterpenes α- pinene, β-myrcene, and

Table 1 Volatile compounds collected in headspace of *P. pinea* bark and shoots and antennal activity in GC–EAD experiments on *T. destruens* adults

Compounds	Bark (μg/h)[a]	Bark (%)[b]	GC–EAD[c] activity on mature adults	Shoots (μg/h)	Shoots (%)	GC–EAD[c] activity on callow adults
Alcohols						
(*Z*)-3-hexen-1-ol	–	–		0.41	0.45	*
Hydrocarbons						
Tridecane	–	–		0.25	0.28	
Tetradecane	–	–		0.49	0.54	
Pentadecane	–	–		0.38	0.42	
Monoterpenes						
α-Pinene	0.24	1.42	*	7.63	8.27	*
β-Pinene	0.03	0.19		1.07	1.16	
β-Myrcene	1.08	6.43	*	2.08	2.26	*
Limonene	19.90	100.00	*	92.30	100.00	*
Sabinene[d]	0.02	0.15		3.87	4.20	
α-terpinolene	0.03	0.18	*	–	–	
(*E*)-limonene oxide[d]	0.02	0.12		–	–	
(*Z*)-limonene oxide[d]	0.08	0.48		–	–	
(*E*)-furanic-linalool oxide	–	–		0.81	0.88	
(*Z*)-Furanic-linalool oxide	–	–		0.69	0.75	
Sesquiterpenes						
α-Longipinene[d]	0.02	0.13		–	–	
Junipene [d]	0.03	0.23		0.90	0.98	
β-Caryophyllene	0.15	0.92	*	2.04	2.22	*
Humulene	0.02	0.13		0.30	0.33	
Germacrene-D	–	–		1.81	1.97	

[a] Average amounts collected from 150 g of fresh shoots and 800 g of a cut branch were 92.3 ± 13.8 μg/h and 16.9 ± 4.4 μg/h, respectively.
[b] Reported percentages are relative to limonene (100).
[c] Asterisks: compounds eliciting responses in *T. destruens* antennae during GC–EAD experiments (see Table 2).
[d] Compound identified with Wiley mass spectral database.

α-terpinolene were the most active (Table 2). These were subsequently tested in the olfactometer in synthetic blends with and without α-terpinolene (see "Methods and Materials"). Four active compounds were common to both mature and callow adults (Table 1), but only mature individuals detected α-terpinolene released from bark, while (*Z*)-3-hexen-1-ol released by shoots was detected only by callow individuals (Table 1). Both sexes of callow and mature individuals showed similar EAD responses for all the tested sources (Table 2).

Behavioral Responses Because in both callow ($F1, 98 = 0.79$, $P=0.38$; Fig. 1, a) and mature adults ($F1, 98=1.52$, $P=0.24$; Fig. 1, b), males and females showed similar responses when exposed to the same source, data for the sexes were pooled. Callow insects were most attracted to shoots and their extracts ($F4, 96=7.83$, $P<0.01$; Fig. 1, a), while mature insects were more attracted to bark and its extracts ($F4, 96=8.29$, $P<0.01$; Fig. 1, b). The responses of callow adults to bark, and mature insects to shoots, did not differ from controls (Fig. 1). With increased concentrations of blends A and B, both callow and mature adults showed an increased response (Fig. 2), which became significantly higher than controls with concentrations equal to or higher than 1 ($F5, 58=6.22$, $P<0.05$ and $F5, 58=3.46$, $P<0.001$, respectively; Fig. 2).

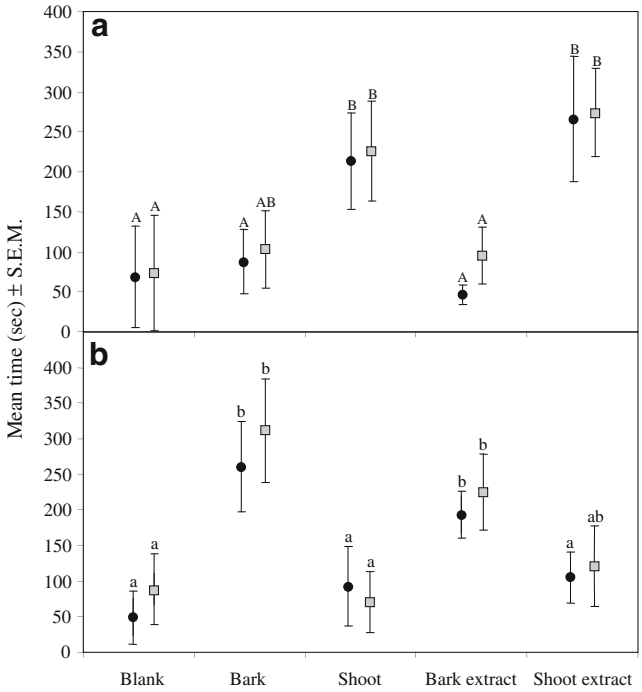

Fig. 1 Mean time spent by males and females of callow (*a*) and mature (*b*) *T. destruens* in the olfactometer test arm containing different odors (arm 1; ten insects per sex, degree of maturation, and odor, for a total of 200 insects). *Black dots*: females; *gray squares*: males. Differences analyzed by two-way ANOVA on odors and sexes, separately for callow and mature adults. Different *letters*: statistical differences by Tukey test ($P<0.05$)

Discussion

Maturation feeding in bark beetles often has been neglected, except in a few species that vector diseases, such as *Scolytus* spp. associated with Dutch Elm Disease and *Phloeosinus* spp. associated with Cypress Canker Disease (Webber and Brasier 1984; Webber and Gibbs 1989). Even in these cases, studies have focused mainly on the role of insects as vectors rather than on the semiochemical interactions between host tree and callow adults. Our results indicate that the behavior of *T. destruens* is governed by specific volatile cues and affected by adult maturation, with callows showing higher responses to shoot extracts and matures to bark volatiles. It has been demonstrated that the behavior of many insects may be affected by previously encountered odors, but the clear

Table 2 Antennal responses of *T. destruens* males and females to volatile compounds emitted from *P. pinea* as determined by GC–EAD with headspace collections from bark and shoots on both callow and mature adults

GC–EAD active compounds	EAD responses (mV) ± SD							
	Mature adults				Callow adults			
	Males	Females	Student's *t* test *df*=4		Males	Females	Student's *t* test *df*=4	
(Z)-3-hexen-1-ol	–	–	–	–	0.04 ± 0.01	0.05 ± 0.02	*t*=0.9	*P*=0.4
α-Pinene	0.12 ± 0.02	0.11 ± 0.02	*t*=0.7	*P*=0.5	0.13 ± 0.02	0.13 ± 0.01	*t*=0.0	*P*=1.0
β-Myrcene	0.11 ± 0.02	0.11 ± 0.03	*t*=0.2	*P*=0.9	0.10 ± 0.02	0.11 ± 0.02	*t*=0.5	*P*=0.7
Limonene	0.06 ± 0.01	0.07 ± 0.01	*t*=1.0	*P*=0.4	0.06 ± 0.02	0.05 ± 0.02	*t*=0.8	*P*=0.5
α-Terpinolene	0.11 ± 0.03	0.12 ± 0.01	*t*=0.4	*P*=0.7	–	–	–	–
β-Caryophyllene	0.09 ± 0.01	0.09 ± 0.02	*t*=0.3	*P*=0.8	0.08 ± 0.02	0.09 ± 0.03	*t*=0.7	*P*=0.5

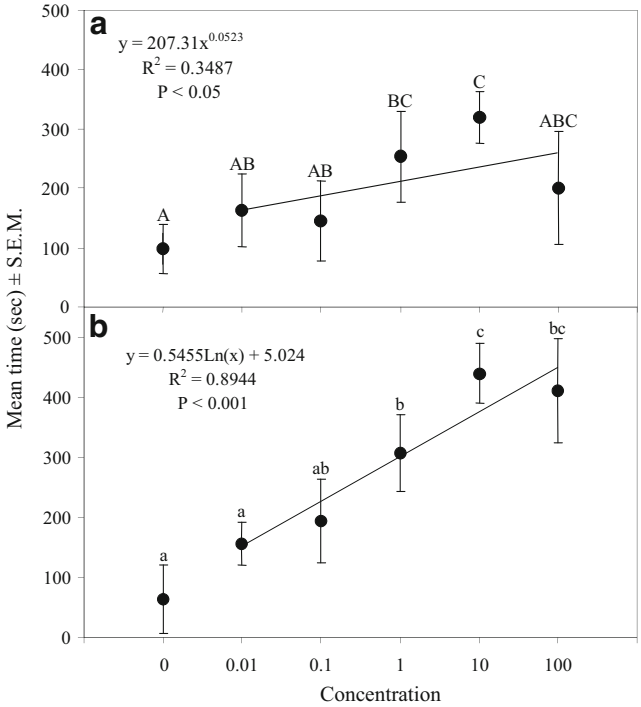

Fig. 2 Mean time spent by callow *T. destruens* in the arm containing the "blend A" (α-pinene and β-myrcene; *a*) and mature individuals in the arm with the "blend B" (α-pinene, β-myrcene, and α-terpinolene; *b*; ten insects per degree of maturation, blend, and concentration, for a total of 120 insects). 0 is the blank (empty olfactometer), whereas 1 is the concentration of volatiles collected from 150 g of fresh shoots and 800 g of a cut branch (Table 1). Values followed by different *letters* are statistically different (Tukey test; $P<0.05$)

responses shown by naive newly emerged callow adults excludes a similar mechanism in this species. Also the intensity of the odor can affect its activity, and several species fail to respond to either low or high concentrations of a kairomone (Jones et al. 1971). However, in our study, concentrations equal to or higher than those naturally released by bark and shoots caused significant alterations in behavior compared with control (clean air). The responses were always positive, although high concentrations of the monoterpenes tested in the blend B may have a repellent effect, as observed in other bark beetle species (Faccoli et al. 2005b).

In many species of scolytids, the host selecting sex (pioneer) tend to be the most sensitive to host volatiles. For example, the males of *Ips typographus* responded more than females (Schlyter et al. 1989; Zhang and Schlyter 2004; Faccoli et al. 2005b, c), and females, which have fewer α-pinene receptors, locate host plant by following male pheromones (Dickens 1981). Similar results were found also in *Scolytus rugulosus* (Ascher et al. 1975) and *Pityogenes* species (Byers et al. 1998, 2004). However, the system is different in *Tomicus* species. Both sexes of *T. piniperda* are equally attracted to pine logs or shoots and host monoterpenes (Långström 1980, 1983; Byers et al. 1985; Lanne et al. 1987), and our results suggest this is also true for *T. destruens*.

An aggregation pheromone has not been found for *T. destruens* or in the more frequently studied species *T. piniperda* (Perttunen et al. 1970; Byers et al. 1985; Lanne et al. 1987). In our experiments, the only compounds that elicited antennal responses in *T. destruens* were four monoterpenes (a-pinene, β-myrcene, a-terpinolene, limonene), one sesquiterpene (β- caryophyllene), and one alcohol ((*Z*)-3-hexen-1-ol). These are common plant volatiles and, depending on the species of bark beetle, may act as attractants (Byers 1995; Poland et al. 2004) or repellents (Poland and Haack 2000; Wallin and Raffa 2000; Byers et al. 2004). Our results suggest that α-pinene, α-terpinolene, and β-myrcene are compounds that modulate host location in *T. destruens* but that the specific responses are different for callow and mature beetles. We propose that this discrimination is based on the presence of α-terpinolene only in bark and on the different ratios of α-pinene and β-myrcene released from needles and bark (see Table 1). In particular, β-myrcene, inducing responses in both mature and callow adults, may have a major role in host selection, as it is one of the most common components of oleoresins of Mediterranean pines (Macchioni et al. 2003) but only occurs in small amounts in continental pine species (Idzojtic et al. 2005). In addition, β-myrcene is not attractive to *T. piniperda* (Byers et al. 1985), and although recent papers reported positive responses of some Chinese populations of *T. piniperda* to β-myrcene released by *P. yunnanensis* (Borg-Karlson et al. 1999; Zhao et al. 2002), these populations were later ascribed to a new species (*Tomicus yunnanensis*; Duan et al. 2004; Kirkendall et al. 2008). It is possible that visual cues also play a role in the location of specific sites on the host plant (Långström et al. 2002), but this was not a parameter considered in our study. Additional studies to examine this possibility, as well as the role of ethanol released by weakened trees and known to attract the congeneric *T. piniperda* (Schröeder and Lindelöw 1989) need to be performed.

In summary, mature adults of *T. piniperda* and *T. destruens* respond to similar blends, but for *T. piniperda* β-myrcene is replaced by 3-carene (Byers et al. 1985; Schröeder and Eidmann 1987), a monoterpene common on continental pines (Idzojtic et al. 2005). This, together with different climatic requirements, may explain the preference of *T. destruens* for Mediterranean pines and *T. piniperda* for the continental ones. Our results show promising possibilities for the use of infochemicals in the protection of Mediterranean pine forests from bark beetle attacks, but additional research on the odor signals that mediate host location by *T. destruens* is still necessary.

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References

ARN, H., STÄDLER, E., and RAUSCHER, S. 1975. The electroantennographic detector—a selective and sensitive tool in the gas chromatographic analysis of insect pheromones. *Z. Naturforsch.* 30:722–725.

ASCHER, K. R. S., GUREVITZ, E., RENNEH, S., and NEMNY, N. E. 1975. The penetration of females of the fruit bark beetle *Scolytus mediterraneus* Eggers into antifeedant-treated twigs in laboratory tests. *Zeit. Pflanz. Pflanz.* 82:378–383.

BENGTSSON, M., BÄCKMAN, A.-C., LIBLIKAS, I., RAMIREZ, M. I., BORG-KARLSON, A. K., ANSEBO, L., ANDERSON, P., LÖFQVIST, J., and WITZGALL, P. 2001. Plant odor analysis of apple: antennal response of codling moth females to apple volatiles during phenological development. *J. Agric. Food Chem*. 49:3736–3741.

BORG-KARLSON, A. K., PERSSON, M., CHRISTIANSSON, A., FÄLDT, J., LÅNGSTRÖM, B., LI, L., LIU, H., ZHOU, N., and LIEUTIER, F. 1999. Relative amounts and enantiomeric compositions of monoterpene hydrocarbons in *Pinus yunnanensis* and *Pinus sylvestris*, pp. 101–110, in F. Lieutier, W. J. Mattson, and M. R. Wagner (eds.). Physiology and Genetics of Tree–Phytophage Interactions. INRA Editions, Versailles.

BYERS, J. A. 1989. Chemical ecology of bark beetles. *Experientia* 45:271–283.

BYERS, J. 1995. Host-tree chemistry affecting colonization in bark beetles, pp. 154–193, in R. T. Cardè, and W. J. Bell (eds.). Chemical Ecology of Insects, 2. Chapman & Hall, New York.

BYERS, J. A., LANNE, B. S., LÖFQVIST, J., SCHLYTER, F., and BERGSTRÖM, G. 1985. Olfactory recognition of host-tree susceptibility by pine shoot beetles. *Naturwissenschaften* 72:324–326.

BYERS, J. A., ZHANG, Q. H., SCHLYTER, F., and BIRGERSSON, G. 1998. Volatiles from nonhost birch trees inhibit pheromone response in spruce bark beetles. *Naturwissenschaften* 85:557–561.

BYERS, J. A., ZHANG, Q. H., and BIRGERSSON, G. 2004. Avoidance of nonhost plants by a bark beetle, *Pityogenes bidentatus*, in a forest of odors. *Naturwissenschaften* 91:215–219.

CARLE, P. 1974. Mise en évidence d'une attraction secondaire d'origine sexuelle chez *Blastophagus destruens* Woll. (Col. Scolytidae). *Ann. Zool. et Écol. Anim.* 6:539–550.

CARLE, P. 1978. Essais d'attraction en laboratoire et en forêt de *Blastophagus* (*piniperda* L. et *destruens* Woll.). *Colloques de l'INRA* 38:92–101.

CARLE, P., DESCOINS, C., and GALLOIS, M. 1978. Phéromones des *Blastophagus* (*piniperda* L. et *destruens* Woll.). *Colloques de l'INRA* 38:87–91.

CHARARAS, C. 1962. Scolytides des Conifères. Encyclopédie Entomologique 37. Lechevalier, Paris.

Czokajlo, D., and Teale, S. A. 1999. Synergistic effect of ethanol to α-pinene in primary attraction of the larger pine shoot beetle, *Tomicus piniperda*. *J. Chem. Ecol.* 25:1121–1130.

DICKENS, J. C. 1981. Behavioral and electrophysiological responses of the bark beetle *Ips typographus* to potential pheromone components. *Phys. Entomol.* 6:251–261.

DUAN, Y., KERDELHUÉ, C., YE, H., and LIEUTIER, F. 2004. Genetic study of the forest pest *Tomicus piniperda* (Coleoptera, Scolytidae) in Yunnan Province (China) compared to Europe: new insights for the systematic and evolution of the genus *Tomicus*. *Heredity* 93:416-422.

EIDMANN, H. H. 1977. Recognition of the trophic environment in requisite governed forest insects. *Coll. Internat. C.N.R.S Paris* 265:151–163.

FACCOLI, M. 2006. Morphological separation of *Tomicus piniperda* and *T. destruens* (Coleoptera, Curculionidae, Scolytinae): new and old characters. *Eur. J. Entomol.* 103:433–442.

FACCOLI, M. 2007. Breeding performance and longevity of *Tomicus destruens* on Mediterranean and continental pine species. *Ent. Exp. Appl.* 123:266–269.

FACCOLI, M., BATTISTI, A., and MASUTTI, L. 2005a. Phenology of *Tomicus destruens* (Wollaston) in northern Italian pine stands, pppp. 185–193, in F. Lieutier, and D. Ghaioule (eds.). Entomological Research in Mediterranean Forest Ecosystems. INRA Editions, Paris.

FACCOLI, M., BLAŽENEC, M., and SCHLYTER, F. 2005b. Feeding response to host and non-host compounds by males and females of the spruce bark beetle *Ips typographus* in a tunnelling microassay. *J. Chem. Ecol*. 31:745–759.

FACCOLI, M., PISCEDDA, A., SALVATO, P., SIMONATO, M., MASUTTI, L., and BATTISTI, A. 2005c. Phylogeography of the pine shoot beetles *Tomicus destruens* and *T. piniperda* (Coleoptera Scolytidae) in Italy. *Ann. Forest Sci.* 62:361–368.

GUERRERO, A., FEIXAS, J., PAJARES, J., WADHAMS, L. J., PICKETT, J. A., and WOODCOCK, C. M. 1997. Semiochemically induced inhibition of behavior of *Tomicus destruens* (Woll.) (Coleoptera Scolytidae). *Naturwissenschaften* 84:155–157.

IDZOJTIC, M., KAJBA, D., and FRANJIC, J. 2005. Differentiation of F1 hybrids *P. nigra* J. F. Arnold x *P. sylvestris* L., *P. nigra* J. F. Arnold x *P. densiflora* Siebold et Zucc., *P. nigra* J. F. Arnold x *P. thunbergiana* Franco and their parental species by needle volatile composition. *Biochem. Syst. Ecol.* 33:427–439.

JONES, R. L., LEWIS, W. J., BOWMAN, M. C., BEROZA, M., and BIERL, B. A. 1971. Host-seeking stimulant for a parasite of the corn earworm: isolation, identification and synthesis. *Science* 173:842–843.

KAISSLING, K.-E. 1987. R. H. Wright Lectures on Insect Olfaction. Colbow K., Simon Fraser University, Burnaby, B.C., Canada.

KIMMERER, T. W., and KOZLOWSKI, T. T. 1982. Ethylene, ethane, acetaldehyde and ethanol production by plants under stress. *Plant Phys.* 69:840–847.

KIRKENDALL, L. R., FACCOLI, M., and YE, H. 2008. Description of the Yunnan shoot borer *Tomicus yunnanensis* (Curculionidae, Scolytinae), an unusually aggressive pine shoot beetle from southern China, with a key to all *Tomicus*. *Zootaxa* 1819:25–39.

LÅNGSTRÖM, B. 1980. Distribution of pine shoot beetle attacks within the crown of Scots pine. *Studia For. Suec.* 154:1–25.

LÅNGSTRÖM, B. 1983. Life cycles and shoot-feeding of the pine shoot beetles. *Studia For. Suec.* 163:1–29.

LÅNGSTRÖM, B., LISHA, L., HONGPIN, L., PENG, C., HAORAN, L., HELLQVIST, C., and LIEUTIER, F. 2002. Shoot feeding ecology of *Tomicus piniperda* and *T. minor* (Coleoptera Scolytidae) in southern China. *J. Appl. Entomol.* 126:333–342.

LANNE, B. S., SCHLYTER, F., BYERS, J. A., LÖFQVIST, J., LEUFVÉN, A., BERGSTRÖM, G., VAN DER PERS, J. N. C., UNELIUS, R., BAECKSTROM, P., and NORIN, T. 1987. Differences in attraction to semiochemicals present in sympatric pine shoot beetles *Tomicus minor* and *T. piniperda*. *J. Chem. Ecol.* 13:1045–1067.

MACCHIONI, F., CIONI, P. L., FLAMINI, G., MORELLI, I., MACCIONI, S., and ANSALDI, M. 2003. Chemical composition of essential oils from needles, branches and cones of *Pinus pinea*, *P. halepensis*, *P. pinaster* and *P. nigra* from central Italy. *Flavour Fragr. J.* 18:139–143.

PERTTUNEN, V., OKSANEN, H., and KANGAS, E. 1970. Aspects of the external and internal factors affecting the olfactory orientation of *Blastophagus piniperda* (Coleoptera, Scolytidae). *Contrib. Boyce Thompson Inst.* 24:293–297.

PETTERSSON, J. 1970. An aphid sex attractant, I. Biological studies. *Entomol. Scand.* 1:63–73.

POLAND, T. M., and HAACK, R. A. 2000. *Tomicus piniperda* (Coleoptera: Scolytidae): is shoot-feeding required for reproductive maturation? *The Great Lakes Entomol.* 33:9–15.

POLAND, T. M., dE GROOT, P., BURKE, S., WAKARCHUK, D., HAACK, R. A., and NOTT, R. 2004. Semiochemical disruption of the pine shoot beetle, *Tomicus piniperda* (Coleoptera: Scolytidae). *Environ. Entomol.* 33:221–226.

SABBATINI PEVERIERI, G., FAGGI, M., MARZIALI, L., PANZAVOLTA, T., BONUOMO, L., and TIBERI, R. 2004. Use of attractant and repellant substances to control *Tomicus destruens* (Coleoptera: Scolytidae) in *Pinus pinea* and *P. pinaster* pine forests of Tuscany. *Entomologica* 38:91–102.

SCHLYTER, F., BIRGERSSON, G., and LEUFVÉN, A. 1989. Inhibition of attraction to aggregation pheromone by verbenone and ipsenol. Density regulation mechanisms in bark beetle *Ips typographus*. *J. Chem. Ecol.* 15:2263–2277.

SCHRÖEDER, L. M., and EIDMANN, H. H. 1987. Gallery initiation by *Tomicus piniperda* (Coleoptera: Scolytidae) on Scots pine trees baited with host volatiles. *J. Chem. Ecol.* 13:1591–1599.

SCHRÖEDER, L. M., and LINDELÖW, A. 1989. Attraction of scolytids and associated beetles by different absolute amounts and proportions of alpha-pinene and ethanol. *J. Chem. Ecol*. 15:807–817.

TASIN, M., ANFORA, G., IORIATTI, C., CARLIN, S., DE CRISTOFARO, A., SCHMIDT, S., BENGTSSON, M., VERSINI, G., and WITZGALL, P. 2005. Antennal and behavioral responses of grapevine moth *Lobesia botrana* females to volatiles from grapevine. *J. Chem. Ecol.* 31:77–87.

VASCONCELOS, T., BRANCO, M., GONCALVES, M., and CaBRAL, M. T. 2005. Periods of flying activity of *Tomicus* spp. in Portugal, pppp. 177–184, in F. Lieutier, and D. Ghaioule (eds.). Entomological Research in Mediterranean Forest Ecosystems. INRA Editions, Paris.

VET, L. E. M., VAN LENTEREN, J. C., HEYMANS, M., and MEELIS, F. 1983. An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. *Phys. Entomol.* 8:97–106.

WALLIN, K. F., and RAFFA, K. F. 2000. Influences of host chemicals and internal physiology on the multiple steps of post-landing host acceptance behavior of *Ips pini* (Coleoptera: Scolytidae). *Environ. Entomol.* 29:442–453.

WEBBER, J. F., and BRASIER, C. M. 1984. The transmission of Dutch Elm Disease: a study of the process involved, pp. 271–307, in J. Anderson, A. D. M. Rayner, and D. Walton (eds.). Invertebrate Microbial Interactions. Cambridge University Press, Cambridge.

WEBBER, J. F., and GIBBS, J. N. 1989. Insect dissemination of fungal pathogens of trees, pp. 161–189, in N. Wilding, N. M. Collins, P. M. Hammond, and J. F. Webber (eds.). Insect–Fungus Interactions. Academic, London.

WOOD, D. L. 1982. The role of pheromones, kairomones, and allomones in the host selection and colonization behavior of bark beetles. *Annu. Rev. Entomol*. 27:411–446.

YE, H. 1996. Studies on the biology of *Tomicus piniperda* (Col., Scolytidae) in the shoot feeding period. *Acta Entomol. Sin.* 39:58–62.

YE, H., and LIEUTIER, F. 1997. Shoot aggregation by *Tomicus piniperda* (L.) (Coleoptera Scolytidae) in Yunnan, South-western China. *Ann. Forest Sc.* 54:635–641.

ZAR, J. H. 1999. Biostatistical Analysis. Prentice Hall, New Jersey.

ZHANG, Q.-H., and SCHLYTER, F. 2004. Olfactory recognition and behavioural avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agr. For. Entomol.* 6:1–20.

ZHANG, Q., LIU, G., SCHLYTER, F., BIRGERSSON, G., ANDERSON, P., and VALEUR, P. 2001. Olfactory responses of *Ips duplicatus* from inner Mongolia, China to nonhost leaf and bark volatiles. *J. Chem. Ecol.* 27:995–1009.

ZHANG, Q.-H., SCHLYTER, F., LIU, G., SHENG, M., and BIRGERSSON, G. 2007. Electrophysiological and behavioral responses of *Ips duplicatus* to aggregation pheromone in Inner Mongolia, China: amitinol as a potential pheromone component. *J. Chem. Ecol.* 33:1303–1315.

ZHAO, T., LI, L. S., and ZHOU, N. 2002. The attraction of Yunnan pine to pine shoot beetle and tree volatile compositions. *J. Northeast For. Univ.* 30:47–49.

often longer. An effective portable insect trap baited with a semiochemical attractant that could provide earlier detection of infestations has not yet been developed. Although large-panel, sticky traps that make use of the color purple as a visual cue and are baited with ash logs are attractive to emerald ash borer (Francese et al. 2005); they are not sufficiently sensitive or easy to use for this purpose.

Adult beetles feed on ash leaves for a week or more before mating (Lyons et al. 2007). Studies by Otis et al. (2005) and Lelito et al. (2007) revealed that males use visual cues to locate females, and that they actively search for females on ash leaflets in the tree canopy. To date, there is no evidence that the emerald ash borer uses long-range pheromones to locate mates (Rodriguez-Saona et al. 2006; Lelito et al. 2007), but the borer is attracted by host volatiles (Rodriguez-Saona et al. 2006; Crook et al. 2008). Manuka oil, which contains high amounts of several antennally active sesquiterpenes found in ash bark, is attractive to emerald ash borer and shows promise in baited traps (Crook et al. 2008). Rodriguez-Saona et al. (2006) demonstrated that at least 16 compounds emitted by Manchurian ash (*F. mandshurica* Rupr.) were antennally active to both sexes of emerald ash borer, which feeds on Manchurian ash in northeast Asia (Yu 1992). The compounds that elicited the strongest and most consistent antennal activity in coupled gas chromatography-electroantennogram detection (GC-EAD) bioassays were the green leaf volatiles (GLVs). These C_6 aldehydes, alcohols, and acetates are produced in large amounts by angiosperm trees (Zhang and Schlyter 2004). Their release rates are elevated by various stress factors, including damage caused by insect feeding (Ruther et al. 2000; Cossé et al. 2006; Rodriguez-Saona et al. 2006). Our preliminary chemical analysis of leaf volatiles from green ash and white ash hosts by using the solid phase micro-extraction (SPME) sampling technique, and analysis of homogenated foliage extracts, revealed the presence of several GLVs that consistently provided strong GC-EAD responses (unpublished data, Poland et al. 2004, 2006). We hypothesized that the emerald ash borer would show attraction to GLVs emitted by ash trees native to North America. GLVs serve as key components in host attractants, as pheromone synergists or as sexual kairomones for a number of coleopteran folivores (Dickens et al. 1990; Dickens 2000; Ruther et al. 2000, 2002; Ruther 2004; Reinecke et al. 2002, 2006; Cossé et al. 2006; Fernandez et al. 2007). The antennal chemoreceptors of some species are tuned specifically to individual GLVs and respond to them with very high, pheromone-like sensitivity (Hansson et al. 1999; Larsson et al. 2001).

In this study, we examined the antennal and behavioral responses of male and female emerald ash borer to GLVs in leaf volatiles from green and white ash trees. We collected volatiles on Super-Q® by aeration of trees, used GC-EAD and GC-MS (gas chromatography-mass spectrometry) to screen and identify candidate compounds, and conducted a series of field experiments over 3 years to evaluate their attractiveness.

Methods and Materials

Aeration of Leaf Volatiles and Chemical Analysis Leaf volatiles were collected from four healthy green ash trees in Sault Ste. Marie, ON on 2, 5, and 23 August 2005, and from a single healthy white ash tree on 8 August and 1 September 2005. Sampling of volatiles took place between 12:30 and 16:30 h; sampling dates were chosen when the weather was warm and sunny. Green ash was received as nursery stock (35 mm diam) from Winkelmolen Nursery Ltd. (Lynden, ON) on 29 April 2005 and out-planted on 5 May 2005. The white ash tree was about 18 years old and located on a residential property.

Foliage was enclosed in bags (approximately 45×25 cm) made from Tedlar® (Richmond Aircraft Products, Norwalk, CA, USA). Two holes were cut at opposite ends of the bag, one fitted with an activated carbon filter for air intake and one fitted with a Super-Q volatile collection trap (VCT) (Analytical Research Systems, Inc., Gainesville, FL, USA), containing 30 mg Alltech Super-Q® adsorbent material. The traps were held tightly inside 1/4 inch Teflon® tubing that was secured to the Tedlar bag with twist-ties and/or hose-clamps. Foliar headspace was sampled at 0.5 l/min with the aid of a Gilair-5 Personal Air Sampler (Sensidyne, Inc., Clearwater, FL, USA). Sampling periods varied from 0.5 to 2 h. Empty Tedlar bags placed in close proximity to the trees were sampled as controls.

The Super-Q VCTs were extracted with 3×50 µl of hexane (>99% purity, Acros) and analyzed by GC-MS on an HP5890 Series II GC fitted with a DB-1 column (25 m× 0.2 mm, with a 0.33-µm film thickness; J&W Scientific), and equipped with an HP5989A mass spectrometer. The GC temperature program started at 40°C held for 1 min, increased to 80°C at 8°C/min, and then to 260°C at 10°C/min, and finally to 325°C at 15°C/min, held for 5 min. The helium flow rate was 1 ml/min. GLVs were identified by comparing EI mass spectra with those from commercially available standards and against Wiley and National Institute of Standards and Technology libraries (Wiley275.L and Nist98.l), and by comparison of retention times with authentic standards. Extracts and GLV standards were also analyzed on a Varian 3500 GC fitted with a SPI injector and a DB-17 column (30 m×0.25 mm with a 0.25-µm film thickness, J&W Scientific) with helium as the carrier gas (2.4 ml/min) for further confirmation of the identity of the GLV compounds. The temperature program started at 40°C

for 1 min, increased at 2°C/min to 70°C, and then increased to 250°C at 15°C/min and held for 1 min. The following GLV standards were used for chemical and GC-EAD analysis: hexanal (98% purity), (*E*)-2-hexenal (98%), hexanol (99%), (*Z*)-2-hexenol (95%), (*Z*)-3-hexenol (98%), (*E*)-3-hexenol (98%), (*E*)-2-hexenyl acetate (98%), (*Z*)-3-hexenyl acetate (98%), and hexyl acetate (99%), obtained from Sigma-Aldrich Canada, and (*Z*)-3-hexenal (92% with 1–2% *E* isomer); (*E*)-2-hexenol (95%); and (*E*)-3-hexenyl acetate (98%) obtained from Bedoukian Research Inc (Danbury, CT, USA).

GC-EAD Detection Green and white ash extracts were analyzed by GC-EAD bioassay to identify GLVs that elicited electrophysiological responses from antennae of 10–40 days-old virgin male and female adults. Newly emerged adults were segregated by sex to provide virgin beetles, which do not mate until a week or more old.

GC-EAD bioassays were performed as previously described (Rodriguez-Saona et al. 2006; Grant et al. 2007). Two microliter of an extract were injected into a Varian 3400 GC fitted with a nonpolar HP-1 capillary column (25 m×0.20 mm with a 0.33-μm film thickness, Hewlett-Packard) with helium as the carrier gas. The GC temperature program started at 60°C for 1 min, increased at 10°C/min to 190°C, and then increased to 265°C at 35°C/min and held for 4 min. The column effluent was split 1:1, with one part going to the flame ionization detector of the GC and the other through a heated (225°C) transfer line (Syntech, Hilversum, Netherlands) into a humidified airstream (800 ml/min) directed at an excised emerald ash borer antenna. The cut ends of the antenna were inserted into small droplets of electrode gel (Signa Gel, Parker Labs, NJ, USA) held in small loops at the ends of gold wire electrodes connected to a Syntech portable INR-2 amplifier.

To compare the relative stimulatory effectiveness of the GLVs identified in the extracts, a synthetic mixture, which included hexanal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, hexanol, (*Z*)-3-hexenyl acetate, and hexyl acetate, was prepared in methylene chloride at a concentration of 7 ng/μl for each component. Two microliter of the mixture were injected into the GC operated with a temperature program that started at 60°C for 1 min, increased at 7°C/min to 80°C, and then increased to 120°C at 25°C/min and held for 1 min. Recordings were obtained from each of five male and female antennae from virgin adults. EAD responses were subjected to a two-way analysis of variance (ANOVA) with GLVs and sex as factors after square root transformation of data to meet the assumptions of normality and equality of variance. Significantly different ($P<0.05$) means were identified with the Holm-Sidak test (SigmaStat for Windows, version 3.5).

Field Studies Based on continuing results from our chemical and GC-EAD analyses, we conducted a series of six factorial, randomized, complete block design experiments to investigate the attractiveness of various GLVs. From 2004 to 2006, experiments 1–5 were conducted in Essex County near Windsor, Ontario, and experiment 6 was undertaken in Livingston County, Michigan. All experiments were conducted in open areas within or along natural woodlots containing green ash (*Fraxinus pennsylvanica* Marsh), also known as red ash in Canada.

Prior to these experiments, we had conducted a preliminary field trial in 2003 (Poland et al. 2004) that indicated 12-unit funnel traps (Phero Tech Inc., Delta, BC, CA, USA) or Japanese beetle traps (Great Lakes IPM Inc., Vestaburg, MI, USA) hung in the canopies of trees were ineffective in capturing emerald ash borer. As previously noted, research conducted in 2003 by Francese et al. (2005) on trap color revealed that glue-coated purple corrugated plastic panels were attractive to emerald ash borer. Thus, in all of our field experiments we used 4.0-mm-thick purple corrugated plastic (Champion Box Company, Inc, Cedaredge, CO, USA; same material and source as Francese et al. (2005)) that was coated in the field with Pestick™ insect trapping glue (Phytotronics Inc, Earth City, MO, USA) just before the installation of the various semiochemical treatments. Traps were hung from 2-m-tall free-standing vertical metal poles (with a 90° bend 45 cm from the top) constructed from 1-cm-diameter concrete-reinforcing bar and inserted 50–60 cm into the ground. Traps were placed 15–20 m apart and 2–4 m clear of the forested edges of woodlots or plantations infested with emerald ash borer.

In Experiment 1, we compared three traps of different design (flat, triangular, or cross-vane) that were unbaited (control) or baited with three GLVs ((*Z*)-3-hexenol, hexanal, and (*E*)-2-hexenal). These were the intial GC-EAD active GLVs we identified. Flat traps were constructed from corrugated plastic panels (30 cm wide×150 cm long) and glued on both sides. Triangular traps were constructed from three 30×150-cm-long panels fastened together with plastic cable ties to form a triangle (prism) and glued on the outside. Cross-vane traps were constructed from two 30×150-cm panels with corresponding slots to allow them to fit together to form a cross-vane, and glued on all exposed surfaces. The traps were baited with the semiochemical lures on 3 June 2004, lures were replaced on 8 July, and the experiment terminated on 28 July when it was evident that most of the beetle flight was over. Treatments were replicated 10 times. Details on the compounds, release device, and release rates are listed in Table 1. As in all experiments, traps were serviced every 10–14 days and the captured emerald ash borers were removed from the traps, washed in Histo-Clear II® (National Diagnostics, Atlanta,

Table 1 Release devices and release rates of compounds used in field experiments as potential attractants for *Agrilus planipennis*

Experiment	Compound (% purity)[a]	Release device	Release rate (mg/24 h)[b]
1	Hexanal (98.1)	1 bubble cap	24
	(E)-2-hexenal (99.1)	1 bubble cap	38
	(*Z*)-3-hexenol (98.0)	1 bubble cap	17
2	Hexanol (99.5)	2 bubble caps	24
	(*E*)-2-hexenol (99.7)	2 bubble caps	32
	(*Z*)-3-hexenol (98.0)	2 bubble caps	34
3	Nonanal (95.6)	2 bubble caps	84
	Hexanal (98.1)	2 bubble caps	48
	(*E*)-2-hexenal (99.1)	2 bubble cap	76
4	(*Z*)-3-hexenol (99.5)	Polyethylene pouch	330
	(*E*)-2-hexenal (99.1)	15-ml polyethylene bottle	110
5	(*Z*)-3-hexenol (99.5)	Polyethylene pouch	330
	(*E*)-2-hexenal (99.1)	Two 15-ml polyethylene bottles	220
6	(*Z*)-3-hexenol (99.5)	Polyethylene pouch	48
	(*Z*)-3-hexenol (99.5)	Polyethylene pouch	330
	(*E*)-2-hexenal (99.1)	15-ml polyethylene bottle	110
	(*E*)-2-hexenal (99.1)	Three 15-ml polyethylene bottles	330

[a] All compounds and release devices purchased from Phero Tech Inc., Delta, British Columbia, Canada

[b] Release rates determined at 30°C in the laboratory by Phero Tech Inc. All compounds released from closed and separate devices

GA, USA) to remove the glue, and preserved in 70% ethanol. All beetles were sexed by examining the internal genitalia.

Experiment 1 had combined (*Z*)-3-hexenol, hexanal, and (*E*)-2-hexenal as one treatment and, in 2005, we conducted two experiments to examine the separate effects of various alcohols (Experiment 2) and aldehydes (Experiment 3). Each of these experiments included a control consisting of an unbaited (blank) sticky trap, as did all remaining experiments. In Experiment 2, we compared binary and tertiary combinations of hexanol, (*E*)-2-hexenol, and (*Z*)-3-hexenol. Thus, the treatments consisted of (1) hexanol+(*E*)-2-hexenol, (2) hexanol+(*Z*)-3-hexenol, (3) hexanol+(*E*)-2-hexenol+(*Z*)-3-hexenol, (4) (*E*)-2-hexenol+(*Z*)-3-hexenol, and (5) unbaited or blank trap. In Experiment 3, we compared binary and tertiary combinations of (*E*)-2-hexenal, nonanal, and hexanal, that is: (1) (*E*)-2-hexenal+nonanal, (2) (*E*)-2-hexenal+hexanal, (3) (*E*)-2-hexenal+nonanal+hexanal, (4) nonanal+hexanal, and (5) blank trap. Nonanal (an aldehyde by product of the GLV biosynthesis pathway) was detected in our early SPME analysis of host volatiles and was GC-EAD active (unpublished data, Poland et al. 2007). In 2005, we used purple traps consisting of two 45×60-cm corrugated purple plastic panels with corresponding slots that allowed them to fit together in a cross-vane shape, and glued on all surfaces. Each trap was fitted with a flat top and bottom (45×45 cm) and was suspended from the cross bar of the metal trap poles by an umbrella rig spreader (Zing Products, Westport MA, USA). The spreader not only provided a means to suspend the trap and keep the trap panels in place, it also allowed the trap to move with the wind and, thus, be less rigid and prone to wind damage. The two field experiments were initiated on 4 June 2005 and were terminated on 20 July 2005. Experiment 2 had five replicates and experiment 3 had 10 replicates of each treatment.

In 2006, the last three experiments were conducted to assess the efficacy of (*Z*)-3-hexenol and (*E*)-2-hexenal, but at higher release rates than used previously. Experiment 4 evaluated traps baited with (*Z*)-3-hexenol (released at 330 mg/day) or (*E*)-2-hexenal (110 mg/day), or the two combined. In Experiment 5, the same volatiles were used as in experiment 4, but the release rate of (*E*)-2-hexenal was increased to 220 mg/day. Experiment 6 examined four single semiochemical treatments consisting of (*Z*)-3-hexenol (released at 48 or 330 mg/day), (*E*)-2-hexenal (released at 110 or 330 mg/day), and the unbaited control. Traps used in 2006 were constructed from a single purple corrugated plastic panel folded into a three-sided prism measuring 40.0-cm wide and 60.0-cm tall on each side, and held together by cable ties. Traps were glued on all exposed surfaces and suspended from the metal trap stand as in 2005. Experiments were conducted from 14 June to 27 June (experiment 4), 28 June to 18 July (experiment 5), and 12 June to 19 July 2006 (experiment 6). Experiments 4 and 5 had six replicates of each treatment and experiment 6 had 12 replicates.

The trap catches of male and female emerald ash borers from each experiment were analyzed independently by using analysis of variance. In each experiment, we had a priori hypotheses about the treatments and they were used to structure orthogonal contrasts for the ANOVA. For example, in the first experiment, our goal was to determine whether or not emerald ash borer trap catch numbers may be influenced by (*a*) an artificial lure, (*b*) sex, and (*c*) trap type, in particular, a triangular-arrayed surface as compared to conventional flat and cross-vane surfaces. As such, the

11 degrees of freedom (*df)* associated with the treatments tested in this experiment [2 lures (baited and unbaited)×2 sexes×3 trap types—1], were broken down into contrasts within the ANOVA to directly address each of these questions:

Contrast	d.f.
Bait	1
Sex	1
Trap	2
Bait×sex	1
Bait×trap	2
Sex×trap	2
Bait ×sex×trap	*2*
Total	11

In the above, the tests for interaction determine whether the levels of one factor behave consistently across the levels of another, such that generalized statements may be made about the individual factors. For example, if the bait×sex interaction term were insignificant ($P>0.10$), then conclusions may be drawn about the effectiveness of the lure, irrespective of sex. The two *df* for trap type was further broken down into a contrast that compared cross-vane and flat traps, and another that compared the average of these two types to the triangular array. With this factorial treatment arrangement, this type of analysis is more powerful and appropriate than multiple comparison procedures, and better suited to specifically address the a priori hypotheses that we posed (Steel and Torrie 1980; Mize and Schultz 1985; Warren 1986).

All of our subsequent experiments focused on lure development and generally followed the treatment arrangement of a 2^2 factorial design (Montgomery 2004). In the simplest cases (experiments 4 and 5), two types of lures were tested (e.g., *a* and *b*), producing four treatment combinations: *a*, *b*, *ab*, and *0* (blank). In a manner parallel to the first experiment, orthogonal contrasts were used to test for interaction between the two lures and then generalized effects associated with the presence of each. Experiments 2 and 3 followed this same form, but involved three compounds, producing the four treatment combinations: *ac*, *bc*, *abc*, and *0*. In these experiments, we contrasted a fifth treatment, *ab*, with *abc* to test for the effect of the third compound *c*. In Experiment 6, two lures were tested, each at three levels (0, 1, and 2×), without combination. In this case, a contrast was used to compare the average effects of the two lures, and polynomial contrasts were used to test for dose responses in each [e.g., response proportional to dose (linear) and disproportional to dose (quadratic)] (Montgomery 2004).

In experiment 1, trap catches were standardized to the same trap surface area as the cross-vane traps (1.8 m^2): all remaining experiments used the same trap size. Residuals from the analyses were examined to verify normality and homogeneity of variance assumptions and, where necessary, trap catch numbers were increased by one to avoid zero counts, and natural log transformed to satisfy these assumptions. Case-by-case, this transformation was identified from the family of power transformations as being the most effective through the procedures developed by Box and Cox (1964). Where data were transformed, we present the untransformed least squares means and the standard errors of the least square means, along with the statistics ($P>F$) from ANOVA of the transformed data. Analyses were conducted by using PROC GLM of the SAS® System (SAS Institute Inc. 2004).

Results

Aeration of Leaf Volatiles and Chemical Analysis Eight GLVs were detected in both white and green ash foliar aeration extracts. (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenol, (*E*)-2-hexenol, hexanol, (*Z*)-3-hexenyl acetate, and hexyl acetate were identified in the white ash extract by GC-MS. The presence of hexanal was determined by its retention times on the DB-17 and HP-1 columns. Green ash extracts were considerably less concentrated than the white ash extract, possibly due to differences in age and maturity of the sampled trees. Only (*E*)-2-hexenal, (*Z*)-3-hexenol, and (*Z*)-3-hexenyl acetate were identified by GC-MS. (*Z*)-3-hexenal, hexanal, (*E*)-2-hexenol, hexanol, and hexyl acetate were identified by their retention times on the DB-17 and HP-1 columns. In both the green and white ash extracts, (*Z*)-3-hexenol and (*Z*)-3-hexenyl acetate represented over 80% of the GLV volatiles emitted by both tree species. No GLVs were detected in the control extracts.

Gas Chromatography-Electroantennogram Detection All of the GLVs identified in both green and white ash extracts elicited EAD responses from both male and female antennae. However, during the initial bioassays of white ash extracts, which were more concentrated than the green ash extracts, the male antennal responses to the GC peaks corresponding to the GLVs, particularly the (*Z*)-3-hexenol GC peak, were unusually small until the extract was diluted 15-fold. This resulted in the expected higher response levels (Fig. 1 inset). Male EAD responses to the aldehydes and alcohols in extracts of both tree species were typically larger than female responses to these compounds, with the GC peak corresponding to (*Z*)-3-hexenol always eliciting the largest male EAD response (Fig. 1 inset). Similar results were obtained previously with the GLVs occurring in Manchurian ash volatiles (Rodriguez-Saona et al. 2006). In our initial GC-EAD bioassays of the ash extracts, the

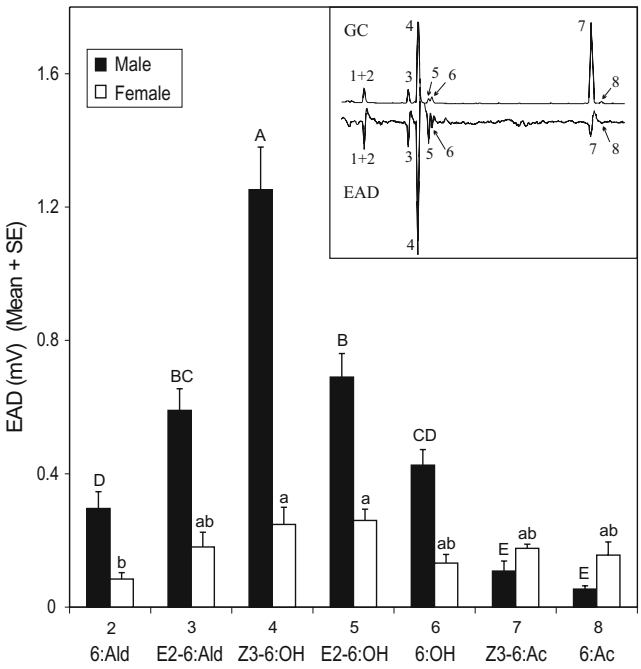

Fig. 1 Mean EAD responses from male and female antennae of *Agrilus planipennis* (N=5 each) to equal quantities (7 ng each) of seven host GLVs (6:Ald=hexanal; E2–6:Ald=(*E*)-2-hexenal; Z3–6:OH=(*Z*)-3-hexenol; E2–6:OH=(*E*)-2-hexenol; 6:OH=hexanol; Z3–6:Ac=(*Z*)-3-hexenyl acetate; 6:Ac=hexyl acetate). *Numbers* above the compound names correspond to the numerically labeled GC and EAD peaks in the *inset figure*. *Male bars* with *different capital letters* are different and *female bars* with *different small case letters* are different (P<0.05, Holm–Sidak test). Differences between male and female responses were significant for all GLVs (P<0.05, Holm–Sidak test) except Z3–6:Ac. Fig. 1 *inset*: GC-EAD recording of male antennal response to GLVs in the diluted white ash aeration extract (1+2, (*Z*)-3-hexenal plus hexanal; 3, (*E*)-2-hexenal; 4, (*Z*)-3-hexenol; 5, (*E*)-2-hexenol; 6, hexanol; 7, (*Z*)-3-hexenyl acetate; 8, hexyl acetate)

male response to the GC peak thought to be only hexanal was larger than expected based on the size of the GC peak and the response to synthetic hexanal. Subsequent closer examination of this peak by using a slower temperature program (40°C to 75°C at 6°C/min) revealed a second peak that was identified as (*Z*)-3-hexenal by GC-MS and its retention time on the HP-1 and DB-17 columns.

Because there were substantial differences in the amounts of GLVs in the ash extracts, we confirmed the relative stimulating effectiveness of the host GLVs by testing a synthetic mixture of GLVs held at a constant concentration of 7 ng/μl. There was a difference between male and female responses (F=101.3, df=1, 6; P<0.001) with male antennae producing larger EAD responses to all of the alcohols and aldehydes than females (P<0.05, Holmes-Sidak test) (Fig. 1). Females were more responsive to hexyl acetate than males, but there was no difference in their respective responses to (*Z*)-3-hexenyl acetate. As with the ash extracts, (*Z*)-3-hexenol was by far the most stimulating compound for males (Fig. 1), often eliciting responses more than twofold larger than either (*E*)-2-hexenal or (*E*)-2-hexenol at the same concentration. Only slight differences were observed among female responses to the GLVs (Fig. 1). The synthetic GLV mixture did not include (*Z*)-3-hexenal because it was discovered late in this study and after the synthetic mixture was bioassayed. However, the EAD response from male antennae to synthetic (*Z*)-3-hexenal was larger than the EAD response to the same concentration of synthetic hexanal, but comparable to that elicited by synthetic (*E*)-2-hexenal.

Field Studies In experiment 1, trap catches for males and females were consistent across both baited and unbaited traps and trap types (two- and three-way interactions, $P\geq$ 0.38). In general, 3.7 times more males were attracted to the traps baited with the three GLVs ((*Z*)-3-hexenol+hexanal+(*E*)-2-hexenal) than females (P<0.01) (Fig. 2a). Also, these traps resulted in an average 76% increase in total catches of both sexes (P<0.01). While there was little difference in the performance of cross-vane and flat traps (P=0.86), triangular traps provided a 40% increase in catches over the other trap types (P=0.07), suggesting the need for further evaluation.

In experiment 2, the combination of hexanol+(*Z*)-3-hexenol (lures 2 and 3) resulted in increased catches of males (P<0.01), but not females (P=0.18) (Fig. 2b). Hexanol+(*E*)-2-hexenol had no effect as an attractant for either sex, either as a binary combination (lure 3) ($P\geq$0.14), or as a tertiary combination with (*Z*)-3-hexenol (lure 4) ($P\geq$ 0.27). Adding hexanol to the combination with (*Z*)-3-hexenol+(*E*)-2-hexenol (lure 3) had no effect on male catches (P=0.30), but appeared to increase female numbers (P=0.06). In general, the presence of (*Z*)-3-hexenol in the various lures resulted in a 3.8-fold increase in males captured with no conclusive effect on females.

In experiment 3, variation in female trap catch was high. The combination of hexanal+nonanal (lures 1 and 3) increased catches of males (P=0.04), but not females (P= 0.08) (Fig. 2c). The combination of (*E*)-2-hexenal+nonanal had no effect as an attractant, either by itself (lure 4) ($P\geq$ 0.35), or in combination with hexanal (lure 3) ($P\geq$0.55) for either sex. Also, adding nonanal to the combination of hexanal+(*E*)-2-hexenal had no effect on catches of either sex ($P\geq$0.58).

In experiment 4, variation in female trap catch was also high. Nonetheless (*Z*)-3-hexenol increased trap catches of both sexes (lures 1 and 3) (P<0.01) (Fig. 2d). In contrast, (*E*)-2-hexenal had no effect as an attractant, either by itself ($P\geq$0.38), or in combination with (*Z*)-3-hexenol ($P\geq$0.22). The presence of (*Z*)-3-hexenol resulted in an average 2.3-fold increase in total catch of beetles. In contrast to the previous experiments, substantially more females were trapped than males in response to (*Z*)-3-hexenol.

TREATMENTS		CONTRAST	*p*-value		
Experiment 1					
1. Cross vane trap (C)		sex	<0.01†		
2. Cross vane trap with (*Z*)-3-hexenol, hexanal, and (*E*)-2-hexenal		bait	<0.01†		
3. Flat trap (F)		trap	0.18†		
4. Flat trap with (*Z*)-3-hexenol, hexanal, and (*E*)-2-hexenal		C *vs* F	0.86†		
5. Triangular trap (T)		C, F *vs* T	0.07†		
6. Triangular trap with (*Z*)-3-hexenol, hexanal, and (*E*)-2-hexenal		sex **x** bait	0.82†		
		sex **x** trap	0.38†		
		bait **x** trap	0.83†		
		sex **x** bait **x** trap	0.67†		
				Male	**Female**
Experiment 2					
1. Hexanol + (*E*)-2-hexenol		(1, 3) *vs* (2, 5)		0.14†	0.73†
2. Hexanol + (*Z*)-3-hexenol		(2, 3) *vs* (1, 5)		<0.01†	0.18†
3. Hexanol + (*E*)-2-hexenol + (*Z*)-3-hexenol		interaction		0.33†	0.27†
4. (*E*)-2-hexenol + (*Z*)-3-hexenol		3 *vs* 4		0.30†	0.06†
5. Blank trap					
Experiment 3					
1. (*E*)-2-hexenal + nonanal		(1, 3) *vs* (2, 5)		0.82	0.35
2. Hexanal + nonanal		(2, 3) *vs* (1, 5)		0.04	0.08
3. Hexanal + (*E*)-2-hexenal + nonanal		interaction		0.93	0.55
4. Hexanal + (*E*)-2-hexenal		3 *vs* 4		0.98	0.58
5. Blank trap					
Experiment 4					
1. (*Z*)-3-hexenol (330 mg/d)		(1, 3) *vs* (2, 4)		<0.01	<0.01
2. (*E*)-2-hexenal (110 mg/d)		(2, 3) *vs* (1, 4)		0.38	0.61
3. (Z)-3-hexanol (330 mg/d) + (*E*)-2-hexenal (110 mg/d)		interaction		0.22	0.79
4. Blank trap					
Experiment 5					
1. (*Z*)-3-hexenol (330 mg/d)		(1, 3) *vs* (2, 4)		<0.01	0.05
2. (*E*)-2-hexenal (220 mg/d)		(2, 3) *vs* (1, 4)		0.73	0.06
3. (Z)-3-hexanol (330 mg/d) + (*E*)-2-hexenal (220 mg/d)		interaction		0.52	0.66
4. Blank trap					
Experiment 6					
1. (*Z*)-3-hexenol (48 mg/d)		(5, 1, 2) linear		0.67	0.26†
2. (*Z*)-3-hexanol (330 mg/d)		(5, 1, 2) quadratic		0.01	0.39†
3. (*E*)-2-hexenal (110 mg/d)		(5, 3, 4) linear		0.83	0.94†
4. (*E*)-2-hexenal (330 mg/d)		(5, 3, 4) quadratic		0.59	0.64†
5. Blank trap		(1, 2) *vs* (3, 4)		0.06	0.06†

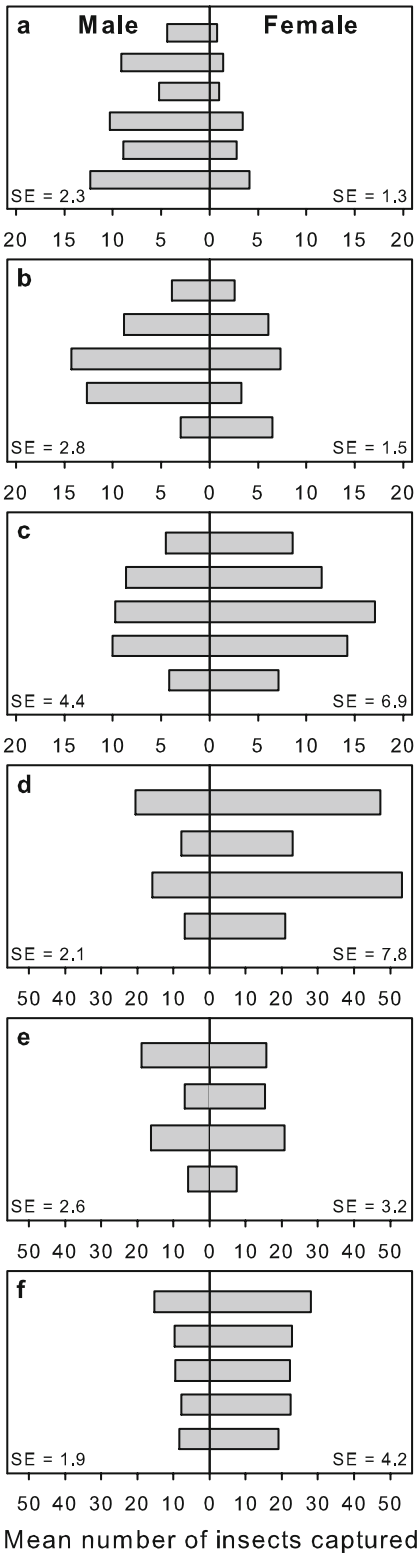

Fig. 2 Response of male and female *Agrilus planipennis* to various combinations of green leaf volatiles in six field experiments. Data were analyzed independently using analysis of variance (ANOVA) followed by orthogonal contrasts. Statistics ($P>F$) are shown for each contrast and those marked with a *dagger* apply to the natural log-transformed data following ANOVA. The standard error (SE) shown is the standard error of the least squares means

In experiment 5, doubling the release rate of (*E*)-2-hexenal used in Experiment 4 had no effect as an attractant for males (lures 2 and 3) ($P=0.73$), but may have had some effect on females ($P=0.06$) (Fig. 2e). (*Z*)-3-Hexenol again resulted in an increased catch of both sexes (lures 1 and 3) ($P\leq0.05$): an average 2.7-fold increase in males and a 1.6-fold increase in females. The presence of (*E*)-2-hexenal did not influence the effects of (*Z*)-3-hexenol ($P\geq 0.52$).

In experiment 6, (*Z*)-3-hexenol resulted in an increased catch of males only, and only at the low release rate (48 mg/day) ($P=0.01$) (Fig. 2f). Female numbers also increased, but not significantly ($P=0.39$). (*E*)-2-hexenal had no effect as an attractant for males or females ($P\geq0.59$). Low dosages of (*Z*)-3-hexenol resulted in total trap catches of beetles that were 50% greater than unbaited traps.

Discussion

The most promising GLV attractant found in this study was (*Z*)-3-hexenol. It was one of the most abundant GLVs in the aeration extracts of both green ash and white ash. In the GC-EAD bioassays, males showed the strongest response to (*Z*)-3-hexenol, whereas for females it was no more stimulating than the other GLVs. In field experiments, males consistently responded positively ($P<0.05$) to the presence of (*Z*)-3-hexenol (experiments 2, 4, 5, and 6), and females responded positively in two (experiments 4 and 5), but not in two other experiments (experiments 2 and 6). In experiment 6 where the two release rates of (*Z*)-3-hexenol were compared directly, males showed a positive ($P<0.05$) response to the low rate (48 mg/day) but not to a rate nearly seven times higher (330 mg/day). This suggests that there is a dose effect, but in experiments 4 and 5 the high release rate lures were attractive. In our GC-EAD bioassays, however, we found that the initial white ash extracts were too concentrated and had to be diluted 15-fold before eliciting full antennal responses from the GLVs, particularly (*Z*)-3-hexenol. Male antennae were sensitive to low dosages (<1 ng) of aldehyde and alcohol GLVs, as observed in other beetle species (Hansson et al. 1999; Larsson et al. 2001), so that excessively high dosages probably saturated the male olfactory receptors which resulted in the reduced EAD responses with the undiluted white ash extracts. Thus high doses of GLVs in traps may well be unattractive or even repellent at close range. In laboratory bioassays involving the Colorado potato beetle, *Leptinotarsa decemlineata* (Say), Dickens (2000) found high dosages of (*Z*)-3-hexenol and (*E*)-2-hexenol in lure blends were repellent but low levels in the same blend were attractive. Further investigation of the effect of lure dosage or release rates of GLVs on emerald ash borer may be worthwhile.

Although hexanol and (*E*)-2-hexenol readily elicited antennal responses from male and female antennae in the GC-EAD bioassays, these compounds were not attractive in the field, when tested at release rates of 24–32 mg/day, within the lower range where (*Z*)-3-hexenol shows activity (17–48 mg/day), nor did they enhance the attractiveness of (*Z*)-3-hexenol. Similarly, the aldehydes (hexanal, (*E*)-2-hexenal, nonanal) were active in GC-EAD bioassays but, with one exception (experiment 2, hexanal+nonanal), they were not attractive to male or female emerald ash borer in the field. We tested (*E*)-2-hexenal in several different experiments. In all cases, it was not attractive to either sex, and did not noticeably increase or decrease trap catch when traps were co-baited with (*Z*)-3-hexenol. The combination of hexanal+nonanal may warrant further attention for its effects on males, either alone or in combination with (*Z*)-3-hexenol.

There are practical and statistical constraints in the number of treatments (chemicals, blends and release rates), layout of traps, and replications possible in field experiments, especially in forests. Consequently, not all host GLVs or their blends were tested, which may account for our failure to detect any behavioral effects for some GLVs, such as (*E*)-2-hexenal. Also, the GLV acetates, which had the weakest EAD activity, were not included in our field tests. Second, it may also be that GLVs are not particularly attractive to females. Aeration extracts from insect damaged Manchurian ash were attractive to female emerald ash borers, but not to males (Rodriguez-Saona et al. 2006). These extracts contained relatively low levels of (*Z*)-3-hexenol and other GLVs and could explain the lack of male behavioral response to these extracts, thus suggesting that females were responding to other induced host compounds. (*Z*)-3-Hexenal was discovered too late in our GC-EAD study for inclusion in the field tests. Further tests are planned to broaden our evaluation of host ash GLVs and to include other EAD active host compounds in combination with them.

Little is known about effective methods to trap buprestids, and this can present difficulties in evaluating attractants. In general, an important component of the development of a trap-based detection program for insects is the design and placement of the trap itself. Attractive chemicals placed in an ineffective trap may result in poor trap catches, and conversely, a well-designed trap, baited with unattractive chemicals or incorrect release rates may also result in poor trap catches. With this in mind, we chose to keep trap color (purple) constant throughout the 3 years of our field tests, and in 2005 and 2006 to keep trap design the same within the year of the experiment. Our results from 2004 showed that flat, cross-vane, and triangular traps were essentially the same in capturing emerald ash borers, although the triangular trap appeared to offer an advantage. In 2005, we chose to use the purple cross-vane trap because of its availability from USDA APHIS. This trap had

tendencies to rotate easily in the wind because of the vanes; therefore, in 2006 we reverted to the triangular (prism), but smaller trap. Further development of the trap silhouette, optimal, but practical positioning of the trap, and perhaps revisiting trap color will no doubt help improve trapping effectiveness for emerald ash borer and buprestids in general.

Green leaf volatiles are well known to be negative olfactory signals used by several species of conifer inhabiting bark beetles to indicate the presence of non-hosts or unsuitable hosts at the tree-species and habitat level (Zhang and Schlyter 2004). On the other hand, several coleopteran folivores feeding on angiosperms use these same GLVs, especially the C_6-alcohols, to help locate hosts, or synergistically enhance their response to pheromones as mentioned in the Introduction. To our knowledge, our results provide the first evidence that buprestids use GLVs as attractants. Because the release of GLVs from plant tissue is elevated by insect feeding damage, we hypothesize that foliage feeding by adult emerald ash borer may increase the apparency and attractiveness of their host trees, *Fraxinus* spp. Because (*Z*)-3-hexenol is most attractive to males and elicits an exceptionally strong male antennal response, it may also serve as an important cue along with other visual and olfactory stimuli for finding females on host foliage. Similar male biased antennal and behavioral responses to (*Z*)-3-hexenol have been reported in other beetle species, particularly chafers (Ruther et al. 2000, 2002; Ruther 2004), where it serves as a sexual kairomone in conjunction with a female pheromone.

In summary, our laboratory and field experiments demonstrate that the GLV (*Z*)-3-hexenol increased trap catch of the emerald ash borer when placed on purple traps in open areas or along the edges of woodlots containing ash. It had greater influence on males than females, and dosage may be a factor determining its effectiveness. This work represents a step towards understanding the role of GLVs for host-plant finding by *A. planipennis*, and for development of an attractant-based trapping systems for early detection of this serious invasive insect pest.

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References

BOX, G. E. P., and COX, D. R. 1964. An analysis of transformations. *J. Royal Stat. Soc.* 26:211–243.

Canadian Food Inspection Agency. 2007. Emerald ash borer—*Agrilus planipennis*. http://www.inspection.gc.ca/english/plaveg/pestrava/agrpla/tech/agrplae.shtml

Chinese Academy of ScienceS, Institute of Zoology 1986. *Agrilus marcopoli* Obenberger, p. 445. China Agriculture, Beijing.

COSSÉ, A. A., BARTLET, R. J., ZILKOWSKI, B. W., BEAN, D. W., and ANDRESS, E. R. 2006. Behaviorally active green leaf volatiles for monitoring the leaf beetle, *Diorhabda elongata*, a biocontrol agent of saltcedar, *Tamarix* spp. *J. Chem. Ecol.* 32:2695–2708.

CROOK, D. J., KHRIMIAN, A., FRASER, I., FRANCESE, J.A., Poland, T.M., and MASTRO, V.C. 2008. Electrophysiological and behavioral responses of *Agrilus planipennis* (Coleoptera: Buprestidae) to host bark volatiles. *Environ. Entomol*. 37:356–365.

DE GROOT, P., BIGGS, W. D., LYONS, D. B., SCARR, T., CZWERWINSKI, E., EVANS, H. J., INGRAM, W., and Marchant, K. 2006. A visual guide to detecting emerald ash borer damage. Natural Resources Canada and Ontario Ministry of Natural Resources, Sault Ste. Marie, Ontario, Canada.

DICKENS, J. C. 2000. Orientation of Colorado potato beetle to natural and synthetic blends of volatiles emitted by potato plants. *Agric. For. Entomol.* 2:167–172.

DICKENS, J. C., JANG, E. B., LIGHT, D. M., and ALFORD, A. R. 1990. Enhancement of insect pheromone response by green leaf volatiles. *Naturwissenschaften.* 77:29–31.

EAB Info. 2007. Emerald Ash Borer. http://www.emeraldashborer.info/index.cfm

FERNANDEZ, P. C., MEINERS, T., BJÖRKMAN, C., and HILKER, M. 2007. Electrophysiological responses of the blue willow leaf beetle, *Phratora vulgatissima*, to volatiles of different *Salix viminalis* genotypes. *Entomol. Exp. Appl.* 125:157–164.

FRANCESE, J. A., MASTRO, V. C., OLIVER, J. B., LANCE, D. R., and YOUSSEF, N. 2005. Evaluation of colors for trapping *Agrilus planipennis* (Coleoptera: Buprestidae). *J. Entomol. Sci.* 40:93–95.

GRANT, G. G., GUO, J., MACDONALD, L., and COPPENS, M. D. 2007. Oviposition response of spruce budworm (Lepidoptera: Tortricidae) to host terpenes and nonhost green leaf volatiles. *Can. Entomol.* 139:564–575.

HAACK, R. A., JENDEK, E., LUI, H., MARCHANT, K. R., PETRICE, T. R., POLAND, T. M., and YE, H. 2002. The emerald ash borer: a new exotic pest in North America. *Newsl. Mich. Entomol. Soc.* 47:3&41–5.

HANSSON, B. S., LARSSON, M. C., and LEAL, W. S. 1999. Green leaf volatile-detecting olfactory receptor neurons display very high sensitivity and specificity in a scarab beetle. *Physiol. Entomol.* 24:121–126.

LARSSON, M. C., LEAL, W. S., and HANSSON, B. S. 2001. Olfactory receptor neurons detecting plant odors and male volatiles in *Anomala cuprea* beetles (Coleoptera: Scarabaeidae). *J. Insect Physiol.* 47:1065–1076.

LELITO, J. P., FRASER, I., MASTRO, V. C., TUMLINSON, J. H., BÖRÖCZKY, K., and BAKER, T. C. 2007. Visually mediated 'paratrooper copulations' in the mating behavior of *Agrilus planipennis* (Coleoptera: Buprestidae), a highly destructive invasive pest of North American ash trees. *J. Insect Behav.* 20:537–552.

LYONS, D. B., and JONES, G. C. 2005. The biology and phenology of the emerald ash borer, pp 62–63, in K. W. Gottschalk (ed.) Proceedings, 16th U.S. Department of Agriculture interagency research forum on gypsy moth and other invasive species 2005; January 18–21, Annapolis, MD. Gen. Tech. Rep. NE-337.

LYONS, D. B., CAISTER, C., DE GROOT, P., HAMILTON, B., MARCHANT, K., SCARR, T., and TURGEON, J. 2007. Survey guide for detection of emerald ash borer. Government of Canada, Sault Ste. Marie, Ontario, Canada.

MCCULLOUGH, D. G., POLAND, T. M., and CAPPAERT, D. L. 2006. Attraction of emerald ash borer to trap trees: effects of stress agents and trap height, pp. 61–62, in V. Mastro, R. Reardon, and G. Parra

(Comp.) Emerald Ash Borer Research Technology Development Meeting, Pittsburgh, Pennsylvania, 26–27 September 2006. USDA Forest Service, FHTET-2005–16.

MONTGOMERY, D. C. 2004. Design and Analysis of Experiments. 6th edn.Wiley, New Jersey.

MIZE, C. W., and SCHULTZ, R. C. 1985. Comparing treatment means correctly and appropriately. *Can. J. For. Res.* 15:1142–1148.

OTIS, G. W., YOUNGS, M. E., and UMPHREY, G. 2005. Effects of colored objects and purple background on emerald ash borer trapping, pp. 31–32, in V. Mastro and R. Reardon (Comp.). Emerald Ash Borer Research and Technology Development Meeting. 4–6 October 2004. Romulus Michigan, 30 September–1 October 2003. USDA Forest Service, FHTET-2004–15.

POLAND, T. M. 2007. Twenty million ash trees later: current status of emerald ash borer in Michigan. *Newsl. Mich. Entomol. Soc.* 52:1&210–14.

POLAND, T. M., dE GROOT, P., GRANT, G., MACDONALD, L., and MCCULLOUGH, D.G. 2004. Developing attractants and trapping techniques for the emerald ash borer, pp. 15–16, *in* V. Mastro and R. Reardon (Comp.). Emerald Ash Borer Research and Technology Development Meeting. 30 September–1 October 2003. Port Huron, Michigan. USDA Forest Service, FHTET-2004–02.

POLAND, T. M., RODRIGUEZ-SAONA, C., GRANT, G., BUCHAN, L., DE GROOT, P., MILLER, J., and MCCULLOUGH, D. G. 2006. Trapping and detection of emerald ash borer: Identification of stress-induced volatiles and tests of attraction in the lab and field, pp. 64–65, *in* V. Mastro, R. Reardon, and G. Parra (Comp.). Emerald Ash Borer Research Technology Development Meeting, Pittsburgh, Pennsylvania, 26–27 September 2005. USDA Forest Service, FHTET-2005–16.

POLAND, T. M., CROOK, D., FRANCESE, J., OLIVER, J., OTIS, G., dE GROOT, P., GRANT, G., MACDONALD, L., MCCULLOUGH, D., FRASER, I., LANCE, D., MASTRO, V., YOUSSEF, N., TURK, T., and YOUNGS, M. 2007. Towards the development of survey trapping technology for the emerald ash borer, pp. 4–5, *in* K.W. Gottschalk (ed.). Proceedings 17th U.S. Department of Agriculture Interagency Research Forum on Gypsy Moth and Other Invasive Species, 2006, Annapolis, Maryland, 10–13 January 2006. USDA Forest Service, Gen. Tech. Report NRS-P-10.

REINECKE, A., RUTHER, J., MAYER, C. J., and HILKER, M. 2006. Optimized trap lure for male *Melolontha* cockchafers. *J. Appl. Entomol.* 130:171–176.

REINECKE, A., RUTHER, J., TOLASCH, T., FRANCKE, W., and HILKER, M. 2002. Alcoholism in cockchafers: orientation of male *Melolontha melolontha* towards green leaf alcohols. *Naturwissenschaften* 89:265–269.

RODRIGUEZ-SAONA, C., POLAND, T. M., MILLER, J. R., STELINSKI, L. L., GRANT, G. G., dE GROOT, P., BUCHAN, L., and MACDONALD, L. 2006. Behavioral and electrophysiological responses of the emerald ash borer, *Agrilus planipennis*, to induced plant volatiles of Manchurian ash, *Fraxinus mandshurica*. *Chemoecology* 16:75–86.

RUTHER, J. 2004. Male-biased response of garden chafer, *Phyllopertha horticola*, to leaf alcohol and attraction of both sexes to floral plant volatiles. *Chemoecology* 14:187–192.

RUTHER, J., REINECKE, A., THIEMANN, K., TOLASCH, T., FRANCKE, W., and HILKER, M. 2000. Mate finding in the forest cockchafer, *Melolontha hippocastani*, mediated by volatiles from plants and females. *Physiol. Entomol.* 25:172–179.

RUTHER, J., REINECKE, A., and HILKER, M. 2002. Plant volatiles in the sexual communication of *Melolontha hippocastani*: response toward time dependent bouquets and novel function of (Z)-3-hexen-1-ol as a sexual kairomone. *Ecol. Entomol.* 27:76–83.

SAS Institute Inc. 2004. SAS OnlineDocÒ 9.1.3. Cary, NC: SAS Institute Inc.

SIEGERT, N. W., MCCULLOUGH, D. G., and TLUCZEK, A. R. 2007. Two years under the bark: towards understanding multiple-year development of emerald ash borer, pp. 20, in V. Mastro, D. Lance, R. Reardon and G. Parra (Comp.). Emerald Ash Borer and Asian Longhorned Beetle Research Technology Development Meeting, 29 October-2 November 2006. USDA Forest Service, FHTET-2007–04.

STEEL, R. G. D., and TORRIE, J. H. 1980. Principles and procedures of statistics. McGraw-Hill, New York.

United States Department of Agriculture, Animal and Plant Health Inspection Service. 2007. Emerald Ash Borer http://www.aphis.usda.gov/plant_health/plant_pest_info/emerald_ash_b/background.shtml

WARREN, W. G. 1986. On the presentation of statistical analyses: reason or ritual. *Can. J. For. Res.* 16:1185–1191.

YU, C. -M. 1992. *Agrilus macropoli* Obenberger (Coleoptera: Buprestidae), pp. 400–401, in G.-R. Xiao (ed.). Forest Insects of China2nd edn.China Forestry, Beijing.

ZHANG, Q. -H., and SCHLYTER, F. 2004. Olfactory recognition and behavioral avoidance of angiosperm nonhost volatiles by conifer-inhabiting bark beetles. *Agric. For. Entomol.* 6:1–19.

Over the last two decades, an increasing number of studies has reported on the importance of host-plant volatile chemicals in mediating host recognition by herbivores (Visser 1986; Bernays and Chapman 1994; Schoonhoven et al. 1998; Bruce et al. 2005). This volatile-mediated response has been suggested to be more pronounced in specialist than generalist herbivores (Bernays and Chapman 1994), and can occur to very low concentrations of chemicals (Angioy et al. 2003). Several studies have investigated optimal combinations of host volatiles to improve monitoring or trapping of insect pests (e.g., Zhang et al. 1999; Hammack 2003; Blackmer et al. 2004; Leskey et al. 2005; Pinero et al. 2006). Within tortricid moth pests, research on the use of host volatiles has focused on species with moderate to broad diet breadths such as codling moth, Oriental fruit moth, and European grape berry moth. In these cases, a number of relatively common volatile compounds show behavioral activity in the laboratory (Ansebo et al. 2004; Coracini et al. 2004; Hern and Dorn 2004; Natale et al. 2004; Tasin et al. 2005, 2006a, b, 2007). However, lures based on host-plant volatiles have not been successful in the field (e.g., Ansebo et al. 2004; Coracini et al. 2004; Mitchell et al. 2008). One possibility is that the role of odors in location of hosts may be a stronger selective force for specialists than for generalists (Bernays and Chapman 1994). Therefore, the development of a lure based on host-plant volatiles to improve monitoring and management decisions for insect pests may be a more suitable strategy for specialist herbivores such as GBM.

Here, we present results of chemical, electrophysiological, and behavioral experiments that used solid phase microextraction (SPME), gas chromatography–electroantennogram detection (GC-EAD), and flight-tunnel assays, to identify the key volatile chemicals from grape shoots used by female GBM to locate a host. In our previous behavioral study, grape shoots were the most attractive grape tissue to female GBM (Cha et al. 2008). Our goal was to develop a host-plant-based synthetic lure that is as attractive as live grape shoots to female GBM. We also present data from a field trial in which we tested the efficacy of traps baited with synthetic lures.

Methods and Materials

Insects GBM were reared in cages placed in walk-in environmental chambers at 26°C and 60% RH under a 18:6 (L:D) photoperiod. Adult moths were fed with 50% honey and water soaked on cotton. Adults mated freely in rearing cages (45 cm H×77 cm W×45 cm D) and oviposited on seedless grape (*Vitis vinifera*, red flame variety). First and second instars were transferred to a diet cup (30 ml, WinCup Inc.) and reared on semi-synthetic diet (Nagarkatti et al. 2000) that consisted of grapes, pinto beans, and commercially available tobacco hornworm diet (Bio-Serve). To minimize any potential effect of laboratory rearing on the behavior of moths, colonies were re-established once a year with larvae and pupae collected from commercial vineyards in July or August.

Plants We used a native host species of GBM in north-eastern USA, *Vitis riparia*, to identify candidate volatile host compounds for the synthetic lure. Cuttings were made from *V. riparia* growing in a research vineyard at the Cornell University, New York State Agricultural Experiment Station in Geneva, NY, USA in December 2005. Cuttings were dipped in rooting hormone (Green Light Co., San Antonio, TX, USA) and placed in a planting box filled with moistened Perlite® in a cold room (4°C), equipped with a heating pad, to facilitate root growth while retarding development of shoots. Once roots were developed, all plants were transplanted to 1-gal pots filled with a mixture of sand, peat moss, and Vermiculite® and moved to a greenhouse with temperatures maintained between 21–26°C. Supplemental light was provided to extend the day length to 16 h and pots were fertilized weekly with water-soluble fertilizer (Peters 20–20–20, Scotts-Sierra Horticultural Products Co.). For sampling of volatile chemicals and flight-tunnel assays (see below), we used plants that were 2–4 weeks post-bloom, without any flowers or berries, since grape shoots were most attractive to female GBM in our previous flight-tunnel study (Cha et al. 2008).

Adsorbent Sampling We used a push–pull collection system to collect headspace volatiles of live grape shoots. The system was a custom-made, bell-shaped glass chamber (18 cm ID, 10 L) with two air-in adapters (7 mm ID) on the top and four air-out adapters (7 mm ID) equally distributed at the bottom wall of the chamber. To accommodate a whole, live potted plant, the glass chamber was placed on two pieces of Pyrex glass with a hole (2 cm) in the middle so that the vegetative portion of the plant could be sampled. After a plant was set up in the chamber, the chamber was flushed with filtered air (3 L min^{-1}) for an hour to replace air inside the chamber with filtered air and to stabilize volatile emission from the plant, because we noticed that handling of the plant during set up temporarily induced release of green leaf volatiles. During the collection, flow meters were used to insure that more filtered air was pushed into the chamber than pulled out through the charcoal filters so as to eliminate possible contamination from outside air. Filtered clean air from a wall-mounted air filtering system (ARS Inc., Gainesville, FL, USA) was pushed into the chamber at 2.5 L min^{-1} and volatiles from the headspace of grape shoots were drawn by a vacuum pump onto four activated charcoal filters (ORBO32-small, Supelco Inc., Bellefonte,

PA, USA) at 0.5 L/min/filter. Adsorbent samplings were made from four plants, with collections for each grape plant made over 4 days at room temperature with supplemental light (18:6 L:D). The chamber was washed with acetone, and new ORBO filters were used for a new plant. The volatiles were eluted with 1 ml hexane every 24 h and then combined. The combined extract was concentrated to 1 ml under a gentle stream of nitrogen gas and kept in a freezer (−20°C) and subjected to GC-EAD and gas chromatography–mass spectrometry (GC-MS) analyses, and flight-tunnel bioassay.

SPME Sampling We used the same glass chamber that was used for adsorbent sampling to collect headspace volatiles of live grape shoots using SPME. Filtered air was pushed into the chamber at 1 L min^{-1}. Three of the four air outlets were blocked with rubber septa, while one was 70% blocked with Teflon tape allowing the SPME fiber to be inserted into a thin stream of headspace volatiles for increased collection efficiency. After a plant was set up in the chamber, we flushed the chamber with filtered air as described above. We conditioned a carboxen/poly-dimethyl siloxane-coated SPME fiber (film thickness 85 μm; Supelco Inc., Bellefonte, PA, USA) in the GC injector (280°C) for 5 min and then inserted the fiber through a small hole on the air-outlet. The fiber was exposed for up to 24 h to absorb the volatiles, and immediately subjected to GC-EAD or GC-MS analyses. The chamber was washed with acetone before sampling a new plant.

Coupled GC-EAD Analysis Coupled GC-EAD analyses were performed following procedures described previously (Zhang et al. 1999; Nojima et al. 2003). A Hewlett-Packard 5890 Series II gas chromatograph, equipped with a non-polar EC-1 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech Associates, Inc., Deerfield, IL, USA) or a polar EC-Wax Econo-Cap capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech) was used for GC-EAD analyses. The oven temperature was programmed from 40°C for 5 min then increased by 15°C min^{-1} to 250°C. Injector and detector temperatures were set at 280°C and 270°C, respectively. A 0.75-mm ID glass inlet liner (Supelco) was used for SPME sample injection and a 4-mm ID liner (Supelco) for liquid samples. Splitless injection was used with nitrogen as the carrier gas at a flow of 2 ml min^{-1}. The column effluent was split in a ratio of 1:1 in the oven to the flame ionization detector and to the heated (270°C) EAD port.

A whole head was removed from a 3-day-old virgin female GBM and both antennae were positioned between two gold wire electrodes that were immersed in two saline-filled micropipettes in a small acrylic holder. We used an Ephrussi–Beadle insect Ringer as saline (Ephrussi and Beadle 1936). The moth's head was placed into the tip of one micropipette, and the tips of both antennae, which were wetted with saline containing surfactant (0.02% Triton X-100) for easy insertion, were maneuvered to make contact with the saline in the other micropipette tip. The antennal holder was placed inside a humidified cooling condenser maintained at 10°C. To compare relative magnitudes of antennal responses, the millivolt values for EAD-active compounds were calculated from GC-EAD recordings performed with SPME and adsorbent sample runs. A minimum of five different antennal pairs (two to three runs/pair) were used to analyze volatiles from shoots of the same four *V. riparia* plants that were subjected to adsorbent sampling.

Chemical Analysis GC-MS was carried out with a Shimadzu GCMS-QP5050A quadrupole mass spectrometer running in the EI (at 70 eV) scan mode coupled with a Shimadzu GC-17A equipped with a nonpolar DB-1 capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; J&W Scientific, Folsom, CA, USA) or a polar EC-Wax Econo-Cap capillary column (30 m×0.25 mm ID, 0.25 μm film thickness; Alltech Assoc.). Helium was used as the carrier gas at a constant flow of 1.0 ml min^{-1}. GC conditions and temperature program were as for the GC-EAD analyses. Volatile compounds were identified by mass spectral matches to library spectra as well as by retention time matches to available authentic standards. The EAD-active compounds were verified by GC-EAD analysis with authentic standards. Quantification of the relative ratio of the EAD-active compounds was made from the adsorbent collection based on ion abundances from GC-MS analyses.

Chemicals (*Z*)-3-hexen-1-yl acetate, linalool oxide (1:1 mixture of (*E*)- and (*Z*)-isomers), nonanal, linalool, methyl salicylate, decanal, β-caryophyllene, and α-farnesene, were obtained from Sigma-Aldrich Inc. (St. Louis, MO, USA), Alfa Aesar (Ward Hill, MA, USA), Fluka (Buchs, Switzerland) or TCI America (Portland, OR, USA). All, except α-farnesene (a mixture of various isomers) were greater than 97% purity. The 4,8-dimethyl-1,3(*E*),7-nonatriene was synthesized by oxidation of (*E*)-citral (Zhang et al. 2002) and then by Wittig reaction (Greenwald et al. 1963), and was a mixture of 95% (*E*)-isomer and 5% (*Z*)-isomer. Germacrene-D was isolated from golden rod as 91% germacrene-D and 9% β-caryophyllene (by USDA Chemistry Research Unit, Gainesville, FL, USA).

For all flight-tunnel bioassays and field tests, mixtures were prepared in ratios that corresponded to the ratios of compounds found in the collections of *V. riparia* shoots. For flight-tunnel bioassays, the blends with all identified compounds were mixed according to the ratios indicated by live plant samplings and diluted with hexane to 0.108 μg of total compounds in 1 μl of hexane. Preliminary flight-

tunnel experiments showed adequate moth responses to this concentration. All subsets of this blend were mixed at the same concentration as the blend with all identified compounds. For the field trial, the blend of compounds tested was diluted to 0.875 μg μl^{-1}. All the blends were stored in a freezer (−20°C) between tests.

Flight-Tunnel Assays Five-day-old females from a mating cage were used for flight-tunnel bioassays. Each cohort was set up using 10–15 female pupae (near eclosion) in a Plexiglas mating cage (30 cm H×30 cm W×30 cm D) and provided with 50% honey and water. Fifteen to 20 males (one antenna clipped for distinction from females) were added to the cage with a grape cluster. On average, 70% of females were mated by the fifth day, with copulation peaking on the second day (G. Loeb, unpublished data). The responses of female moths from the mating cage to grape shoots and volatile blends were assessed in a flight tunnel as described in Cha et al. (2008). After each flight-tunnel trial, the male and female moths were returned to the colony to maintain a consistent population level. We did not confirm mating status of females in the flight-tunnel experiments. The flight tunnel was 2 m in length by 0.6 m in width and 0.6 m in height, with a fan installed at the upwind end to create a steady airflow into the tunnel and an exhaust hood at the downwind end to evacuate odor from the flight tunnel. Wind speed was set at 0.25 m s^{-1} at the point of release of moths. A pattern made of dark green paper circles (10 cm diameter) was randomly presented both on a white background glass floor and on the glass ceiling below the light source. The upwind and downwind ends of the tunnel consisted of two layers of cheesecloth to prevent escape of moths. Light was provided from above by eight 25-W incandescent bulbs. Light intensity in the tunnel was 25 lx so as to mimic dusk conditions. During the experiments, the temperature and relative humidity of the flight tunnel were 23.3°C (±0.72 SD) and 23.2% (±0.32 SD).

All female moths were flown around dusk for the laboratory colony (over a 2-h period, from 1 h before to 1 h after dark). We tested individual moths in the flight tunnel, recording behavior for 8 min per moth. For each moth, we noted whether it left the release cage and made an upwind flight (more than 50 cm of tight, zigzag flight to within 10 cm of the target) as well as if it landed on (made contact with) the target. For data analysis, we categorized each moth based on the most complete behavior the moth displayed within an 8-min observation period. Thus, the behavioral responses of moths were categorized as 'no upwind flight' (no directed flight toward the target), 'upwind flight' or 'landing'.

We used freshly cut grape shoots and rubber septa (Thomas Scientific, Swedesboro, NJ, USA) loaded with synthetic blends as targets in the flight tunnel. A grape shoot (15 cm) was cut and immediately placed into a water pick just prior to flights as described in Cha et al. (2008). Freshly cut shoots were used because we found no difference in upwind flight or landing responses of females to freshly cut shoots or potted grape plants and because we wanted to minimize target size and eliminate possible contamination from pot or soil. In the flight tunnel, the cut shoot was apparent to female GBM. The target for a particular trial was placed at the upwind end in the center of the tunnel (30 cm from upwind end), affixed on an acetone-washed holder constructed of a glass rod and Teflon tubing. Female moths were introduced in the target plume (determined by using smoke from incense) at about 1.5 m downwind from the target in the center of the tunnel (30 cm from downwind end) in a small metal screen cage.

Field Experiment The field test was conducted during August and September of 2007 in a commercial vineyard near Rushville, NY, USA that had a history of severe GBM damage. We chose a custom-made plastic sheet trap for the field test, based on preliminary flight-tunnel tests of three different trap designs, including the Bio-lure Scenturion delta trap (Suterra LLC, Bend, OR, USA), a red ball trap (Zhang et al. 1999) and our custom plastic sheet trap, and by using the same seven-component blend we used in the field test. The Delta trap and red ball trap baited with the seven-component blend did not catch any upwind flying moths in the flight tunnel, whereas the plastic sheet trap captured 20% of the moths. The plastic sheet trap was constructed by overlaying 11 layers of plastic sheets (30 cm×30 cm; Kittrich Co., La Mirada, CA, USA) coated with STP Oil Treatment as the sticky substance. Each week one layer of plastic sheet was removed from each side, allowing us to monitor GBM for up to 6 weeks without applying additional oil. Each trap had six holes (2.5×2.5 cm) cut in two rows (three holes in top row and three holes in bottom row), with a rubber septum attached at each hole. Each hole was 5 cm apart and 6.25 cm from an edge of the trap. Each septum was loaded with 300 μl of one of the synthetic blends or hexane, and attached to the trap with an insect pin. For each hole, a pin was pressed through the middle plastic layer (2.5 cm below hole) and woven back out again in a manner that left the point of pin in the center of each hole. Rubber septa were replaced every 3 weeks.

We compared attractiveness of two different lures, the seven- and 11-component blends, as well as a hexane control, to female GBM. Each treatment was replicated four times in four blocks, with two traps installed (one trap per block) at the edge of a vineyard planted with *V. labrusca* (variety niagara) and two traps installed (one trap per block) at the edge of a forest contacting the vineyard. Traps were hung approximately 1 m from the ground and spaced at least 10 m apart. Traps were monitored twice weekly from

August 17, 2007 until September 11, 2007, yielding a total of seven assessments over a 25-day period. Each captured GBM was transferred to the laboratory, and its sex was determined under a dissecting microscope. Since the captured moths were usually drenched with oil, we could not determine whether the female moths were mated or not.

Statistical Analysis The attractiveness of different lure mixtures to female GBM was analyzed by using generalized linear models, with upwind flight or landing as dependent variables and different mixtures as a fixed independent variable, with binomial distribution with logit link function and maximum likelihood estimation (Proc Glimmix; SAS Institute 2006). The effect of the different type of lures (hexane control vs. seven-component vs. 11-component) and location of trap (forest edge vs. vineyard edge) on the number of male and female GBM captured over a 25-day period was analyzed by using repeated mixed model analysis of variance with Kenward–Roger degrees of freedom estimation and AR(1) as a covariance structure for repeated measures (Proc Mixed; SAS Institute 2000). Block was a random factor nested in location. Lure type, location, and time were fixed factors.

Results

Identification of Candidate Host Volatiles from V. riparia Shoots A total of four shoot samples were analyzed by SPME and GC-EAD by using at least five female GBM antennae for each sample. SPME sampling consistently revealed the same 11 significant antennal responses (Table 1). The EAD-active compounds were identified by comparison of mass spectra and GC-MS retention times with those of synthetic standards. The adsorbent collection of volatiles was made from four *V. riparia* shoots and the relative ratios of the 11 EAD-active chemicals determined by GC-MS and used in the flight-tunnel tests.

Flight-Tunnel Experiments for Identification of Key Host Volatiles: Negative and Positive Controls Responses of female GBM in the flight tunnel to hexane-loaded rubber septa and grape shoots were used as negative and positive controls, respectively. A total of 42 females were tested individually to a rubber septum loaded with 300 μl of hexane; the majority of these did not leave the cage, and those that did flew to the ceiling, sides, or back screen of the flight tunnel. A total of 144 females were flown to grape shoots and 73.6% (±3.7 SE) of them showed upwind flight. Thus, we used an upwind flight rate of 0% as our negative control and 73.6% as our positive control. Some 55.6% (±11.7 *SE*; $N=51$) of females tested exhibited upwind flight to the shoot extract; this was not significantly different from the responses to the shoots ($t_{24}=1.57$; $P=0.129$).

Flight-Tunnel Experiment 1 Based on the strength of EAD responses (Table 1), we first tested a blend composed of seven compounds, (*Z*)-3-hexenyl acetate, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, β-caryophyllene, germacrene-D, and α-farnesene (treatment 1, Fig. 1). This blend elicited 51.6% (±8.9 SE) of females to exhibit upwind flight responses, but this was not as attractive as actual grape shoots ($t_{36}=2.37$; $P=0.023$). Next, we tested whether there were possible deterrents in this blend by sequentially removing each of the components (treatments 2–8, Fig. 1). No deterrent was detected, as no blend with one component removed had significantly greater attraction

Table 1 EAD-active chemicals from shoots of *Vitis riparia* determined by SPME, GC-EAD, and GC-MS

Antennae active compounds	GC-MS (%)	EAD response (mV±SD)	Relative ratio in mix (%)	First seven-compo mix	Second seven-compo mix
(*Z*)-3-hexen-1-yl acetate	14.6	0.23±0.14	10	•	
(*Z*)-Linalool oxide	0.7	0.14±0.07	3		•
(*E*)-Linalool oxide	1.2	0.09±0.06	3		•
Nonanal	2.2	0.06±0.04	6		•
Linalool	1.7	0.50±0.40	6	•	
(*E*)-4,8-dimethyl 1,3,7-nonatriene	21.2	0.58±0.42	13	•	•
Methyl salicylate	1.5	0.60±0.51	6	•	
Decanal	2.9	0.08±0.08	6		•
β-caryophyllene	10.4	0.36±0.20	3	•	•
Germacrene-D	10.5	0.26±0.19	11	•	•
α-Farnesene	33.0	0.35±0.21	34	•	

Relative ratio (%) of peak areas of active compounds in the shoot extract and the strength of antennal response (mV±SD) to each of the active compounds are shown. All synthetic blends were mixed based on the relative ratio of compounds shown here. In addition, volatile components in the first seven-component blend (see "Flight-tunnel experiment 1") and second seven-component blend (see Flight-tunnel experiment 2) are shown

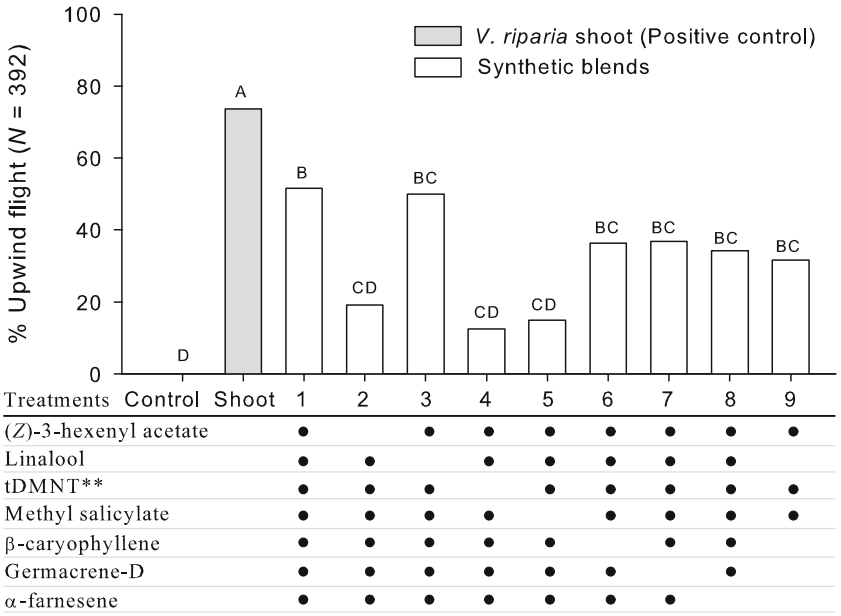

Fig. 1 Upwind flight responses (%) of female grape berry moth (N=392) in flight tunnel to hexane control (0%), *Vitis riparia* shoots (*gray bar*), and various synthetic blends (*white bars*). Treatment 1 was a mixture of seven compounds listed below the graph. Treatments 2–9 were subsets of treatment 1. At least 20 individual moths were flown to each synthetic blend tested. Different *letters on bars* indicate significant differences (P<0.05; *tDMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene)

compared to the original seven-component blend. However, from this experiment, (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate appeared to be important components, since removing any of these resulted in significantly fewer upwind flights compared to the original blend (Fig. 1). The blend composed only of (*Z*)-3-hexenyl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and methyl salicylate (treatment 9) elicited GBM flight responses not statistically different from those to the original blend (t_{36}=1.37; P=0.179). Three hundred and ninety two females were tested in this experiment.

Flight-Tunnel Experiment 2 The second set of flight-tunnel trials used all 11 EAD-active compounds listed in Table 1. This full blend elicited 68.6% (±7.8 SE) of females to exhibit upwind flight; this was not statistically different from that to grape shoots (t_{38}=0.60; P=0.55; treatment 10, Fig. 2). The removal from the blend of linalool, β-

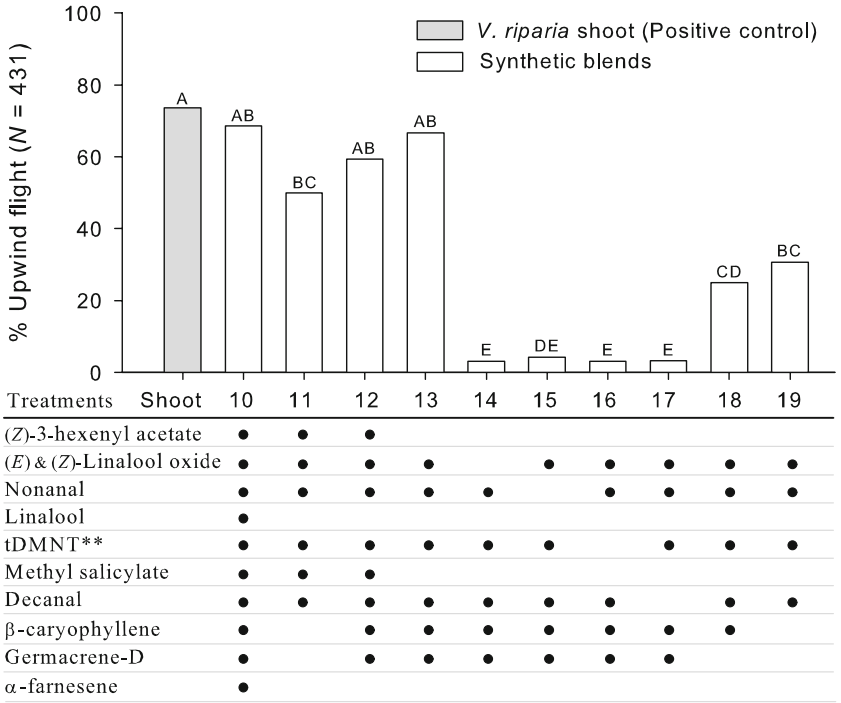

Fig. 2 Upwind flight responses (%) of female grape berry moth (N=431) in flight tunnel to *Vitis riparia* shoots (*gray bar*) and various synthetic blends (*white bars*). Treatment 10 was a mixture of 11 compounds listed below the graph. Treatments 11–19 were various subsets of treatment 10. At least 20 individual moths were flown to each synthetic blend tested. Different *letters on bars* indicate significant differences (P<0.05), except that P=0.051 for both "shoot vs. treatment 11" and "treatment 17 vs. treatment 18" (*tDMNT: (*E*)-4,8-dimethyl-1,3,7-nonatriene)

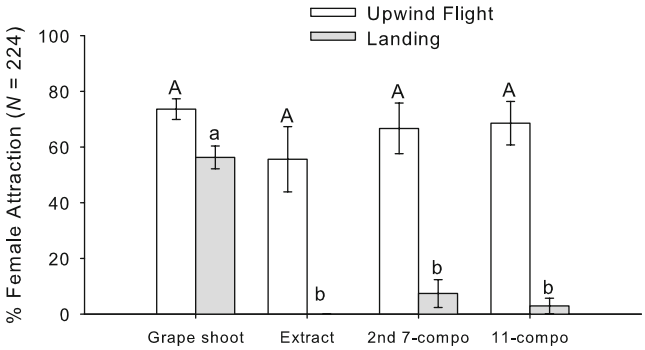

Fig. 3 Comparison of upwind flight (%) and landing (%) responses of female grape berry moth to *Vitis riparia* shoots, shoot extract and two synthetic blends. 11-component blend=(*Z*)-3-hexen-1-yl acetate, (*E*)-linalool oxide, (*Z*)-linalool oxide, nonanal, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, decanal, β-caryophyllene, germacrene-D, and α-farnesene; second seven-component blend=11-component blend minus (*Z*)-3-hexen-1-yl acetate, linalool, methyl salicylate and α-farnesene. Different *letters* (*capital letters* for orientation response and *small letters* for landing response) *on bars* indicate significant differences ($P<0.05$). *Error bars*=±1 SE

caryophyllene, germacrene-D, and α-farnesene, the four compounds that appeared not to be important based on the results of the first experiment, resulted in 50% (±11.8 SE) of GBM tested exhibiting upwind flight; this was not statistically different from the responses exhibited to the 11-component blend ($t_{38}=1.31$; $P=0.198$), but was marginally lower than that of the shoots ($t_{38}=2.02$; $P=0.051$). Adding β-caryophyllene and germacrene-D back to the blend (treatment 12) increased attractiveness to the level comparable to shoots.

In a further treatment, (*Z*)-3-hexen-1-yl acetate and methyl salicylate (treatment 13) were removed from the nine-component blend (i.e., 11-component blend minus linalool and α-farnesene) without reducing the attractiveness (66.7%±9.1 SE) of the mixture relative to that of the shoots or the original 11-component blend (Fig. 2). Linalool oxide, nonanal, (*E*)-4,8-dimethyl-1,3,7-nonatriene, and decanal were key components in this second seven-component blend. The removal of linalool oxide (as a 1:1 mixture of (*E*)- and (*Z*)-linalool oxide), nonanal, decanal, or (*E*)-4,8-dimethyl-1,3,7-nonatriene individually resulted in significantly lower (3.1~4.2%; treatments 14–17, Fig. 2) flight responses from females. In contrast, the removal of germacrene-D (treatment 18) or β-caryophyllene and germacrene-D (treatment 19) from the second nine-component blend resulted in only a 41.7% and 36.1% decrease (relative to that to the second seven-component blend), respectively, in upwind flight responses of females. Since the germacrene-D contained 9% of β-caryophyllene, however, we could not conclude that both germacrene-D and β-caryophyllene were essential compounds in the second seven-component blend. Four hundred and thirty one females were tested in this experiment.

Although the upwind flight responses of females to the 11- and second seven-component synthetic blends (treatments 10 and 13 in Fig. 2, respectively) were not significantly different ($F_{3,24}=0.93$; $P=0.442$) from those to grape shoots or shoot extract, there were significant differences among these treatments in terms of landing ($F_{3,24}=8.71$; $P<0.001$). In particular, the extract and synthetic blends elicited significantly lower landings from females in the flight tunnel compared to the shoots (Fig. 3). Two hundred and twenty four females were tested in this experiment.

Field Experiment In a late-season field trial, conducted in a high GBM-pressure commercial vineyard, traps baited with either the 11- or second seven-component blends caught significantly ($F_{2,15}=7.22$; $P=0.006$) higher numbers of female GBM than traps baited with a hexane control (Fig. 4); there was no significant effect of location (i.e., forest edge vs. vineyard) or time on catch of female moths. In contrast, there were no differences among treatments in the numbers of male moths captured ($F_{2,24.9}=0.08$; $P=0.925$). For males, however, we found a significant interaction among time, location, and lure ($F_{12,27}=2.47$; $P=0.025$). Overall, the total number of female and male moths captured was low (0.25 moths per day for best treatment), especially considering that the trial was performed in a high GBM-pressure vineyard. The best blend caught an average of four female moths/trap during the 25-day trapping period.

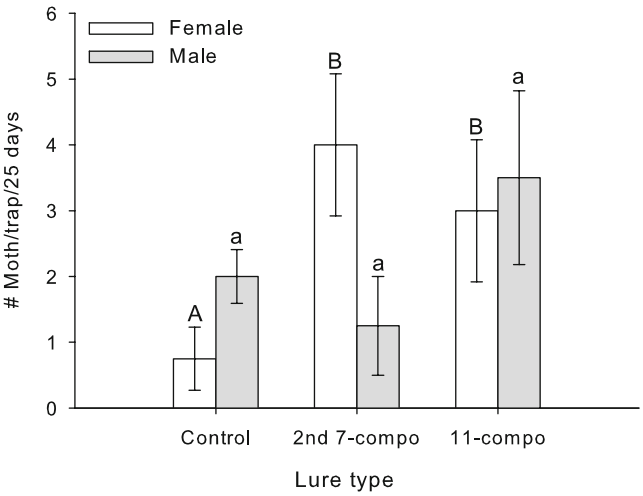

Fig. 4 Number of female and male grape berry moth captured in plastic-sheet traps, each baited with six rubber septa impregnated with a hexane control, the second seven-component blend or an 11-component blend in a commercial vineyard over a 25-day period. Eleven-component blend=(*Z*)-3-hexen-1-yl acetate, (*E*)-linalool oxide, (*Z*)-linalool oxide, nonanal, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, decanal, β-caryophyllene, germacrene-D, and α-farnesene; second seven-component blend=11-component blend minus (*Z*)-3-hexen-1-yl acetate, linalool, methyl salicylate and α-farnesene. *Error bars*=±1 SE

Discussion

We identified a blend of 11 volatile compounds [(*Z*)-3-hexen-1-yl acetate, (*E*)-linalool oxide, (*Z*)-linalool oxide, nonanal, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, decanal, β-caryophyllene, germacrene-D, and α-farnesene] from the shoots of *V. riparia*, a native host, that elicited an equivalent percentage of upwind flight by female GBM as that by intact shoots. Traps baited with this blend successfully caught female GBM in a vineyard. Subtraction of (*Z*)-3-hexen-1-yl acetate, linalool, methyl salicylate, and α-farnesene from this blend did not affect efficacy of the mixture with respect to eliciting both upwind flight responses of female GBM in a flight tunnel and trap catch in the field.

We originally hypothesized that the magnitude of antennal response in the GC-EAD analysis may indicate the relative importance (i.e., behavioral activity) of each of the compounds. However, this approach resulted in a blend that was not as attractive as grape shoots in our first set of flight-tunnel experiments. Addition of the four compounds that elicited the smallest antennal responses resulted in improved levels of upwind flight, comparable to that to grape shoots in the second set of experiments. This result suggests that the strength of antennal responses should not be assumed to correspond directly to behavioral activity.

Although both the 11- and second seven-component blends elicited upwind flight responses of female GBM comparable to that to grape shoots, the lower level of landing and low trap catches in the field to the synthetic blends suggest that their compositions may not be optimal for trapping female GBM. Several, non-mutually exclusive factors may be responsible for this. GBM could use both olfactory and visual cues for landing. For example, in *Lygus hesperus*, visual cues were shown to enhance the response to host-plant volatiles (Blackmer and Cañas 2005). GBM, a crepuscular species, oviposits dramatically less in the absence of light (Clark and Dennehy 1988), suggesting that females may use visual cues in this behavior. In addition, other chemicals, such as fatty acids, with low volatility present on grape leaf or berry surfaces could also elicit landing. For example, Wallace et al. (2004) reported that mated females of the spruce budworm preferentially oviposited on substrates treated with waxes from the needles of a host plant. Finally, CO_2 and moisture gradients released by grape shoots could affect close-range landing behaviors (references in Thom et al. 2004; Hilker and McNeil 2008).

The low-landing responses we obtained to the synthetic mixtures could also be due to a sub-optimal ratio of components in the blend. It has been suggested that many herbivores and parasitoids perceive a specific mixture of plant volatiles to locate their host reliably (Schoonhoven et al. 1998; Hilker and McNeil 2008). Such specificity can be mediated by species-specific host chemicals. However, the compounds in our two blends are relatively ubiquitous plant volatiles. In such a case, a species-specific ratio of common compounds could be a key to locating hosts within a complex environment of plant volatiles (Bruce et al. 2005; Hilker and McNeil 2008). Although we based the ratio of compounds in the blends on our adsorbent collections from live, potted plants, it is possible that the ratio of components we tested might not correspond exactly to that released by live grape shoots due to the adsorbent characteristics (Tasin et al. 2006b), and also the differential release rate of components from the rubber septa (Butler and McDonough 1979; Heath et al. 1986). The fact that we also observed low levels of landings on septa with the extract supports this contention, although other factors may also be involved.

Headspace volatiles were collected from live potted grapes to ensure that they were consistent in quality and quantity with that released by plants in the field, since physical wounding, such as cutting, could cause changes in volatile emissions both locally and systemically (Davies 1987; Rhodes et al. 1999). In the flight tunnel, however, we found both cut shoots and potted plants were equally attractive to female GBM, suggesting that cut-plant materials did not significantly affect behavior. However, immediately after excision, there was a six-fold increase in (*Z*)-3-hexenyl acetate released by shoots. This chemical, a green leaf volatile, is well recognized as a wound-induced compound (e.g., Engelberth et al. 2004), and also elicited a GC-EAD response from female GBM. Thus, the ratio of at least one GC-EAD-active compound, (*Z*)-3-hexenyl acetate, that was also considered essential in one of our blends (see below), appears to be not critical for female GBM behavior. Our future research will investigate explicitly the role of blend ratio of GC-EAD-active compounds to determine how GBM, a specialist herbivore, recognizes its host by using common, ubiquitous volatile compounds, and, if ratio is important, whether the ratios of all the GC-EAD compounds are important or just that of a subset of the compounds.

The results from the field-trapping experiment showed no significant differences in the number of males captured between traps with synthetic lures based on host-plant volatiles and control traps. This suggests that males may not be responding to the same blend of host-plant volatiles as females, similar to the findings in flight-tunnel trials for the European berry moth (e.g., Masante-Roca et al. 2007). However, definitive conclusions cannot be drawn since our field trial was conducted late in the season, and relatively few moths were captured.

Not all the volatile compounds isolated by GC-EAD were essential for eliciting upwind flight from female

GBM. Moreover, what compounds are essential appears to be context specific (e.g., Mumm and Hilker 2005; Hilker and McNeil 2008). For example, removal of (*Z*)-3-hexen-1-yl acetate, linalool, methyl salicylate, and α-farnesene from the 11-component blend gave equivalent levels of attraction as shoots. Removal of any of the remaining seven compounds, individually, significantly reduced attractiveness of the blend, suggesting that some or all of these seven compounds [(*E*)-linalool oxide, (*Z*)-linalool oxide, nonanal, (*E*)-4,8-dimethyl-1,3,7-nonatriene, decanal, β-caryophyllene, and germacrene-D] are essential for optimal attraction. However, with the first seven-component blend of (*Z*)-3-hexen-1-yl acetate, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, methyl salicylate, β-caryophyllene, germacrene-D and α-farnesene that we tested, we found that (*Z*)-3-hexen-1-yl acetate, (*E*)-4,8-dimethyl-1,3,7-nonatriene and methyl salicylate were essential components of the blend. Therefore, the finding that some compounds, such as (*Z*)-3-hexen-1-yl acetate and methyl salicylate in this study, acted as essential components in one blend (i.e., the first seven-component blend; treatment 1 in Fig. 1) but not in another (i.e., the second seven-component blend; treatment 13 in Fig. 2) suggests that some plasticity exists in the volatile-based host recognition system of GBM. Such a context dependency may have important implications both for understanding the evolution of volatile-based host recognition of herbivores and for developing a synthetic lure that works in the field. Plasticity in recognition of host plants with different volatile blends could have been selected for to allow host location under seasonal and daily variation in host chemistry (e.g., Vallat and Dorn 2005; Hilker and McNeil 2008).

The long-term goal of our research is to develop a synthetic lure based on host-plant volatiles that can be used to monitor female GBM populations in the field as a way to improve the timing of management decisions. Our late-season field trial provides some reason for optimism that this approach is feasible. However, the low capture of moths indicates that significant hurdles remain. One problem is that synthetic lures may not stand out to females from the background volatile noise produced by the crop plant and the many other plants in the environment. Ultimately, we may need to discover some novel compounds that are uniquely attractive, either temporally or spatially. Furthermore, information on close-range visual and chemosensory cues may help improve the utility of volatile chemical-based lures for pest management.

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References

ANGIOY, A. M., DESOGUS, A., BARBAROSSA, I. T., ANDERSON, P., and HANSSON, B. S. 2003. Extreme sensitivity in an olfactory system. *Chem. Sens.* 28:279–284.

ANSEBO, L., CORACINI, M. D. A., BENGTSSON, M., LIBLIKAS, I., RAMIREZ, M., BORG-KARLSON, A. K., TASIN, M., and WITZGALL, P. 2004. Antennal and behavioural response of codling moth *Cydia pomonella* to plant volatiles. *J. Appl. Entomol.* 128:488–493.

BERNAYS, E. A., and CHAMMAN, R. F. 1994. Host plant selection by phytophagous insects. Chapman & Hall, New York.

BLACKMER, J. L., and CAÑAS, L. A. 2005. Visual cues enhance the response of *Lygus hesperus* (Heteroptera: Miridae) to volatiles from host plants. *Environ. Entomol.* 34:1524–1533.

BLACKMER, J. L., RODRIGUEZ-SAONA, C., BYERS, J. A., SHOPE, K. L., and SMITH, J. P. 2004. Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. *J. Chem. Ecol.* 30:1547–1564.

BRUCE, T. J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2005. Insect host location: a volatile situation. *Trend. Plant Sci.* 10:269–274.

BUTLER, L. I., and MCDONOUGH, L. M. 1979. Insect sex-pheromones—evaporation rates of acetates from natural-rubber septa. *J. Chem. Ecol.* 5:825–837.

CHA, D. H., HESLER, S. P., MOSER, C. L., NOJIMA, S., LINN, C. E., ROELOFS, W. L., and LOEB, G. M. 2008. Flight tunnel responses of female grape berry moth (*Paralobesia viteana*) to host plants. *J. Chem. Ecol.* 34:622–627.

CLARK, L. G., and DENNEHY, T. J. 1988. Oviposition behavior of grape berry moth. *Entomol. Exp. Appl.* 47:223–230.

CORACINI, M., BENGTSSON, M., LIBLIKAS, I., and WITZGALL, P. 2004. Attraction of codling moth males to apple volatiles. *Entomol. Exp. Appl.* 110:1–10.

DAVIES, E. 1987. Wound responses in plants, pp, pp. 243–264, in D. Davies (ed.). The Biochemistry of PlantsAcademic, London.

DOZIER, H. L., and BUTLER, H. G. 1929. Life history and control of the grape berry moth in Delaware. *J. Econom. Entomol.* 22:132–136.

ENGELBERTH, J., ALBORN, H. T., SCHMELZ, E. A., and TUMLINSON, J. H. 2004. Airborne signals prime plants against insect herbivore attack. *PNAS USA* 101:1781–1785.

EPHRUSSI, B., and BEADLE, G. W. 1936. A technique for transplantation of *Drosophila*. *Am. Nat*. 70:218–225.

GLEISSNER, B. D. 1943. Biology and control of berry moth in the Erie grape belt. *Pennsylvania State College School of Agriculture Bulletin* 451:1–74.

GOODWIN, W. H. 1916. The grape berry moth. *Ohio Agricultural Experiment Station Bulletin* 293:259–307.

GREENWALD, R., CHAYKOVSKY, M., and COREY, E. J. 1963. TheWittig reaction using methylsulfinyl carbanion–dimethyl sulfoxide. *J. Org. Chem.* 28:1128–1129.

HAMMACK, L. 2003. Volatile semiochemical impact on trapping and distribution in maize of northern and western corn rootworm beetles (Coleoptera: Chrysomelidae). *Agri. For. Entomol.* 5:113–122.

HEATH, R. R., TEAL, P. E. A., TUMLINSON, J. H., and MENGELKOCH, L. J. 1986. Prediction of release ratios of multicomponent pheromones from rubber septa. *J. Chem. Ecol.* 12:2133–2143.

HERN, A., and DORN, S. 2004. A female-specific attractant for the codling moth, *Cydia pomonella*, from apple fruit volatiles. *Naturwissenschaften* 91:77–80.

HILKER, M., and MCNEIL, J. 2008. Chemical and behavioral ecology in insect parasitoids: how to behave optimally in a complex odourous environment, pp. 92–112, in E. Wajnberg, C. Bernstein, and J. van Alphen (eds.). Behavioral Ecology of Insect ParasitoidsBlackwell, Malden, MA.

HOFFMAN, C. J. 1990. Development and validation of a risk assessment program for the management of grape berry moth, *Endopiza viteana* (Clemens), in New York state. PhD Dissertation, Cornell University, Ithaca, NY.

LESKEY, T. C., ZHANG, A. J., and HERZOG, M. 2005. Nonfruiting host tree volatile blends: novel attractants for the Plum curculio (Coleoptera: Curculionidae). *Environ. Entomol.* 34:785–793.

MASANTE-ROCA, I., ANTON, S., DELBAC, L., DUFOUR, M.-C., and GADENNE, C. 2007. Attraction of the grapevine moth to host and non-host plant parts in the wind tunnel: effects of plant phenology, sex, and mating status. *Entomol. Exp. Appl.* 122:239–245.

MITCHELL, V. J., MANNING, L.- A. , COLE, L., SUCKLING, D. M., and EL-SAYED, A. M. 2008. Efficacy of the pear ester as a monitoring tool for codling moth *Cydia pomonella* (Lepidoptera: Tortricidae) in New Zealand apple orchards. *Pest Manage. Sci.* 64:209–214.

MUMM, R., and HILKER, M. 2005. The significance of background odour for an egg parasitoid to detect plants with host eggs. *Chem. Sens.* 30:337–343.

NAGARKATTI, S., MUZA, A., and SAUNDERS, M. 2000. Meridic diet for *Endopiza viteana* (Lepidoptera: Tortricidae). *Can. Entomol.* 132:259–261.

NATALE, D., MATTIACCI, L., PASQUALINI, E., and DORN, S. 2004. Apple and peach fruit volatiles and the apple constituent butyl hexanoate attract female oriental fruit moth, *Cydia molesta*, in the laboratory. *J. Appl. Entomol.* 128:22–27.

NOJIMA, S., LINN, C., MORRIS, B., ZHANG, A. J., and ROELOFS, W. 2003. Identification of host fruit volatiles from hawthorn (*Crataegus* Spp.) attractive to hawthorn-origin *Rhagoletis pomonella* flies. *J. Chem. Ecol.* 29:321–336.

PINERO, J. C., JACOME, I., VARGAS, R., and PROKOPY, R. J. 2006. Response of female melon fly, *Bactrocera cucurbitae*, to host-associated visual and olfactory stimuli. *Entomol. Exp. Appl.* 121:261–269.

RHODES, J. D., THAIN, J. F., and WILDON, D. C. 1999. Evidence for physically distinct systemic signalling pathways in the wounded tomato plant. *Ann. Bot.* 84:109–116.

ROELOFS, W. L., TETTE, J. P., TASCHENBERG, E. F., and COMEAU, A. 1971. Sex pheromone of the grape berry moth: identification by classical and electroantennogram methods, and field tests. *J. Insect Physiol.* 17:2235–2243.

SAS Institute. 2000. SAS/STAT User's Guide. SAS Institute, Cary, North Carolina, USA.

SAS Institute. 2006. The GLIMMIX Procedure. <http://www.sas.com>.

SCHOONHOVEN, L. M., JERMY, T., and VAN LOON, J. J. A. 1998. Insect–Plant Biology. Chapman & Hall, London, UK.

SLINGERLAND, M. V. 1904. The grape berry moth. *Cornell University, Agricultural experiment station of the college of agriculture Bulletin* 223:43–59.

TASCHENBERG, E. F. 1945. The biology and control of the grape berry moth *Polychrosis vineana* (Clemens). PhD Dissertation, Cornell University, Ithaca, NY.

TASIN, M., ANFORA, G., IORIATTI, C., CARLIN, S., DE CRISTOFARO, A., SCHMIDT, S., BENGTSSON, M., VERSINI, G., and WITZGALL, P. 2005. Antennal and behavioral responses of grapevine moth *Lobesia botrana* females to volatiles from grapevine. *J. Chem. Ecol.* 31:77–87.

TASIN, M., BACKMAN, A. C., BENGTSSON, M., IORIATTI, C., and WITZGALL, P. 2006a. Essential host plant cues in the grapevine moth. *Naturwissenschaften* 93:141–144.

TASIN, M., BACKMAN, A. C., BENGTSSON, M., VARELA, N., IORIATTI, C., and WITZGALL, P. 2006b. Wind tunnel attraction of grapevine moth females, *Lobesia botrana*, to natural and artificial grape odour. *Chemoecology* 16:87–92.

TASIN, M., BACKMAN, A.-C., CORACINI, M., CASADO, D., IORIATTI, C., and WITZGALL, P. 2007. Synergism and redundancy in a plant volatile blend attracting grapevine moth females. *Phytochemistry* 68:203–209.

THOM, C., GUERENSTEIN, P. G., MECHABER, W. L., and HILDEBRAND, J. G. 2004. Floral CO_2 reveals flower profitability to moths. *J. Chem. Ecol.* 30:1285–1288.

VALLAT, A., and DORN, S. 2005. Changes in volatile emissions from apple trees and associated response of adult female codling moths over the fruit-growing season. *J. Ag. Food Chem.* 53:4083–4090.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WALLACE, E. K., ALBERT, P. J., and MCNEIL, J. N. 2004. Oviposition behavior of the eastern spruce budworm *Choristoneura fumiferana* (Clemens) (Lepidoptera: Tortricidae). *J. Insect Behavior* 17:145–154.

WEIGLE, T., BIXBY, J., and ENGLISH-LOEB, G. 1999. Reexamination of grape berry moth management practices in the Lake Erie region. 1998 New York State Fruit Project Reports Relating to IPM. NYS IPM Publication #216. Cornell University Cooperative Extension.

ZHANG, A. J., LINN, C., WRIGHT, S., PROKOPY, R., REISSIG, W., and ROELOFS, W. 1999. Identification of a new blend of apple volatiles attractive to the apple maggot, *Rhagoletis pomonella*. *J. Chem. Ecol.* 25:1221–1232.

ZHANG, A., OLIVER, J. E., ALDRICH, J. R., WANG, B., and MASTRO, V. C. 2002. Stimulatory beetle volatiles for the Asian longhorned beetle, *Anoplophora glabripennis* (Motschulsky). *Z. Naturforsch.* 57c:553–558.

(HIPVs) have been used to attract and retain beneficial insects into vineyards and hopyards (James 2003a,b, 2005), which has led to a reduction in pest densities (James and Price 2004; James and Grasswitz 2005). To date, however, little is known about practical application of HIPVs in cotton agroecosystems.

Lygus lineolaris and *Lygus hesperus* (Heteroptera: Miridae) are important pests of cotton and other crops in North America (Jackson and Graham 1983; Leigh et al. 1988; Wheeler 2001). The feeding mechanics of *L. hesperus* can be categorized as 'mechanical cell rupture-enhanced maceration' that involves stylet maceration of plant cells, injection of saliva, and ingestion of the saliva-cell content slurry (Backus et al. 2007). Selection of oviposition sites by mirids typically is preceded by probing with the mouthparts (Cobben 1978; Wheeler 2001), and we have observed this behavior by *L. hesperus* (Williams, unpublished data). Rodriguez-Saona et al. (2002) showed that feeding and oviposition by *L. hesperus* females induced emission of significant amounts of volatiles from cotton, and the level of emission was positively associated with the number of eggs laid on a plant. Williams et al. (2005) reported that feeding by virgin females of *L. hesperus* induced 2.6-fold higher emission of cotton volatiles than feeding by mated females, and treatment of maize plants with *L. hesperus* salivary gland extracts and artificial mechanical injury resulted in 2.7-fold higher emission of volatiles than artificial mechanical injury alone.

Anaphes iole (Hymenoptera: Mymaridae) is an egg parasitoid that attacks *Lygus* and other mirids in North America (Huber and Rajakulendran 1988). Parasitism rates can exceed 90%, and thus, this wasp is an important natural enemy of *Lygus* species that has potential for pest suppression (Ruberson and Williams 2000). Conti et al. (1996, 1997) demonstrated that chemical and physical cues were important for host acceptance (i.e., recognition and oviposition) by *A. iole* of *L. hesperus* eggs. Wasps on plants harboring host eggs utilized chemical cues from the host and injured host plant, as well as physical cues associated with eggs and oviposition sites for host acceptance. Oviposition behavior of *A. iole* was also influenced by the experience of female wasps (Conti et al. 1997). Naïve females spent more time examining host eggs than did females preconditioned by exposure to host eggs embedded in plant tissue. Subsequent behavioral studies demonstrated that *A. iole* females were attracted to odors derived from plants infested with *L. hesperus* in a four-arm olfactometer (Manrique et al. 2005). However, wasp perception of individual synthetic plant volatiles and their role in attraction to and parasitism of host eggs by *A. iole* have not been previously investigated.

The goal of the present study was to evaluate the responses of *A. iole* wasps to individual HIPVs by using a combination of electroantennogram (EAG), behavioral, and field studies. Because associative learning often increases the response of parasitoids to host-related volatiles (Vet and Groenewold 1990; Turlings et al. 1991; Steinberg et al. 1992; Conti et al. 1997; Drukker et al. 2000; Santolamazza-Carbone et al. 2004), we investigated the effects of experience on *A. iole* response to HIPVs. Specifically, we asked: (1) Do female and male *A. iole* antennae respond differentially to HIPVs? (2) Does experience influence *A. iole* response to (*Z*)-3-hexenyl acetate, a common green leaf volatile? (3) Does *A. iole* respond to individual HIPVs in a wind tunnel? and (4) Do HIPVs increase field parasitism by *A. iole*?

Methods and Materials

Insects *A. iole* used in this study were originally obtained from a laboratory colony maintained on *L. hesperus* Knight eggs at the US Department of Agriculture-Agricultural Research Service (USDA-ARS) Biological Control and Mass Rearing Research Unit, Mississippi State, MS, USA. After emergence, wasps were provided with distilled water and 1 M sucrose ad libitum. *A. iole* were reared in Plexiglass cages (26×26×20 cm) at 25±1°C, 60–85% relative humidity (RH), and 14:10 L/D photoperiod until experimentation (3-day-old adults).

Electroantennography Concentration Responses The compounds included in this study (Table 1) were tested individually as olfactory stimuli. For the EAG study, serial dilutions (0.1, 1.0, 10, and 100 μM) of each compound were made with paraffin oil (E. Merck, Darmstadt, Germany). Stimulus applicators were prepared by pipetting 25 μl of a test solution onto a 6×0.5 cm strip of Whatman no. 1 filter paper (Whatman International Ltd., Maidstone, Kent, UK), after which the filter paper was placed inside a 14.5-cm long glass Pasteur pipette. Fresh stimulus applicators were prepared after 2 h of use. Three controls were used: (1) an empty pipette, (2) a pipette containing 25 μl paraffin oil only on filter paper, and (3) a pipette containing 25 μl 100 μM octanal in paraffin oil on filter paper (octanal standard). Differences in volatilities (see Kovats indices, Table 1) of the test compounds permitted only relative comparisons between test chemicals, except for closely related compounds.

The EAG apparatus (Syntech Ltd., Hilversum, The Netherlands) was linked to a desktop computer (with IDAC-02 data acquisition interface board) on which recording, storing, and quantifying EAG responses were performed. The recording and indifferent electrodes were silver wires enclosed in drawn glass capillary tubes filled with phosphate-buffered saline (NaCl, 4 g; Na_2HPO_4,

Table 1 Chemicals used in EAG and olfactometer trials with *A. iole*, their purity, commercial sources, and Kovats' retention indices

Chemical	Purity (%)	Commercial source[a]	Kovats' index[b]
Green leaf volatiles			
1-Hexanol	98	Aldrich	851
(*Z*)-3-Hexen-1-ol[c]	98	Aldrich	858
(*E*)-3-Hexen-1-ol[c]	98	Aldrich	1,038
(*E*)-2-Hexenyl acetate	98	Aldrich	NA
(*Z*)-3-Hexenyl acetate[c]	≥98	Bedoukian	1,009
Monoterpenes			
β-Myrcene[c]	≥99	Aldrich	992
(±)-Linalool[c]	95–97	Sigma	1,100
β-Caryophyllene[c]	90	Sigma	1,467
(*E*,*E*)-α-Farnesene[c,d]	NA	Bedoukian	1,500
(+)-Limonene[c]	97	Aldrich	1,030
(*E*)-β-Ocimene[c]	97	Fluka	1,038
(−)-α-Pinene[c]	98	Aldrich	937
Carboxylate ester			
Methyl salicylate[c]	≥99	Sigma	1,234
Aliphatic aldehyde			
Octanal (positive control)	99	Aldrich	1,006

[a] Aldrich Chemical Co., Milwaukee, WI, USA; Bedoukian Research, Inc., Danbury, CT, USA; Fluka, Buchs, Switzerland; Sigma Corp., St. Louis, MO, USA
[b] Kovats (1958). DB-5 column
[c] Included in preconditioning regime with four-arm olfactometer
[d] A mixture of α-farnesene and isomers

0.57 g; KH_2PO_4, 0.1 g; KCl, 0.1 g in 500 ml distilled water; pH 7.4). Antennal preparations were made by cutting transversely through the mesonotum just anterior to the tegulae with a scalpel and mounting the thorax on the indifferent electrode. Both antennae remained intact, and the recording electrode was placed on the tip of one randomly chosen antenna. The antennal preparation was bathed continuously by a stream of charcoal-filtered and humidified air at a flow rate of 1 l/min. Air temperature and relative humidity was measured approximately 15 cm from the antennal preparation (overall ranges for all trials: 19.2–26.3°C, 28–52% RH).

EAG recording began 6 min after the antennal preparation was mounted. At this time, the following test protocol was used for each recording trial. The controls were tested in the following order: 1, 2, 3, 2, after which six randomly chosen chemical treatments were tested. For each chemical, order of delivery of the four concentrations was random. Delivery of controls (2, 3, 2) was made after each four-concentration series of a chemical treatment. After the final chemical treatment for each recording, controls were presented in the following order: 2, 3, 2, 1. Presentation of controls throughout the recording session permitted standardization of antennal responses. Test compounds and controls were applied (0.5-s pulse) at 30-s intervals separated by a purge of filtered–humidified air via an aluminum tube approximately 5 mm from the antenna. EAGs were measured as maximum amplitude of depolarization (mV). Each chemical was tested on 10 to 35 individuals of each gender.

Short-Range Olfactory Response to HIPVs Short-range walking response of parasitoids to HIPVs was measured with a four-arm olfactometer (Vet et al. 1983). The base of the arena was precision-machined aluminum with a groove for a rubber O-ring, and the top consisted of a circular piece of Plexiglass (30 cm diameter). The Plexiglass top had a hole (5 mm diameter) in the center to facilitate placement of a wasp into the arena. The hole was plugged with a Teflon-wrapped cork. The top of the arena was held securely to the base by spring clamps, and the O-ring seal ensured that the arena did not leak. Each arm of the olfactometer was divided into three regions: release, visit, and selection regions (Manrique et al. 2005). The airflow (25 ml/min per arm) inside the arena was equalized by using one flowmeter (Aalborg, Orangeburg, NY, USA) at each arm and a terminal flowmeter between the olfactometer and the pump (Model 400-3910, Barrant Co., Carrington, IL, USA). Charcoal-filtered and humidified air was passed through four 250 ml Erlenmeyer flasks that contained odor sources. A white 19-l plastic bucket was inverted over the olfactometer. The bottom of the bucket had been removed and covered with a light diffuser upon which a circular light (22 cm diameter) was placed. The outside of the bucket was covered with aluminum foil to ensure that it was opaque.

An observation hole (6×6 cm) was cut in the side of the bucket to facilitate visual recording of data. This hole remained covered except when observations were being made. The use of a white bucket over the olfactometer provided uniform light inside the arena and reduced bias from external visual cues. Smoke tests ensured that the system was working properly (Vet et al. 1983).

In all trials, a single HIPV treatment was compared against an untreated control. (*Z*)-3-hexenyl acetate was chosen because of the high antennal response recorded in the EAG study (Fig. 1). The HIPV stimulus was prepared in the same way that EAG stimuli were prepared (see above). After pipetting 25 μl of the test solution or paraffin oil only (untreated control) onto the strip of filter paper, it was placed inside an Erlenmeyer flask. Two treatment arms were assigned opposite one another, and the other two arms were untreated controls. Individual *A. iole* females were transferred into the center of the arena with a dissecting needle, and the hole in the top of the arena was plugged. Thereafter, the vacuum pump was turned on, and the bucket-light assembly was replaced onto the arena. Wasps not leaving the release site within 2 min of initiating a trial were replaced. Otherwise, each wasp was observed continuously for 5 min, and the time spent in each region of the arena was recorded with a multichannel timer. Individual *A. iole* females were used only once. After each trial, the inside of the arena was wiped with 70% ethanol, the arena was rotated 90° clockwise, and the treatments were randomly reassigned. The HIPV stimulus was replaced after 30 min use. Air temperature was measured inside the chamber (overall range for all trials, 20.8–25.0°C) during the bioassays (April–June; 1000 to 1700 hours CDT).

Trials were conducted to determine the effect of different preconditioning regimes on response of *A. iole* female wasps to a single HIPV. The goal was to partition the preconditioning effects of a single synthetic HIPV, a blend of 11 synthetic HIPVs, a host plant on which *L. lineolaris* had fed and oviposited, and an artificial egg pack of *L. lineolaris* eggs on the response by *A. iole*. Compounds were chosen because their levels increased after *Lygus* feeding (Rodriguez-Saona et al. 2002) and because of the strong concentration response we observed in the EAG trials (see below). *Erigeron annuus* was used because it is an important host of *Lygus* species. *E. annuus* was collected from the vicinity of Stoneville, MS, USA, and stems (6 cm-long) with flowers were caged with mated *L. lineolaris* females for approximately 12 h, thus permitting feeding and oviposition. Artificial egg packs into which *L. lineolaris* had oviposited (<24-h old) were also used as a treatment. For these last two treatments, insects were removed from plants or egg packs prior to

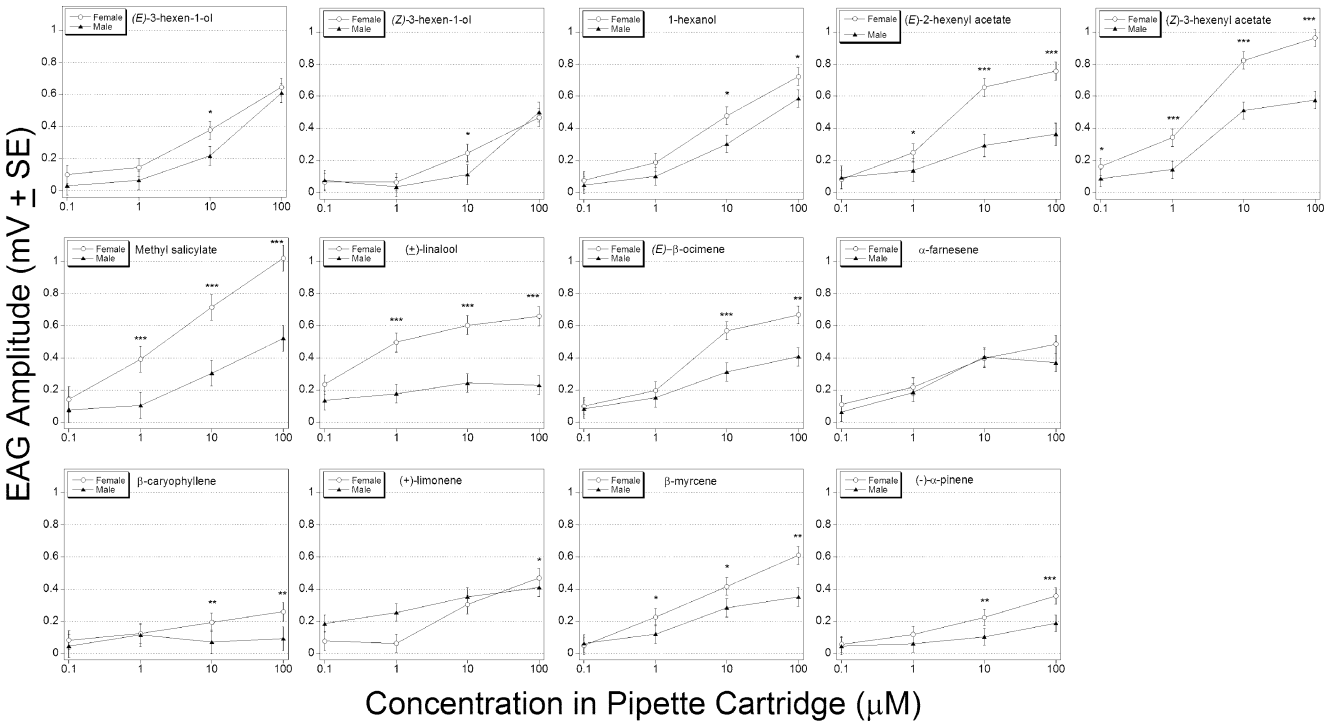

Fig. 1 EAG concentration–response curves of *A. iole* to 13 herbivore-induced plant volatiles. EAG amplitudes are control-adjusted and presented as proportional responses (mean±SE) to the standard, 100 μM octanal. Each compound was tested on 10 to 35 individuals of each gender. Significant differences between genders are noted by asterisks (*single asterisk* P=0.05–0.01, *double asterisk* P=0.01–0.001, *triple asterisk* P<0.001). P values correspond to ANOVA

experiments. Five preconditioning regimes were chosen to represent a range of signals, from simple to complex: (1) 10 μM (*Z*)-3-hexenyl acetate, (2) 1 μM each of 11 HIPVs (refer to Table 1), (3) *E. annuus* with *L. lineolaris* eggs, (4) *E. annuus* with *L. lineolaris* eggs + 10 μM (*Z*)-3-hexenyl acetate, and (5) *E. annuus* with *L. lineolaris* eggs + 1 μM each of 11 HIPVs. The exposure time for all preconditioning regimes was 45 min. For all these trials, wasps were tested with 100 μM (*Z*)-3-hexenyl acetate in the four-arm olfactometer. Different combinations of these four treatments allowed partitioning the effects of different odors on the behavior of *A. iole* wasps.

Long-Range Olfactory Response to HIPVs A horizontal wind tunnel was used to assess the behavioral response of *A. iole* at a larger scale than possible in a four-arm olfactometer. The wind tunnel was constructed of Plexiglass and measured 65 cm long×35 cm high×40 cm wide. It was subdivided into two compartments by a 34-cm-long partition at the upwind end. Each compartment received odors from either a treatment stimulus or untreated control. Charcoal filtered and humidified air was first pushed via pump (Model 400-3910, Barrant Co., Carrington, IL, USA) through 250 ml Erlenmeyer flasks containing either an odor source or blank control, and then into the two upwind wind tunnel compartments. HIPV odor sources were prepared as described for the four-arm olfactometer. Each compartment had four holes (each 5 mm diameter) to allow air flowing from the treatment chambers to enter the cage, and a flowmeter (Aalborg, Orangeburg, NY, USA) was used to regulate the overall airflow at 100 ml/min per hole. At the downwind end of the tunnel, there was a door with a rectangular window (19×15.25 cm) covered with nylon organdy that allowed introduction of wasps. At the downwind end of the tunnel, a piece of Plexiglass (20.3×35 cm) was glued from each side of the window to the side wall of the box, thus helping to funnel air toward the window. A computer muffin fan at the downwind end vented airflow from the tunnel. Smoke tests confirmed that air flowed through the chamber without mixing between treatment compartments. All outside walls of the box were covered with opaque white cardstock to provide uniform lighting and avoid external visual cues. The paper on the top of the wind tunnel had two holes (22 cm diameter) to facilitate illumination provided by two circular lights. The lights were placed parallel to the long axis of the chamber, with the outside edge of each light 10 cm from the edge of the chamber. The wind tunnel was rinsed with 70% ethanol after each bioassay. Temperature and humidity inside the room were measured (overall ranges for all trials, 20.8–24.0°C, 18–49% RH) during the bioassays (1100 to 1700 hours CDT).

In all trials with the horizontal wind tunnel, a single HIPV treatment was compared against an untreated control. (*Z*)-3-hexenyl acetate, methyl salicylate, and α-farnesene were chosen because of the high antennal responses observed in the EAG study (Fig. 1) and the responses in the four-arm olfactometer study. The HIPV stimulus was prepared in the same manner as EAG stimuli (see above); after adding 25 μl test solution or paraffin oil only (untreated control) onto a 6×0.5 cm strip of filter paper, it was placed inside an Erlenmeyer flask. The air pump, muffin fan, and lights were turned on 5 min before each release. Thereafter, 50 naïve *A. iole* females were released from each of four 20-ml glass scintillation vials (Kimble Glass, Inc., Vineland, NJ, USA) on the tunnel floor at the downwind end of the tunnel. The assay was terminated after 2 h, and the number of wasps on all walls of both compartments at the upwind end of the wind tunnel was counted. This procedure was repeated six times for each HIPV treatment, and the stimulus and blank controls were randomly reassigned to the two upwind chamber compartments after each repetition.

Field Parasitism A field investigation was conducted to determine the effect of synthetic HIPVs on parasitism of *L. lineolaris* eggs by *A. iole*. Based on our olfactometry studies and the work of Manrique et al. (2005), we hypothesized that host eggs in proximity to HIPVs would suffer greater parasitism than hosts associated with an untreated control. A cotton field (25 ha, *Gossypium hirsutum* L. var. DPL 215 BG/RR) was selected in Elizabeth, Washington Co., Mississippi (2.8 km E Stoneville; 33°25.4″ N, 90°52.8″ W). Agronomic practices (e.g., pesticide and fertilizer use) were consistent with those used in commercial cotton production, except no foliar insecticides were applied. This field was bordered on two sides by soybean, *Glycine max* (L.) Merr., one side by corn, *Zea mays* L., and on one side by a 12-m wide grass, *Cynodon dactylon* (L.) Pers., alley adjacent to natural vegetation (e.g., *Vitis* spp., *Ampelopsis arborea* (L.) Koehne, *Ambrosia trifida* L., *Quercus nigra* L., *Lonicera japonica* Thunberg, *Sambucus canadensis* L., and *Carya illinoensis* (Wangenheim) K. Koch.

A 6-ha portion of the cotton field was chosen for the study, and the experiment was arranged in a randomized complete block design with ten replicates of each of the following four treatments: (*Z*)-3-hexenyl acetate, α-farnesene, methyl salicylate, and an untreated control. These compounds were chosen because of our previous olfactometry results and previous studies on HIPVs with another mymarid (James 2005; James and Grasswitz 2005). Each plot was approximately 0.04 ha (20×20 m). HIPV bait-host egg stations were used to test the effect of synthetic HIPVs on parasitism of *L. lineolaris* eggs by *A. iole*. Each station consisted of a 45-ml

transparent plastic vial (12 dram crystal, Thornton Plastics, Salt Lake City, UT, USA) with four 1-cm diameter equidistant holes 5 mm from the top of the vial. Nested inside the plastic vial was a 20-ml glass scintillation vial (Kimble Glass, Inc., Vineland, NJ, USA) containing 2 ml of undiluted synthetic HIPV (see above) and a 1×5 cm piece of filter paper (Whatman no. 1, Whatman International, Ltd., Maidstone, England) that acted as a wick and enhanced volatilization of the HIPV. The plastic vial was closed with a white plastic cap to exclude rain and debris, and aluminum foil was wrapped around the vial, taking care not to cover the holes, to reduce ultraviolet light effects on the HIPV. In untreated controls, the scintillation vial contained only the strip of filter paper. The vial assembly was attached to a bamboo garden stake (65 cm long) so that the top of the dispenser was 45 cm above the soil surface. A *L. lineolaris* egg pack (9×9 cm) <2 days old was clipped to the bamboo stake 3 cm above the top of the dispenser.

One HIPV bait-host egg station was established in the center of each plot from 1530 to 1600 hours CST on 9 July 2004. Stations were placed in the furrow such that the top of the HIPV dispenser was 45 cm above the soil surface, and the egg pack was parallel to the row. Plants were removed such that there was no vegetation within 30 cm of the station; the destroyed plants were moved at least 10 m from the station to avoid bias of HIPV production from these plants. Approximately 1,000 naïve mixed gender wasps held in a glass scintillation vial were released 2 h later at each bait-egg station by placing the open vial on the soil beneath the host egg pack. Egg packs were recovered after 2 days and were held in an environmental chamber for 10 days at 25±1°C, 60–85% RH, and 14:10 L/D photoperiod. Egg packs were then observed under a dissecting scope at ×50, and parasitized and unparasitized eggs were counted. Parasitism data were expressed as the proportion of parasitized eggs in each egg pack. There was no difference in total host egg density per pack between HIPV treatments (mean=2,120, SE=101; F=1.73; df=3, 36; P=0.178). Voucher specimens of the wasps that were released are deposited in the National Entomological Collection, National Museum of Natural History, Smithsonian Institution, Washington, D.C., USA.

Data Analysis Maximum EAG responses were control-adjusted with the paraffin oil only control and expressed as proportional responses relative to the octanal standard. These data were then square root-transformed $(0.5(\sqrt{x}+\sqrt{x}+1))$ (Zar 1996), and analysis of variance, PROC MIXED (SAS Institute 2003), was used to compare maximum EAG deflection between gender and HIPV treatment–concentration combinations. Regression analysis was also used to assess the influence of gender and HIPV treatment on EAG amplitude (PROC REG and PROC MIXED; SAS Institute 2003). Due to heteroscedasticity over concentrations, a weighted regression (reciprocal of the variance) was calculated. Data from olfactometry studies were arcsine square root-transformed $(\arcsin(\sqrt{x}+\sqrt{x}+1))$ prior to two-tailed paired t test (selection region in the four-arm olfactometer) or G test for goodness of fit (flight tunnel; SAS Institute 2003). Data on parasitism were arcsine square root-transformed $(\arcsin(\sqrt{x}+\sqrt{x}+1))$ prior to single-factor analysis of variance (ANOVA) followed by mean comparison by using a one-tailed Dunnett's test of each HIPV treatment to the control (SAS Institute 2003). Untransformed values are presented for EAG, behavioral, and parasitism results.

Results

EAG Concentration–Response Curves Overall responses of female *A. iole* were significantly greater than male responses (F=76.52; df=1, 69.7; P<0.001). Female *A. iole* responses to (*E*)-2-hexenyl acetate at 1, 10, and 100 μM (F=2.47, 4.29 and 5.08, respectively; df=1, 1,205; P=0.014, <0.001, and <0.001, respectively) and to (*Z*)-3-hexenyl acetate at 0.1, 1, 10, and 100 μM (F=2.19, 3.41, 3.98, and 4.64, respectively; df=1, 1,200; P=0.029, <0.001, <0.001, and <0.001, respectively) were significantly greater than male responses (Fig. 1). For (*E*)-3-hexen-1-ol, (*Z*)-3-hexen-1-ol, and 1-hexanol, female responses were greater than those of males at 10 μM (F=2.56, 2.32, and 2.46, respectively; df=1, 1,203, 1,203, and 1,202, respectively; P=0.011, 0.021, and 0.014, respectively) and for 1-hexanol at 100 μM (F=2.00; df=1, 1,202; P=0.046). Female responses were greater than those of males at 1, 10, and 100 μM for methyl salicylate (F=3.34, 3.72, and 3.75, respectively; df=1, 1,204; P=<0.001, <0.001, and <0.001, respectively) for (±)-linalool (F=5.11, 5.19, and 6.29, respectively; df=1, 1,204; P<0.001), and for β-ocimene at 10 and 100 μM (F=3.53 and 2.93, respectively; df=1, 1,185 and 1,223, respectively; P=<0.001 and 0.004, respectively; Fig. 1). For β-myrcene, female responses were greater than those of males at 1, 10, and 100 μM (F=2.37, 1.97, and 3.25, respectively; df=1, 1,202; P=0.018, 0.049, and 0.001, respectively). For β-caryophyllene and α-pinene, female responses were greater than those of males at 10 μM (F=2.65 and 2.79, respectively; df=1, 1,205 and 1,199, respectively; P=0.008 and 0.005, respectively) and 100 μM (F=3.01 and 3.92, respectively; df=1, 1,205 and 1,199, respectively; P=0.003 and <0.001, respectively). For (+)-limonene, female response was greater than that of males at 100 μM (F=2.26; df=1, 1,202; P=0.024). For α-farnesene, there was no difference between female and male response at any concentration (Fig. 1).

Regression analysis of the impact of gender and HIPVs on antennal response indicated a linear response between antennal receptiveness (y) and HIPV concentration (log10 x; Table 2). Contrasts of slopes between the genders revealed greater ($P<0.05$) slopes for females for seven of the 13 HIPVs (Table 2). Ranked in order of P values (greatest to least significant), the seven significant compounds were: (*E*)-2-hexenyl acetate < β-myrcene = (±)-linalool < α-pinene = methyl salicylate < β-caryophyllene < β-ocimene (Table 2).

Comparison of slopes across herbivore-induced plant volatiles by gender showed that 42 comparisons were significant ($P<0.05$) for females, and 35 were significant for males (Table 3). For example, in females, responses to (*E*)-2-hexenyl acetate and (*Z*)-3-hexenyl acetate were not significantly different from one another, but each was different from β-caryophyllene, (*Z*)-3-hexen-1-ol, α-farnesene, (±)-linalool, (+)-limonene, and α-pinene. Female response to (*Z*)-3-hexenyl acetate was different from the response to (*E*)-3-hexen-1-ol and β-ocimene. The response of females to β-caryophyllene was significantly different from responses to all HIPVs except (±)-linalool. The matrix layout of Table 3 facilitates additional comparison of slopes for each gender.

EAG analyses clearly revealed that *A. iole* responded to the compounds tested. Although female antennae generally were more responsive than male antennae, both genders responded to most HIPVs. For females, methyl salicylate and (*Z*)-3-hexenyl acetate elicited significantly higher EAG responses than the other compounds tested. Wasps were least responsive to the monoterpenes β-caryophyllene and α-pinene. The two C_6 acetates, (*E*)-2-hexenyl acetate and (*Z*)-3-hexenyl acetate, had similar shapes of concentration–response curves, although wasps were more responsive to (*Z*)-3-hexenyl acetate. Two alcohols, (*E*)-3-hexen-1-ol and (*Z*)-3-hexen-1-ol, had similar-shaped concentration–response curves, but wasps were more responsive to the *E* isomer. At the two lowest concentrations tested, female wasps seemed to be most responsive to linalool and methyl salicylate.

Short-Range Olfactory Response to HIPVs A. iole females spent significantly more time in odor fields (selection regions) containing (*Z*)-3-hexenyl acetate compared to untreated control odor fields for trials using preconditioning regimes 3 (3.08 vs. 1.92 min; $t=2.31$; $df=28$; $P=0.028$), 4 (3.09 vs. 1.91 min; $t=2.24$; $df=25$; $P=0.034$), and 5 (3.29 vs. 1.71 min; $t=3.42$; $df=29$; $P=0.002$; Fig. 2). In contrast, significant responses to (*Z*)-3-hexenyl acetate were not detected for preconditioning regimes 1 (2.40 vs. 2.60 min; $t=-0.47$; $df=46$; $P=0.644$) and 2 (2.87 vs. 2.13 min; $t=1.73$; $df=51$; $P=0.090$).

Generally, responsiveness toward (*Z*)-3-hexenyl acetate increased as complexity of the preconditioning regime increased. For example, preconditioning regimes that included host plant (*E. annuus*) that had been subjected to feeding and oviposition by *L. lineolaris* led to the greatest attraction to (*Z*)-3-hexenyl acetate. The regime with the most complex odor mixture (*E. annuus* with *L. lineolaris* eggs + 1 μM each of 11 HIPVs) led to significantly greater attraction than did *Erigeron* and host eggs alone or 1 μM of

Table 2 Regression equations, F values, and significance levels for contrasts of slopes (female versus male) describing the effects of herbivore-induced plant volatiles on *A. iole* antennal response

	Female		Male		Den. df [b]	F	P
Herbivore-induced plant volatile	Equation[a]	R^2	Equation[a]	R^2			
Green leaf volatiles							
1-Hexanol	1.53+0.31(x)	0.722	1.39+0.26(x)	0.516	168	1.07	0.301
(*Z*)-3-Hexen-1-ol	1.35+0.22(x)	0.446	1.21+0.19(x)	0.231	148	0.02	0.883
(*E*)-3-Hexen-1-ol	1.50+0.27(x)	0.543	1.33+0.24(x)	0.543	156	0.39	0.535
(*E*)-2-Hexenyl acetate	1.61+0.34(x)	0.713	1.40+0.17(x)	0.260	128	17.75	<0.001
(*Z*)-3-Hexenyl acetate	1.78+0.33(x)	0.672	1.49+0.27(x)	0.444	180	2.74	0.100
Monoterpenes							
β-Myrcene	1.51+0.28(x)	0.753	1.39+0.19(x)	0.535	164	9.17	0.003
(±)-Linalool	1.84+0.14(x)	0.353	1.42+0.05(x)	0.031	144	9.20	0.003
β-Caryophyllene	1.37+0.11(x)	0.181	1.20+0.03(x)	0.022	120	4.11	0.045
α-Farnesene	1.56+0.19(x)	0.511	1.45+0.18(x)	0.418	156	0.17	0.678
(+)-Limonene	1.36+0.26(x)	0.565	1.42+0.14(x)	0.060	160	2.16	0.143
β-Ocimene	1.58+0.30(x)	0.510	1.43+0.19(x)	0.355	164	3.95	0.049
α-Pinene	1.38+0.18(x)	0.572	1.22+0.09(x)	0.135	192	8.28	0.005
Carboxylate ester							
Methyl salicylate	1.78+0.33(x)	0.842	1.39+0.25(x)	0.714	76	8.54	0.005

[a] Antennal response=$a+b$ (log10 concentration)
[b] Numerator $df=1$

Table 3 Matrix of *P* values for comparison of slopes for concentration response of *A. iole* antennae to individual herbivore-induced plant volatiles

HIPV	1-Hexanol	(*Z*)-3-Hexen-1-ol	(*E*)-3-Hexen-1-ol	(*E*)-2-Hexenyl acetate	(*Z*)-3-Hexenyl acetate	β-Myrcene	(±)-Linalool	β-Caryophyllene	α-Farnesene	(+)-Limonene	(*E*)-β-Ocimene	α-Pinene	Methyl salicylate
1-Hexanol	XXX	0.008	0.150	0.648	0.501	0.272	<0.001	<0.001	<0.001	0.037	0.197	<0.001	0.533
(*Z*)-3-Hexen-1-ol	*0.247*	XXX	0.231	0.002	<0.001	0.116	0.150	<0.001	0.268	0.631	0.171	0.142	0.006
(*E*)-3-Hexen-1-ol	*0.784*	*0.169*	XXX	0.060	0.032	0.075	0.010	<0.001	0.022	0.493	0.870	0.007	0.075
(*E*)-2-Hexenyl acetate	*0.017*	*0.212*	*0.011*	XXX	0.843	0.122	<0.001	<0.001	<0.001	0.012	0.083	<0.001	0.802
(*Z*)-3-Hexenyl acetate	*0.879*	*0.304*	*0.672*	*0.023*	XXX	0.071	<0.001	<0.001	<0.001	0.005	0.046	<0.001	0.923
β-Myrcene	*0.076*	*0.598*	*0.048*	*0.423*	*0.100*	XXX	0.003	<0.001	0.008	0.295	0.842	0.002	0.132
(±)-Linalool	*<0.001*	*0.007*	*<0.001*	*0.062*	*<0.001*	*0.003*	XXX	0.055	0.720	0.062	0.006	0.918	0.001
β-Caryophyllene	*<0.001*	*<0.001*	*<0.001*	*0.039*	*<0.001*	*0.002*	*0.677*	XXX	0.020	<0.001	<0.001	0.028	<0.001
α-Farnesene	*0.027*	*0.350*	*0.017*	*0.672*	*0.037*	*0.671*	*0.010*	*0.008*	XXX	0.120	0.014	0.776	<0.001
(+)-Limonene	*0.051*	*0.108*	*0.002*	*0.864*	*0.005*	*0.263*	*0.051*	*0.033*	*0.494*	XXX	0.396	0.054	0.021
(*E*)-β-Ocimene	*0.057*	*0.513*	*0.035*	*0.494*	*0.075*	*0.896*	*0.004*	*0.003*	*0.769*	*0.325*	XXX	0.004	0.097
α-Pinene	*<0.001*	*0.011*	*<0.001*	*0.305*	*0.001*	*0.036*	*0.306*	*0.186*	*0.097*	*0.321*	*0.050*	XXX	<0.001
Methyl salicylate	*0.495*	*0.775*	*0.376*	*0.173*	*0.571*	*0.457*	*0.002*	*0.001*	*0.275*	*0.097*	*0.395*	*0.014*	XXX

P values for males are in italics.

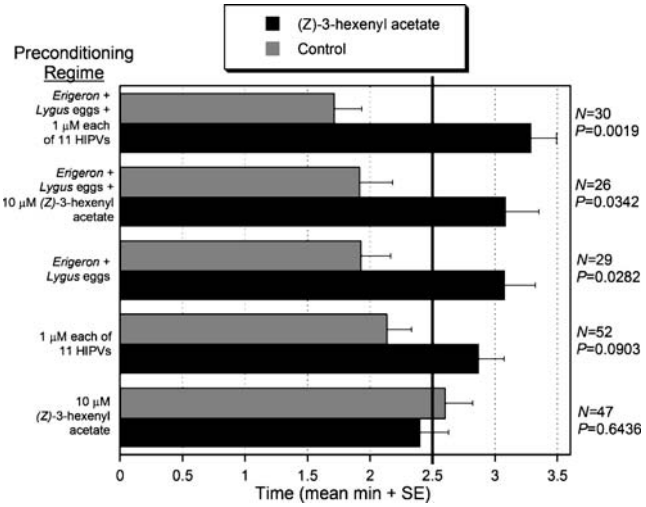

Fig. 2 Mean total time (+SE) that female *A. iole* wasps spent in treatment vs. control fields of four-arm olfactometer after exposure to different preconditioning regimes. Treatment odor was 100 μM (*Z*)-3-hexenyl acetate. *Solid vertical* line at 2.5 min indicates hypothetical value for equal response. *P* values correspond to paired *t* test

11 HIPVs, suggesting an additive or synergistic effect for the combination of *Erigeron* and host eggs alone or 1 μM of 11 HIPVs. In the simplest regime (10 μM (*Z*)-3-hexenyl acetate), the lack of behavioral response contrasted with EAG results that showed that *A. iole* females perceived (*Z*)-3-hexenyl acetate.

Long-Range Olfactory Response to HIPVs More naïve *A. iole* females were recovered from methyl salicylate (G=15.57; df=1; P<0.001) and α-farnesene (G=4.31; df=1; P<0.05) treatment compartments than controls; there was no difference in wasp numbers recovered from the (*Z*)-3-hexenyl acetate treatment and controls (G=3.61; df=1; P>0.05; Fig. 3). For this experiment, approximately 32% of the wasps were recaptured in the treatment and control compartments.

Field Parasitism Single-factor ANOVA revealed a significant HIPV treatment effect on parasitism (F=3.84; df=3, 36; P=0.018). Parasitism was greater in the α-farnesene (q=3.27; df=36; P=0.007) and (*Z*)-3-hexenyl acetate (q=2.42; df=36; P=0.053) treatments than in the untreated control. Mean parasitism ranged from 0.68% in the control to 2.57% in α-farnesene (Fig. 4). Parasitism was numerically but not significantly (P>0.05) greater for host eggs in the methyl salicylate treatment than the control (Fig. 4).

Discussion

An understanding of the chemical ecology of *A. iole* is essential before developing HIPV strategies for enhancing

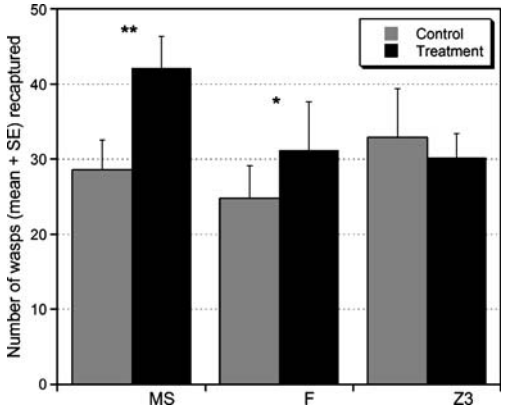

Fig. 3 Mean number (+SE) of female *A. iole* wasps recovered in treatment vs. control compartments of a horizontal wind tunnel. *Empty bars* indicate controls; *solid bars* indicate treatments [*MS* methyl salicylate, *F* α-farnesene, and *Z3* (*Z*)-3-hexenyl acetate]. Significant differences are noted by *asterisks* (*single asterisk* $P<0.05$, *double asterisk* $P<0.001$) using *G* test for goodness of fit. $N=6$ replicates per tested compound

biological control of *Lygus* species. This study used a variety of approaches to study the response of *A. iole* to HIPVs. Our EAG analyses on *A. iole* are the first that we are aware of on a mymarid, and we found that the strongest antennal responses were elicited by (*Z*)-3-hexenyl acetate and methyl salicylate. The greater female antennal responses may be adaptive given that these compounds are associated with the presence of host eggs. Male perception of HIPVs might reflect their use as cues for locating female conspecifics. Behavioral studies revealed that the response to (*Z*)-3-hexenyl acetate was dependent on wasp experience. In further behavioral studies, naïve *A. iole* females were responsive to methyl salicylate and α-farnesene but not to (*Z*)-3-hexenyl acetate. In a field

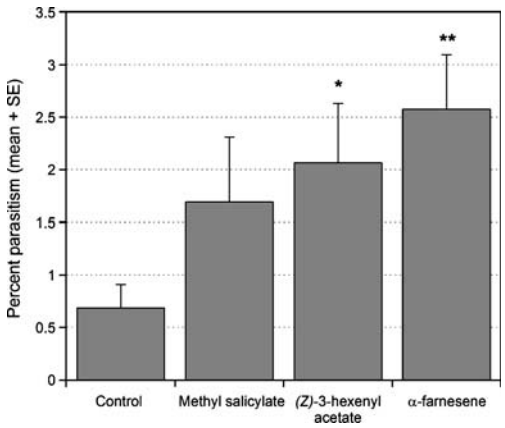

Fig. 4 Percent mean parasitism (+ SE) by *A. iole* of *L. lineolaris* eggs baited with single herbivore-induced plant volatiles in a cotton field. Significant differences for Dunnett's test of each treatment vs. the control are noted by *asterisks* [(*Z*)-3-hexenyl acetate, $P=0.053$; α-farnesene, $P=0.007$). $N=10$ replicates per tested compound and control

experiment, (*Z*)-3-hexenyl acetate and α-farnesene increased egg parasitism by *A. iole*.

Vet and Dicke (1992) proposed a conceptual framework for infochemical use in parasitoid–host and predator–prey interactions. An important issue in this framework is the reliability-detectability problem faced by foraging natural enemies, i.e., that cues from the host or prey are reliable but not easily detectable and, conversely, that signals from plants damaged by the host or prey are more easily detectable but not necessarily reliable. Detection of host-specific cues has important implications for the use of HIPVs in pest management, i.e., HIPVs must be attractive to natural enemies of the herbivore species that cause the injury. Recent studies recognize a growing list of parasitoids that can distinguish between host and non-host HIPVs (Du et al. 1996; Dicke 1999; Meiners et al. 2000; Moraes et al. 2005), including *A. iole*, which is attracted to cotton volatiles emitted after injury by its host, *L. hesperus*, but not by a non-host, *Spodoptera exigua* (Manrique et al. 2005). However, the present study also indicated that a single, relatively ubiquitous HIPV can be attractive to *A. iole* and can lead to increased parasitism. The green-leaf volatile, (*Z*)-3-hexenyl acetate; the sesquiterpene, α-farnesene; and the carboxylate ester, methyl salicylate, are all produced and released by plants in response to injury by *Lygus* species (Rodriguez-Saona et al. 2002; Blackmer et al. 2004; Williams et al. 2005) and by other herbivores (Turlings et al. 1991; Loughrin et al. 1995; Röse et al. 1996; Paré and Tumlinson 1998). Thus, it appears that *A. iole* can use either host-specific or general volatile cues in the process of host habitat location.

Under field conditions, the variation in production and emission of HIPVs by plants likely complicates the signal perceived and utilized by parasitoids for host location. However, associative learning, i.e., association of host-specific volatiles with the presence of hosts, is a possible solution to this problem (Vet and Groenewold 1990; Vet and Dicke 1992; Kaiser et al. 2003; Randlkofer et al. 2007; Schröder et al. 2008). Compared to larval parasitoids, little is known on the learning capabilities of host-plant-derived cues in egg parasitoids. Egg parasitoids are less expected to use plant-derived cues or to learn; instead, they are likely to use host-derived cues (i.e., host adult pheromones) that are highly predictable within and between generations during foraging (Vet et al. 1995). Recent studies have shown that egg parasitoids use not only short-distance (host-associated) cues but also may use long-distance volatiles, such as those associated with the host plant (Meiners and Hilker 1997; Hilker et al. 2002; Hilker and Meiners 2006). Because host-plant volatiles are highly detectable but less reliable than host-associated odors, learning might be critical for egg parasitoids that employ host-plant volatiles in host finding (e.g. Mumm et al. 2005). Schröder et al. (2008) hypothe-

sized a contextual learning sequence by a eulophid that is mediated by both a sugar food source and by the presence of host eggs of a suitable age. The present study and those by Conti et al. (1997) and Manrique et al. (2005) show that *A. iole*, a parasitoid that specializes in *Lygus* spp. eggs, uses host-plant volatiles and that both innate and learned responses are important in host finding. Naïve wasps responded to methyl salicylate and α-farnesene but needed to learn host-plant odors before responding to (*Z*)-3-hexenyl acetate. These three volatile compounds are common in many plant species, and one or more of them are induced in cotton, maize, and alfalfa following herbivory by *L. hesperus* (Rodriguez-Saona et al. 2002; Blackmer et al. 2004; Williams et al. 2005). It is, therefore, not surprising that *A. iole* uses more than one volatile cue to find hosts; still, a single compound was sufficient to attract wasps in both laboratory and field experiments. This was confirmed by the fact that several plant volatile compounds elicited an antennal response from *A. iole*, albeit at different amplitudes. Associative learning, however, was not a requisite for host-habitat location by *A. iole* given that naïve wasps responded to HIPVs under both laboratory and field conditions.

Field studies that address the applicability of using HIPVs for the protection of agricultural crops are limited but promising. Drukker et al. (1995) and Shimoda et al. (1997) reported higher densities of predators in the proximity of host-infested plants than near uninfested plants. Several investigators have applied signal molecules, such as jasmonic acid or methyl jasmonate, to plants and demonstrated increases in oxidative enzymes (Thaler 1999), proteinase inhibitors (Rodriguez-Saona et al. 2005), and HIPVs (Ockroy et al. 2001; Rodriguez-Saona et al. 2001). Thaler (1999) also reported increases in parasitism of *S. exigua* on induced plants versus controls. A series of field studies demonstrated that sachets of individual synthetic HIPVs could be used to attract and retain natural enemies in perennial crop systems (James 2003a,b; James and Price 2004; James and Grasswitz 2005). In particular, these studies showed that (*Z*)-3-hexenyl acetate, farnesene, and methyl salicylate could be used to manipulate behavior of beneficial insects under field conditions. These three compounds were attractive to numerous predators and parasitoids; methyl salicylate attracted the broadest diversity of insects and farnesene the least (James 2005; James and Grasswitz 2005). However, all compounds attracted the mymarid *Anagrus daanei* and other *Anagrus* species, although this effect was inconsistent. In our field study with the mymarid *A. iole*, we found that host eggs baited with (*Z*)-3-hexenyl acetate or α-farnesene suffered greater parasitism than untreated controls. The low overall rate of parasitism (approximately 2.6%) may be due to the unnaturally large number of host eggs present in a single patch and the propensity of *A. iole* to disperse its eggs among host patches. Wasps may also have been more strongly attracted to volatiles emitted by nearby cotton plants.

This study provides evidence that plant volatiles represent key semiochemical signals in the recognition of and attraction to host-associated patches by *A. iole*. Bloem and Yeargan (1982) reported that *Anaphes diana* (=*Patasson lameerei* Debauche) used its antennae to differentiate between volatiles from host-damaged vs. undamaged plants and from host vs. non-host frass. The authors proposed that volatiles from host-damaged plants and from host frass play important roles in the host-finding sequence of *A. diana*. It would be adaptive for host-specific natural enemies of *Lygus* to recognize and walk or fly to host-associated habitats. Prior work by Manrique et al. (2005) found that *A. iole* females can discriminate between plant odors emanating from *Lygus*-damaged vs. non-host damaged plants. Here, we identified several plant-produced compounds that, upon further development, might be useful tools to manipulate *A. iole* behavior to enhance *Lygus* suppression. Future recordings from antennal sensilla might help determine if the structurally similar compounds [(*E*)-3-hexen-1-ol vs. (*Z*)-3-hexen-1-ol] are detected by the same or different types of receptor cells and if specific receptor cells exist for certain compounds. Likewise, further lab and field studies of other plant- and host-associated compounds not included in this study, mixtures of compounds, and identification and behavioral testing of GC-EAG active compounds, would be worthwhile.

Based on our current understanding of *A. iole* host foraging, we propose the following host-finding sequence for this egg parasitoid. The proposed sequence does not exclude the possible roles of other sensory modalities (e.g., vision). This sequence is similar to that proposed for *A. diana* (Bloem and Yeargan 1982) and fits into the conceptual framework of Vet and Dicke (1992): (1) detection of the host habitat via host-specific HIPVs; (2) nonrandom and random searching for host eggs on the plant guided by physical and chemical (volatile and/or tactile) cues from host and plant; (3) recognition and acceptance of host eggs directed by physical and chemical (volatile and/or tactile) cues from host eggs and associated plant tissue.

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References

BACKUS, E. A., CLINE, A. R., ELLERSEICK, M. R., and SERRANO, M. S. 2007. *Lygus hesperus* (Hemiptera: Miridae) feeding on cotton: new methods and parameters for analysis of nonsequential electrical penetration graph data. *Ann. Entomol. Soc. Amer.* 100:296–310.

BIRKETT, A. M., CHAMBERLAIN, K., GUERRIERI, E., PICKETT, J. A., WADHAMS, L. J., and YASUDA, L. 2003. Volatiles from whitefly-infested plants elicit a host-locating response in the parasitoid, *Encarsia formosa*. *J. Chem. Ecol.* 29:1589–1600.

BLACKMER, J. L., RODRIGUEZ-SAONA, C., BYERS, J. A., SHOPE, K. L., and SMITH, J. P. 2004. Behavioral response of *Lygus hesperus* to conspecifics and headspace volatiles of alfalfa in a Y-tube olfactometer. *J. Chem. Ecol.* 30:1547–1564.

BLOEM, K. A., and YEARGAN, K. V. 1982. Host-finding behavior of *Patasson lameerei*, (Hym., Chalcidoidea), a parasitoid of *Sitona* eggs (Col., Curculionidae). *Entomophaga* 1:93–97.

COBBEN, R. H. 1978. Evolutionary trends in Heteroptera. Part II. Mouthpart-structures and feeding strategies. *Meded. Landbouwhogesch. Wageningen.* 78–5:1–407.

COLAZZA, S., MCELFRESH, J. S., and MILLAR, J. G. 2004. Identification of volatile synomones, induced by *Nezara viridula* feeding and oviposition on bean spp., that attract the egg parasitoid *Trissolcus basalis*. *J. Chem. Ecol.* 30:945–964.

CONTI, E., JONES, W. A., BIN, F., and VINSON, S. B. 1996. Physical and chemical factors involved in host recognition behavior of *Anaphes iole* Girault, an egg parasitoid of *Lygus hesperus* Knight (Hymenoptera: Mymaridae; Heteroptera: Miridae). *Biol. Control* 7:10–16.

CONTI, E., JONES, W. A., BIN, F., and VINSON, S. B. 1997. Oviposition behavior of *Anaphes iole*, an egg parasitoid of *Lygus hesperus* (Heteroptera: Miridae; Hymenoptera: Mymaridae). *Ann. Entomol. Soc. Amer.* 90:91–101.

DICKE, M. 1999. Are herbivore-induced plant volatiles reliable indicators of herbivore identity to foraging carnivorous arthropods? *Entomol. Exp. Appl.* 91:131–142.

DICKE, M., VAN BAARLEN, P., WESSELS, R., and DIJKMAN, H. 1993. Herbivory induces systemic production of plant volatiles that attract predators of the herbivore: extraction of endogenous elicitor. *J. Chem. Ecol.* 19:581–599.

DRUKKER, B., SCUTAREANU, P., and SABELIS, M. W. 1995. Do anthocorid predators respond to synomones from *Psylla*-invested pear trees under field conditions? *Entomol. Exp. Appl.* 77:193–203.

DRUKKER, B., BRUIN, J., and SABELIS, M. W. 2000. Anthocorid predators learn to associate herbivore-induced plant volatiles with presence or absence of prey. *Physiol. Entomol.* 25:260–265.

DU, Y.-J., POPPY, G. M., and POWELL, W. 1996. Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of *Aphidius ervii*. *J. Chem. Ecol.* 22:1591–1605.

HILKER, M., and MEINERS, T. 2006. Early herbivore alert: insect eggs induce plant defense. *J. Chem. Ecol.* 32:1379–1397.

HILKER, M., KOBS, C., VARAMA, M., and SCHRANK, K. 2002. Insect egg deposition induces *Pinus* to attract egg parasitoids. *J. Exp. Biol.* 205:455–461.

HUBER, J. T., and RAJAKULENDRAN, V. K. 1988. Redescription of and host-induced antennal variation in *Anaphes iole* Girault (Hymenoptera: Mymaridae), an egg parasite of Miridae (Hemiptera) in North America. *Can. Entomol.* 120:893–901.

HUNTER, M. D. 2002. A breath of fresh air: beyond laboratory studies of plant volatile-natural enemy interactions. *Agric. Forest Entomol.* 4:81–86.

JACKSON, C. G., and GRAHAM, H. M. 1983. Parasitism of four species of *Lygus* (Hemiptera: Miridae) by *Anaphes ovijentatus* (Hymenoptera: Mymaridae) and an evaluation of other possible hosts. *Ann. Entomol. Soc. Amer.* 76:772–775.

JAMES, D. G. 2003a. Field evaluation of herbivore-induced plant volatiles as attractants for beneficial insects: methyl salicylate and the green lacewing, *Chrysopa nigricornis*. *J. Chem. Ecol.* 29:1601–1609.

JAMES, D. G. 2003b. Synthetic herbivore-induced plant volatiles as field attractants for beneficial insects. *Environ. Entomol.* 32:977–982.

JAMES, D. G. 2005. Further field evaluation of synthetic herbivore-induced plant volatiles as attractants for beneficial insects. *J. Chem. Ecol.* 31:481–495.

JAMES, D. G., and PRICE, T. S. 2004. Field-testing of methyl salicylate for recruitment and retention of beneficial insects in grapes and hops. *J. Chem. Ecol.* 30:1613–1628.

JAMES, D. G., and GRASSWITZ, T. R. 2005. Synthetic herbivore-induced plant volatiles increase field captures of parasitic wasps. *BioControl* 50:871–880.

KAISER, L., PEREZ-MALUF, R., SANDOZ, J. C., and PHAM-DELEGUE, M.-H. 2003. Dynamics of odour learning in *Leptopilina boulardi,* a hymenopterous parasitoid. *Anim. Behav.* 66:1077–1084.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. Chicago University Press, Chicago.

KOVATS, E. 1958. Gas-chromatographische Charakterisierung organisher Verbindungen. *Helv. Chim. Acta* 41:1915–1932.

LEIGH, T. F., KERBY, T. A., and WYNHOLDS, T. F. 1988. Cotton square damage by the plant bug, *Lygus hesperus* (Hemiptera: Heteroptera: Miridae), and abscission rates. *J. Econ. Entomol.* 81:1328–1337.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1995. Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol.* 21:1217–1227.

MANRIQUE, V., JONES, W. A., WILLIAMS, L. III, and BERNAL, J. S. 2005. Olfactory responses of *Anaphes iole* (Hymenoptera: Mymaridae) to volatile signals derived from host habitats. *J. Ins. Behav.* 18:89–104.

MEINERS, T., and HILKER, M. 1997. Host location in *Oomyzus gallerucae* (Hymenoptera: Eulophidae), an egg parasitoid of the elm leaf beetle *Xanthogaleruca luteola* (Coleoptera: Chrysomelidae). *Oecologia* 112:87–93.

MEINERS, T., and HILKER, M. 2000. Induction of plant synomones by oviposition of a phytophagous insect. *J. Chem. Ecol.* 26:221–231.

MEINERS, T., WESTERHAUS, C., and HILKER, M. 2000. Specificity of chemical cues used by a specialist egg parasitoid during host location. *Entomol. Exp. Appl.* 95:151–159.

MORAES, M. C. B., LAUMANN, R., SUJII, C. P., and BORGES, M. 2005. Induced volatiles in soybean and pigeon pea plants artificially infested with the neotropical brown stink bug, *Euschistus heros*, and their effect on the egg parasitoid, *Telenomus podisi*. *Entomol. Exp. Appl.* 115:227–237.

MUMM, R., TIEMANN, T., VARAMA, M., and HILKER, M. 2005. Choosy egg parasitoids: specificity of oviposition-induced pine volatiles exploited by an egg parasitoid of pine sawflies. *Entomol. Exp. Appl.* 115:217–225.

OCKROY, M. L. B., TURLINGS, T. C. J., EDWARDS, P. J., FRITZSCHE-HOBALLAH, M. E., AMBROSETTI, L., BASSETTI, P., and DORN, S. 2001. Response of natural populations of predators and parasitoids to artificially induced volatile emissions in maize plants (*Zea mays* L.). *Agric. Forest Entomol.* 3:201–209.

PARÉ, P. W., and TUMLINSON, J. H. 1998. Cotton volatiles synthesized and released distal to the site of insect damage. *Phytochemistry* 47:521–526.

RANDLKOFER, B., OBERMAIER, E., M, and EINERS, T. 2007. Mother's choice of the oviposition site: balancing risk of egg parasitism and need of food supply for the progeny with an infochemical shelter? *Chemoecology* 17:177–186.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDNER, S. J., PARÉ, P. W., and HENNEBERRY, T. J. 2001. Exogenous methyl jasmonate induces volatile emissions in cotton plants. *J. Chem. Ecol.* 27:679–695.

RODRIGUEZ-SAONA, C., CRAFTS-BRANDNER, S. J., WILLIAMS, L. III, and PARÉ, P. W. 2002. *Lygus hesperus* feeding and salivary gland extracts induce volatile emissions in plants. *J. Chem. Ecol.* 28:1733–1747.

RODRIGUEZ-SAONA, C., CHALMERS, J. A., RAJ, S., and THALER, J. S. 2005. Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. *Oecologia* 143:566–577.

RÖSE, U. S. R., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1996. Volatile semiochemicals released from undamaged cotton leaves. *Plant Physiol.* 111:487–495.

RUBERSON, J. R., and WILLIAMS, L. H. 2000. Biological control of *Lygus* spp.: a component of areawide management. *Southwest. Entomol. Suppl.* 23:96–110.

SANTOLAMAZZA-CARBONE, S., ILLAMOLA, A. R., and RIVERA, A. C. 2004. Host finding and host discrimination ability in *Anaphes nitens* Girault, an egg parasitoid of the *Eucalyptus* snout-beetle *Gonipterus scutellatus* Gyllenhal. *Biol. Control* 29:24–33.

SAS INSTITUTE, INC. 2003. *SAS/STAT* User's Guide, Release 9.1 edition. Cary, NC.

SCHRÖDER, R., WURM, L., VARAMA, M., MEINERS, T., and HILKER, M. 2008. Unusual mechanisms involved in learning of oviposition-induced host plant odours in an egg parasitoid? *Anim. Behav.* 75:1423–1430.

SHIMODA, T., TAKABAYASHI, J., ASHIRA, W., and TAKAFUJI, A. 1997. Response of predatory insect *Scolothrips takahashi* towards herbivore induced plant volatiles under laboratory and field conditions. *J. Chem. Ecol.* 23:2033–2048.

STEINBERG, S., DICKE, M., VET, L. E. M., and WANNINGEN, R. 1992. Response of the braconid parasitoid *Cotesia* (=*Apanteles*) *glomerata* to volatile infochemicals: effects of bioassay set-up, parasitoid age and experience and barometric flux. *Entomol. Exp. Appl.* 63:163–175.

THALER, J. S. 1999. Jasmonate-inducible plant defences cause increased parasitism of herbivores. *Nature* 399:686–688.

TUMLINSON, J. H., LEWIS, W. J., and VET, L. E. M. 1993. How parasitic wasps find their hosts. *Sci. Am.* 268:100–106.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

VET, L. E. M., and GROENEWOLD, A. W. 1990. Semiochemicals and learning in parasitoids. *J. Chem. Ecol.* 16:3119–3135.

VET, L. E. M., and DICKE, M. 1992. Ecology of infochemical use by natural enemies in a tritrophic context. *Annu. Rev. Entomol.* 37:141–172.

VET, L. E. M., VAN LENTEREN, J. C., HEYMANS, M., and MEELIS, E. 1983. An airflow olfactometer for measuring olfactory responses of hymenopterous parasitoids and other small insects. *Physiol. Entomol.* 8:97–106.

VET, L. E. M., LEWIS, W. J., and CARDE, R. T. 1995. Parasitoid Foraging and Learning, pp. 65–101, in R. Cardé, and W. J. Bell (eds.). Chemical Ecology of Insects 2Chapman & Hall, New York.

WEI, J., WANG, L., ZHU, J., ZHANG, S., NANDI, O., and KANG, L. 2007. Plants attract parasitic wasps to defend themselves against insect pests by releasing hexenol. PLoS One 2(9):e852.

WHEELER, A. G. Jr. 2001. Biology of the Plant Bugs. Cornell University Press, Ithaca.

WILLIAMS, L. III, RODRIGUEZ-SAONA, C., PARÉ, P. W., and CRAFTS-BRANDNER, S. J. 2005. The piercing-sucking herbivores *Lygus hesperus* and *Nezara viridula* induce volatile emissions in plants. *Arch. Insect Biochem. Physiol.* 58:84–96.

ZAR, J. H. 1996. Biostatistical Analysis. 3rd edn.Prentice-Hall, Inc., Upper Saddle River, New Jersey.

butterflies such as *M. cinxia* lay their eggs in clusters, often depositing a single cluster in a day (Wahlberg et al. 2004; Saastamoinen 2007). Butterflies that lay eggs singly must often seek at least 20–50 oviposition sites per day and thus may be more time limited than egg limited (Courtney 1982), whereas for butterflies that lay their eggs in clusters, the costs of prolonged search or host assessments may be beneficial (Singer 2004).

In general, butterflies are attracted to alight on plants through a combination of visual and olfactory stimuli (Rausher 1978; Feeny et al. 1989; Janz and Nylin 1997). A positive response to visual stimuli is to alight and taste a plant. To assess whether a plant is chemically acceptable, a female checkerspot butterfly scratches the surface of a leaf with the first pair of tarsal claws. This scratching damages the leaf surface and facilitates the release of chemical stimuli that are assessed by the female butterfly. If the plant is accepted for oviposition, the butterfly curls her abdomen to the underside of a leaf and oviposits. In checkerspot butterflies, plant acceptance depends on the physical features of the oviposition site such as the size, shape, and orientation of the leaf (Singer 2004).

Most checkerspot species are oligophagous or even monophagous on plant species that produce iridoid glycosides (IGs) as plant secondary compounds (Bowers 1983; Jensen 1991; Wahlberg 2001; Murphy et al. 2004; Reudler Talsma et al. 2008). In this study, we focus on two IGs, aucubin and catalpol, which are found in more than ten Asteridae plant families (El-Naggar and Beal 1980; Olmstead et al. 2000). These IGs are known to be oviposition cues for the specialist butterfly *Junonia coenia* (Pereyra and Bowers 1988) and feeding stimulants for larvae of at least some checkerspot butterflies (Bowers 1983). *M. cinxia* larvae perform better on diets high in iridoid glycosides (Harvey et al. 2005; Saastamoinen et al. 2007).

Nieminen et al. (2003) examined the natural pattern of oviposition by *M. cinxia* on their food plant, *P. lanceolata* in the field. The authors compared plants used for oviposition with neighboring and random plants in the patch. They found that plants used for oviposition contained significantly higher concentrations of aucubin than neighboring and random plants. Additionally, plants selected for oviposition and random plants had higher catalpol concentrations than neighboring plants. This suggests that ovipositing females selected plants with higher IG concentrations, or at least with higher concentrations of aucubin.

An alternative explanation for the positive association between IG concentrations and oviposition in Nieminen et al. (2003) is that the presence of butterfly eggs on leaf tissues leads to an induction of IGs in the plant. Studies of other insects have reported that allelochemicals in plants can be induced through oviposition and the presence of eggs on, or imbedded in, the leaf surface (Agrawal 2000; Colazza et al. 2004; Hilker et al. 2005). Induction of IGs by *M. cinxia* oviposition has not been investigated thus far, but IG production in *P. lanceolata* can be induced both by fungal infection (Marak et al. 2002) and by herbivory (Darrow and Bowers 1999; Stamp and Bowers 2000). Furthermore, Peñuelas et al. (2006) found that the leaves of *Lonicera implexa* (Camprifoliaceae) with egg clusters of *Euphydryas aurinia* (a close relative of *M. cinxia*) had 15-fold higher concentrations of IGs than leaves directly opposite on the same plant.

Plant size as well as chemistry may influence butterfly oviposition choice. Large plants may receive more eggs than small plants simply because they are visually conspicuous and difficult to leave accidentally after alighting. If the butterfly can perceive size, then large plants may also be used because they are more suitable for larval development. For instance, the gregarious *M. cinxia* larvae seldom move to another individual plant during early instars (although this can happen, see "Results"), so they would benefit from a large oviposition plant (Kuussaari et al. 2004). Indeed, Nieminen et al. (2003) found that naturally occurring egg clusters tended to occur on larger than average plants. More generally, the 'plant vigor hypotheses', elaborated by Price (1991), predicts that vigorous pants that reach a larger than average size should be preferred by herbivores. Both plant biomass and nutritional quality may be higher in vigorous plants (Heisswolf et al. 2005; Lastra et al. 2006).

We performed a set of dual- and multiple-choice experiments in cages and in the field to answer the following questions: (1) What is the effect of *P. lanceolata* plant chemistry on the oviposition behavior of *M. cinxia*? (2) Does oviposition cause induction of IGs in the host plant? (3) What is the effect of plant size (measured as number of leaves) on the oviposition behavior of *M. cinxia* butterflies?

Methods and materials

Study Species M. cinxia (Glanville fritillary) butterflies used for these experiments were the offspring of field-caught butterflies from Åland, SW Finland. In Åland, the butterflies fly in June and lay large clusters (150–200 eggs) on the underside of leaves of *P. lanceolata* and *Veronica spicata* L. (Plantaginaceae) (Kuussaari et al. 2000). Larvae hatch after 2–4 weeks depending on the temperature. The larvae spin a communal web on the host plant and feed gregariously throughout the summer. Because of restricted mobility, small larvae usually depend on the host plant the adult female chose for oviposition. Larvae diapause gregariously as L5 in a silk winter nest and become active

again in spring. In the last instar (L7), larvae disperse and feed individually. They pupate within the vegetation in mid May (Kuussaari et al. 2004).

P. lanceolata (ribwort plantain) is a perennial plant with a worldwide distribution (Sagar and Harper 1964). In natural populations, IG levels range from undetectable to ca. 9% of dry weight (Bowers 1991). In the field in Åland, these concentrations range between 0.6% and 2.2% for aucubin and between 0.7% and 2.0% for catalpol (Nieminen et al. 2003). The variation in the constitutive IG amount in *P. lanceolata* is partially genetically determined (Adler et al. 1995; Bowers and Stamp 1992, 1993). Most plants used for oviposition experiments were derived from an artificial selection experiment, in which plants were selected on the basis of high and low concentrations of total leaf iridoid glycosides for four generations (Marak et al. 2000). For experiment 1 (see below), nine plants, each derived from a different half sib family from the low selection line, and six plants, each derived from a different half sib family from the high selection line, were clonally propagated following root cloning (Wu and Antonovics 1975). This resulted in 15 different genotypes. In addition, for experiments 2 and 3, five new crosses were made between pairs of plants of the low selection line and five crosses between pairs of plants of the high selection line. From each of these crosses, a single offspring was raised and clonally propagated. This resulted in five from the low (L1–L5) and five genotypes originating from the high (H1–H5) IG selection line. Since not all genotypes originating from the high selection line have higher IG concentrations than all genotypes originating from the low selection line under all environmental conditions (see "Results"), we use differences in IG between genotypes, rather than selection lines, as the basis of our experiments. In experiment 1, we included 15 more plants that were collected from the field in Åland to extend the range of variation in concentrations of aucubin and catalpol used in the experiment. Although these plants had similar IG concentrations as genotypes from the selection lines (field plants 4.3±0.5% dry weight (dw); plants from the selection lines 4.5±0.5% dw), they had lower average concentrations of aucubin but higher average concentrations of catalpol (see Fig. 4).

Oviposition Experiments Three experiments were carried out to study the oviposition behavior of *M. cinxia* in response to concentrations of iridoid glycosides and plant size of *P. lanceolata.* In experiments 1 and 2, we offered potted plants to butterflies in cages by using dual (exp. 1) or multiple (exp. 2) choice tests. In experiment 3, we transplanted plants and butterflies to a natural field plot. Experiments were carried out in Finland, except for one of the cage experiments (exp. 2) that was carried out in the Netherlands. In the cage experiments, none of the plants flowered, and *M. cinxia* females usually laid a single egg batch per plant.

Experiment 1: Small Cages, Finland In total, 30 pairs of plants were used in dual-choice oviposition tests, using eight small mesh cages (38×38×44 cm) over the course of 13 days in June 2005, when the weather was sunny and warm. Cages were placed outside the Nåtö Biological Station, Åland, Finland. In 18 trials, we used sets of two *P. lanceolata* plants of different genotypes, one randomly selected from the nine genotypes of the low, and one randomly selected from the six genotypes of the high selection line. In 12 other trials, we used sets of two of the Finnish plants from the field. Before the plants were put in the cage, we counted the number of leaves, and the second fully expanded leaf was taken to determine its IG content by using high-performance liquid chromatography (HPLC) analyses. A mated female *M. cinxia* butterfly was put into each cage along with a sponge saturated with honey water (1:3) to provide a source of water and food for the butterfly. At the end of the day, all plants and butterflies were removed from the cages. Plants were then searched for butterfly egg clusters. The following day, the number of eggs in each cluster was counted. This process was repeated with new plants and butterflies without oviposition experience. If a butterfly died in the cage, and there was no oviposition, the same pair of plants was used again with a new butterfly. In total, 44 different female butterflies were used, and 19 of them oviposited.

To see if there was systemic induction of IGs in the plant after oviposition, we determined the IG concentrations in leaves from 28 plants that received eggs (19 from experiment 1 and an additional nine from a similar unpublished experiment). We compared the IG concentration of the second fully expanded leaf, sampled before oviposition, with that of a leaf from the same rosette ring as the one that bore the egg batch. This leaf was sampled the evening after oviposition.

Statistical Analyses Cage trial was used as the experimental unit. In 19 of the cages, oviposition was observed. In 17 cases, butterflies oviposited on only one of the two plants. In two cases, butterflies oviposited on both plants, but laid a "normal-sized" cluster on one plant (overall clutch size ± SE=121±22) and only a few eggs (18 and 23, respectively) on the other plant. Only plants with the "normal-sized" clusters were considered as oviposition plants in the analyses. Paired *t* tests (STATISTICA version 7.1 (StatSoft Inc., Tulsa, OK, USA) were used to test whether within a cage leaf IG concentrations and plant size (number of leaves) differed between plants with and without oviposition. The number of leaves, the number of eggs, and the aucubin concentration were log10-transformed prior to

analyses to meet the assumptions of normality and homogeneity of variances. We used Pearson's correlations to test for associations of egg cluster size with the iridoid concentrations and plant size (number of leaves).

Experiment 2: Large Cages, the Netherlands Ten cages (1×1×1 m) were placed outside near the Netherlands Institute of Ecology at Heteren. In each cage, we placed ten plants, five genotypes originating from the low (L1–L5) and five originating from the high IG selection line (H1–H5; see study species). Each cage represented a single replicate. We counted the number of leaves on each plant and collected the sixth fully expanded leaf for chemical analyses. A male and female of *M. cinxia* were released into each cage. Over the following days, we checked for evidence of oviposition, but if oviposition occurred, we did not remove the plant or the butterflies. We marked the leaves onto which the female butterflies had oviposited, but did not remove the eggs. If a female died without ovipositing, she was replaced by a new female. The experiment was terminated when all of the butterflies had died. In total, we used 13 female butterflies from 25th May until the 16th June 2003, six of these butterflies laid eggs.

Statistical Analyses Cages were used as the experimental unit. Paired *t* tests were used to test whether average IG concentrations and the number of leaves differed between plants with and without oviposition events within a cage. Aucubin was log10-transformed, and catalpol and total IG values were square-root-transformed prior to analyses to meet assumptions of normality and homogeneity of variances. Associations of plant size with iridoid concentration were compared by using Pearson's correlations.

Experiment 3: Uncaged Field Experiment, Finland We used the same ten genotypes of *P. lanceolata* as in the above experiment, L1–L5 and H1–H5. In the fall of 2001, we made 40 clonal replicates of each genotype by using the root-cloning method from Wu and Antonovics (1975). Plants were maintained over the winter in 11 cm pots filled with potting soil in an unheated greenhouse in Heteren, the Netherlands. In May 2002, roots of the ca. 25-cm tall plants were washed to remove adhering potting soil, put in moist bags, shipped to Finland, and stored at 4°C until transplantation. A small field was selected as transplantation site, in an open, dry rocky area on peat soil with sparse shrubs and trees near Tvärminne Zoological Station, SW Finland (59° 50′35 N, 23°14′54 E). This site represents a suitable habitat within the distribution range of *P. lanceolata* and *M. cinxia*, but neither of the species was present. On May 11, 2002, plants were planted in 40 patches of ten plants, with each patch containing one individual of each genotype. Roots were gently placed in small slits in the soil to minimize disturbance of the natural vegetation. Plants within the patches were planted 10 cm apart, and the patches were at least 2 m apart. They were watered as required.

M. cinxia larvae were collected from Åland in 2001, overwintered as fifth instar in the lab at Nåtö Biological Station, Finland, and pupated in the spring of 2002. From the 300 reared pupae, 269 adult butterflies (134 ♂ and 135 ♀) emerged, and were introduced in the field site the morning after they had emerged. This occurred between June 6 and 9, 2002, when the experimental plants had regrown new leaves under the prevailing conditions.

We harvested the two youngest fully expanded leaves of each plant from seven patches and air-dried them for HPLC analysis of IGs to estimate the average concentration of IGs for each genotype in the field. Starting when the first butterflies were released on June 6, plants were checked daily for the presence of egg clusters. Egg clusters were left in place and we recorded whether eggs hatched and whether caterpillars managed to produce a winter nest. As some caterpillars moved from the oviposition plant to nearby plants, the number of damaged plants exceeded the number of plants on which oviposition occurred, and some winter nests occurred on non-oviposition plants. On June 15, we measured the number of leaves and the length and width of the longest leaf of each experimental plant. All plants in the field were of the same age because they were cloned at the same time.

Statistical Analyses Differences in plant size and iridoid glycoside concentrations among genotypes and patches were analyzed by using generalized linear models (Procedure GENMOD, SAS v. 8.2, SAS Institute, Cary, NC, USA) with a normal error distribution. Leaf length, leaf width, leaf number, and the product of these ("plant size index"), as well as the concentrations of aucubin, catalpol, and total IG were square-root-transformed prior to analyses to meet assumptions of normality and homogeneity of variance. Plant size index was used as a covariate in analyses of iridoid glycosides to estimate size-independent genotypic differences in secondary chemistry. Effects of plant size, genotype, and patch on the number of egg clusters, number of hatched clusters, and number of diapausing clusters per plant were analyzed by using a GLM with a Poisson error distribution and a log-link function after $(x+1)$ transformation of the independent variables. Since concentrations of aucubin and catalpol were measured only in a subset of the plants and yielded insufficient data on phenotypic associations between secondary metabolites and oviposition, the effects of aucubin and catalpol on oviposition were assessed by using the mean genotypic concentration. We performed univariate and multiple regression of genotype means for size and secondary metabolite concentrations on genotype

means for oviposition traits (number of egg clusters laid, hatched, and surviving to diapause).

Chemical Analyses The second fully expanded leaf was taken from all the *P. lanceolata* plants used in experiment 1. They were air-dried in open envelopes as were the leaves collected from the field plot in experiment 3. The sixth fully expanded leaf from the plants in the cages in Heteren (exp. 2) were frozen at −80°C and freeze-dried. After the leaf drying step, the procedure for chemical analyses was the same for all experiments. Leaves were ground with a Laboratory Vibration Mill (MM 301, Retsch GmBH & Co, Germany). Fine ground dry material of the leaves (25 mg) was extracted in 10 ml of 70% MeOH and shaken overnight. The crude extract was filtered on a Whatman #4 filter paper and diluted ten times with Milli-Q water. Concentrations of the IGs aucubin and catalpol were analyzed by HPLC with a Bio-Lc (Dionex Corp., Sunnyvale, CA, USA) equipped with a GP40 gradient pump, a Carbopac PA 1 guard (4×50 mm) and analytical column (4×250 mm), and an ED40 electrochemical detector for pulsed amperimetric detection (PAD). NaOH (1 M) and Milli-Q water were used as eluents (10:90, 1 ml/min). Retention times were 3.25 and 4.40 min for aucubin and catalpol, respectively. Concentrations were analyzed with Chromeleon version 6.60 (Dionex Corp. Sunnyvale, CA, USA).

Results

Experiment 1: Small Cages, Finland Oviposition was observed in 19 cages, with a total of 21 egg batches. Plants onto which *M. cinxia* oviposited had a significantly higher level of aucubin (2.56±0.43) than plants without (1.57±0.37) oviposition within the same cage (paired *t* test, $t=2.78$, $df=16$, $P=0.013$; Fig. 1a). In 71% of the cases, females chose the plant with the higher aucubin concentration. By contrast, there were no consistent differences in the concentration of catalpol between plants selected (2.25±0.35) and ignored (2.59±0.35) for oviposition (paired *t* test, $t=-0.72$, $df=16$, $P=0.48$). There was also no significant difference in the total IG concentration between plants with (4.81±0.58) and without (4.16±0.55) oviposition (paired *t* test, $t=0.81$, $df=16$, $P=0.43$).

The number of leaves on the plants ranged from six to 26. There was no association of leaf number with oviposition choice (paired *t* test, $t=0.45$, $df=15$, $P=0.66$; Fig. 1b). In 43% of the cases, the plant onto which the butterfly oviposited had fewer leaves than the alternative plant, whereas in 50% of the cases it had more leaves. For plants on which eggs were laid, there was no correlation between the egg cluster size and the iridoid concentration

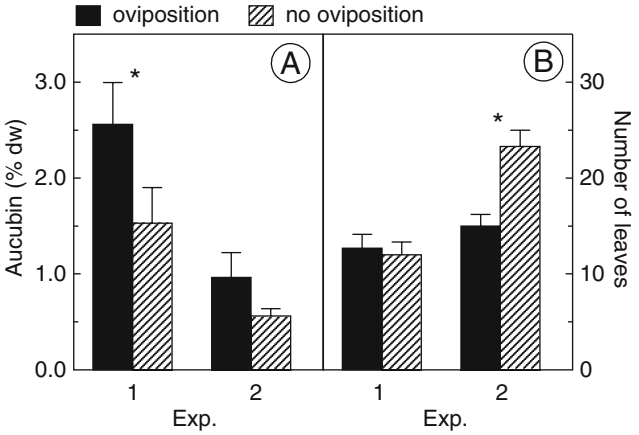

Fig. 1 **a** Average leaf concentration of aucubin (percent of dry weight) and **b** average number of leaves on *Plantago lanceolata* plants with (*black bars*) and without (*hatched bars*) oviposition by *Melitaea cinxia* in experiments 1 and 2. Note that experiment 1 represents a 1-day dual-choice test, whereas experiment 2 represents a multiple-choice test of longer duration. See text for a detailed description of the experiments. *$P<0.05$

or plant size (number of leaves; aucubin: $r=-0.085$, $N=17$, $P=0.75$; catalpol: $r=0.15$, $N=17$, $P=0.56$; total iridoid level: $r=0.19$, $N=17$, $P=0.46$; no. of leaves: $r=0.43$, $N=16$, $P=0.10$).

The observed patterns of associations between plant traits and oviposition did not differ between the cages with plants originating from the field (Finnish plants) and the cages with plants from the cloned Dutch genotypes. Higher concentrations of aucubin in plants chosen for oviposition were observed both for the Finnish (1.8±0.6% vs. 1.0±0.3%) and for the Dutch plants (3.3±0.6% vs. 2.0±0.6%), whereas neither the concentration of catalpol (Finnish: 3.0% vs. 3.4%; Dutch 1.6% vs. 1.7%) nor the number of leaves (Finnish 17.4 vs. 15.3; Dutch 9.4 vs. 9.0) affected oviposition.

We analyzed concentrations of iridoid glycosides in leaves of 28 plants both before and after oviposition. These measures did not differ significantly, suggesting that there was no systemic induction of IGs by the oviposition event within the observed time frame (1 day; paired *t* test, aucubin: $t=-0.15$, $df=27$, $P=0.89$; catalpol: $t=0.22$, $df=27$, $P=0.82$; total iridoid glycosides: $t=1.7$, $df=27$, $P=0.10$).

Experiment 2: Large Cages, the Netherlands Oviposition by *M. cinxia* was observed in six of the ten cages, on one to four plants within a cage, with a total of 15 egg batches on 12 plants. The average iridoid glycoside concentrations of plants with oviposition did not differ from those without oviposition within a cage (paired *t* tests, aucubin: $t=1.35$, $df=5$, $P=0.23$; Fig. 1a; catalpol: $t=1.83$, $df=5$, $P=0.13$; total IG concentration: $t=1.57$, $df=5$, $P=0.18$). Egg batches

were found on plants with significantly fewer leaves than average (t=−3.91, df=5, P<0.05; Fig. 1b). The number of leaves ranged from six to 49 and was negatively correlated with the iridoid concentration (aucubin: r=−0.047, N=59, P<0.001; catalpol: r=−0.045, N=59, P<0.001; total IG level: r=−0.048, N=59, P<0.001).

Experiment 3: Field Site, Finland; Size and Chemistry of Plant Genotypes Plant size differed both among genotypes and patches (Tables 1 and 2). There was a three-fold variation among genotypes. Genotypes also showed a significant, ca. four-fold, variation in their average leaf concentration of iridoid glycosides (Table 1). The range of aucubin concentrations (nine-fold) was larger than the range of catalpol concentrations (four-fold, Table 1). Although genotypes originating from the high IG selection line (H1–H5) on average had higher concentrations of total iridoid glycosides than genotypes originating from the low IG selection line (L1–L5; Table 1), the concentrations of the constituent components aucubin and catalpol varied greatly among genotypes within lines. Part of the variation in total concentrations of iridoid glycosides and catalpol among plants was associated with plant size (Table 2). The total IG concentration of the plant was negatively correlated with both the number of leaves and the size index of the plants (phenotype concentration: no. of leaves: r=−0.55, N=64, P<0.001; size index: r=−0.43, N=64, P<0.001; genotype concentration: no. of leaves: r=−0.77, N=10, P<0.01; size index: r=−0.68, N=10, P<0.05). In other words, larger plants tended to have lower IGs. The concentrations of both aucubin and catalpol were independent of the patch where plants were growing (Table 2).

Experiment 3: Field Site, Finland; Oviposition and Performance of M. cinxia on Different Plant Genotypes Egg clusters of *M. cinxia* were found in 41 (10.3%) plants in the field site. The total number of egg clusters was 63, with a maximum of seven per plant and nine per patch. Forty-eight (76.2%) of the egg clusters hatched, and one-third of them eventually produced winter nests. Of these nests, 13 were on the initial oviposition plant and eight on non-oviposition plants to which the larvae had moved during the season.

The number of egg clusters per plant increased significantly with plant size and differed both among patches and among genotypes (Table 3), ranging from a mean number of 0.025 to 0.50 clusters per plant for different genotypes. Similar effects were found for the number of successful clusters, i.e., clusters that hatched (Table 3). The effects of plant size on the number of egg clusters were mainly due to an increase in oviposition with the number of leaves per plant (Fig. 2a). The association between maximum leaf length and oviposition was non-linear: plants with leaves of an intermediate length of ca. 8 cm were the most often used for oviposition (Fig. 2b), whereas leaf width was not associated with the number of egg clusters on a plant (P> 0.5). Univariate regression analysis (Table 4) showed that at the genotype level, two factors significantly contributed to differences in the number of egg clusters per plant: average concentrations of aucubin and average leaf length (Table 4, Fig. 2b,c). In a multiple regression, the effect of aucubin disappeared (Table 4), indicating that it was partly mediated by genotypic correlations with other factors. In particular, this involved a negative correlation with leaf number (Fig. 3) that tended to have a positive effect on oviposition (Fig. 2a), and a positive correlation with maximum leaf

Table 1 Characteristics of ten *Plantago lanceolata* genotypes selected for low (L1–L5) or high (H1–H5) leaf IG in the Tvärminne field site and occurrence of *Melitaea cinxia* on them (exp. 3)

	Leaf IG (% dw)						Plant size (cm)				*M. cinxia*			
	Total	SE	Aucubin	SE	Catalpol	SE	Size index	Leaf number	Leaf length	Leaf width	P	C	H	D
L1	2.15^{a}	0.11	0.55^{a}	0.05	1.57^{a}	0.09	179.0^{f}	17.9^{c}	10.2^{ab}	0.99^{bc}	11	20	17	5
L2	5.77^{bc}	0.19	1.98^{cd}	0.11	3.77^{bc}	0.15	89.6^{bc}	6.2^{a}	13.4^{c}	1.03^{c}	1	1	1	1
L3	5.79^{bc}	.018	2.93^{d}	0.12	2.80^{b}	0.12	95.9^{bcd}	9.7^{b}	11.8^{bc}	0.85^{ab}	3	3	3	1
L4	5.30^{b}	0.19	2.62^{cd}	0.12	2.67^{b}	0.13	101.1^{cde}	7.7^{ab}	9.7^{a}	1.34^{d}	5	5	1	1
L5	6.29^{bcd}	0.20	0.85^{ab}	0.07	5.41^{d}	0.18	64.9^{ab}	9.8^{b}	9.4^{a}	0.70^{a}	3	4	4	3
H1	7.27^{cde}	0.20	1.85^{c}	0.09	5.39^{d}	0.17	125.3^{de}	9.8^{b}	12.0^{bc}	1.03^{c}	3	3	3	0
H2	8.60^{e}	0.22	4.93^{e}	0.15	3.65^{bc}	0.14	90.1^{bcd}	7.0^{a}	13.0^{c}	0.97^{bc}	1	1	1	1
H3	7.91^{de}	0.23	1.62^{bc}	0.10	6.20^{d}	0.20	83.5^{abc}	8.0^{ab}	10.5^{ab}	0.98^{bc}	4	10	9	5
H4	6.65^{bcde}	0.23	2.00^{cd}	0.12	4.51^{cd}	0.19	56.0^{a}	6.0^{a}	9.0^{a}	0.93^{bc}	7	12	8	2
H5	5.07^{b}	0.17	1.72^{c}	0.09	3.32^{bc}	0.13	138.9^{ef}	9.2^{b}	13.8^{c}	1.07^{c}	3	4	1	2

Values for leaf IG concentrations and plant size are back-transformed least square estimates from GLM with block and genotype effects. Values within columns that do not share a common letter have non-overlapping 95% confidence intervals. Occurrence of *M. cinxia* is summed over the 40 replicate plants per genotype: the observed numbers of plants with egg clusters (P), total numbers of egg clusters (C), hatched clusters (H), and diapausing groups in winter nests (D).

Table 2 Effects of patch and genotype on the size and leaf IG concentration of *Plantago lanceolata* in the Tvärminne field site (exp. 3)

Source	*df*	Size index	Leaf number	Leaf length	Leaf width	*df*	IG total	Aucubin	Catalpol
Covariate						1	35.3***	3.0	30.9***
Patch	39	2.5***	2.5***	1.9**	2.5***	7	1.3	1.1	1.6
Genotype	9	12.0***	22.9***	10.9*	11.5***	9	12.9***	13.5***	10.1***
Error	334					49			

Size index was used as a covariate in analyses of IGs. Values are quasi-*F* values from GLM analyses of deviance.
*$P<0.05$; **$P<0.01$; ***$P<0.001$

length that had a negative effect on oviposition in the range of values for the genotype means (Fig. 2b).

Comparison of Plant Chemistry Between the Three Experiments The total amount of IGs as well as the ratio of aucubin to catalpol differed among the experiments (Fig. 4). Plants originating from Finnish field sites that were used in the small cages (exp. 1) and cloned genotypes transplanted into natural habitat in Finland (exp. 3) had on average more catalpol than aucubin. By contrast, cloned genotypes grown in potting soil in the small cages in Finland (exp. 1) or large cages in the Netherlands (exp. 2) had on average more aucubin than catalpol. In the large cage experiment (exp. 2), the concentrations of IGs were generally very low. Especially striking is the huge difference between the plants used for the experiment in NL (exp. 2) and the field experiment in Finland (exp. 3) because plants used for these two experiments were of the same genotypes. Although the rank order of the genotypes was consistent across both of these experiments (Fig. 5), the absolute concentrations of IGs were significantly higher in the field experiment in Finland (exp. 3). Concentrations of aucubin in these genotypes were ca. three times higher in Finland ($t=5.44$, $df=9$, $P<0.001$), whereas concentrations of catalpol were on average more than 20 times higher in the field experiment in Finland (4.0%) than in the large cage experiment in the Netherlands (0.16%; $t=8.75$, $df=9$, $P<0.001$).

Table 3 Effects of plant size, patch, and genotype of *Plantago lanceolata* in the Tvärminne field site (exp. 3) on the number of oviposited and the number of hatched *Melitaea cinxia* egg clusters per plant

	df	Egg clusters	Hatched
Plant size	1	45.6***	56.5***
Patch	39	2.3***	2.2***
Genotype	9	5.1***	5.4***
Error	333		

Values are quasi-*F* values from GLM analyses of deviance.
***$P<0.001$

Discussion

Butterflies that lay eggs in clusters, such as checkerspots, are expected to spend more time discriminating among potentially suitable host plants than solitary egg laying species (Singer 2004). When a searching *M. cinxia* female alights on a plant, it habitually "tastes" it, rests for a while before moving to another part of the plant or to another plant, and tastes again, continuing this process until she finds a plant chemically acceptable and begins to oviposit. The female selects where to oviposit based on cues she obtains from the plant. The results of the cage experiment in Finland (exp. 1) suggest that oviposition choice in female butterflies is related to concentration of aucubin in the plant. This result is in agreement with those of Nieminen et al. (2003) obtained by sampling from natural populations in the field.

We found no difference in IG concentrations of leaves before and after oviposition, suggesting that the butterflies chose plants with higher aucubin, and that the oviposition event itself did not systemically induce further production of IGs. However, since we did not measure IG concentrations before and after oviposition in the leaf that was actually oviposited on, there remains the possibility that local induction in the leaf selected for oviposition occurs (Peñuelas et al. 2006). Also, our post-oviposition sample occurred the evening after oviposition. It is possible that the induction of the IGs took longer than a single day. Unfortunately, although induction of secondary metabolites following herbivory is well studied, the timing of induction that follows oviposition is poorly studied. We do know that induction of IGs in *P. lanceolata* that follows fungal infection has been observed as early as 6 h after inoculation (Marak et al. 2002), while induction after leaf damage by caterpillars of the specialist *J. coenia* was not observed until 6 days after herbivory (Fuchs and Bowers 2004).

The concentration of aucubin is correlated with the total concentration of IGs. Several experiments have shown that the performance of *M. cinxia* larvae is enhanced when the larvae feed on plants with a higher concentration of IGs than on plants with a lower concentration. They have a shorter development time, higher larval weight, and they

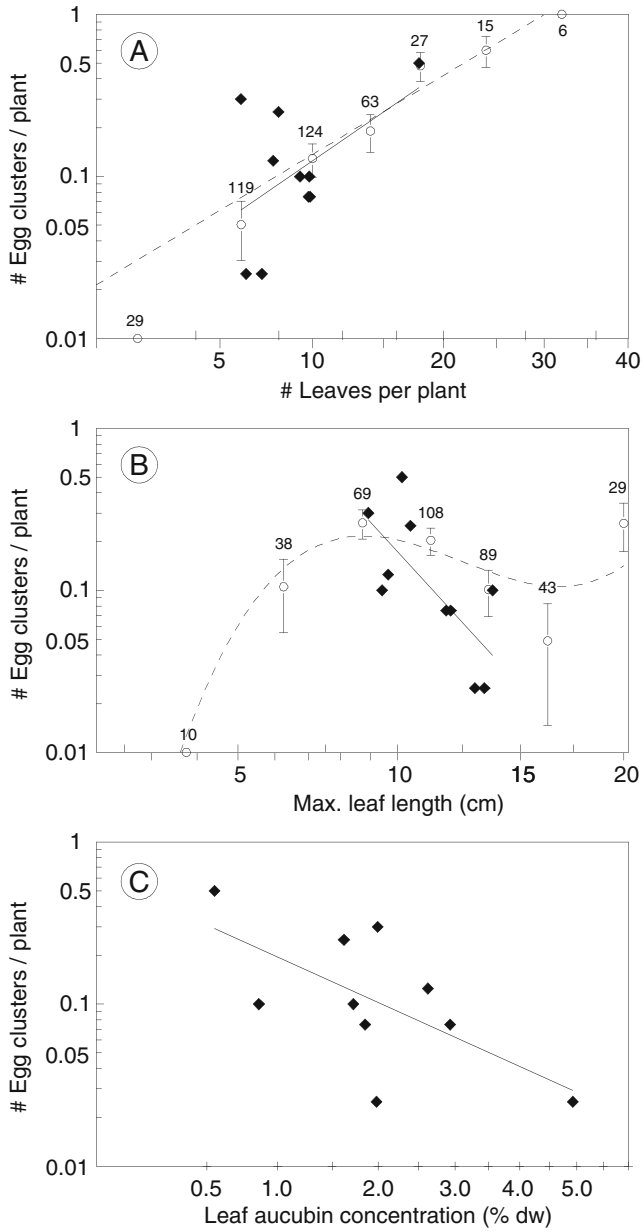

Fig. 2 Number of *Melitaea cinxia* egg clusters (*Y*-axis log scale) per *Plantago lanceolata* plant in experiment 3, as a function of **a** number of leaves per plant, **b** length of the longest leaf per plant, and **c** leaf aucubin concentration (*X*-axis, square root scale). *Black diamonds* represent genotype mean values of traits with corresponding *solid lines* of regression. *Open circles* (sample size on top) represent mean numbers of egg clusters for classes of phenotypic values of leaf number and leaf length ± 1 SE (class limits indicated above the *X*-axis). *Dotted lines* are corresponding polynomial regression lines based on parameter estimates from Poisson regressions of the phenotypic data

tend to have a larger pupal size (Harvey et al. 2005; Saastamoinen et al. 2007). These trait values are usually associated with a higher fitness (Roff 1992). There are many factors that affect larval fitness of *M. cinxia*, but larval size is strongly correlated with overwintering

Table 4 Univariate and multiple regressions of genotype means for morphological and chemical traits on genotype means for the number of *Melitaea cinxia* egg clusters per plant of *Plantago lanceolata* in the Tvärminne field site (exp. 3)

	Univariate	Multivariate
Total IGs	−0.503	–
Aucubin	−0.648*	−0.231
Catalpol	−0.140	–
Plant size index	+0.199	–
No. of leaves per plant	+0.503	+0.278
Max. leaf length	−0.718*	−0.583^{+}
Max. leaf width	−0.005	–
Model R^2		0.71*

Values are standardized regression coefficients.
$^{+}P<0.10$; $*P<0.05$

survival, which can be low (Nieminen et al. 2001; Kuussaari et al. 2004). The shorter development time also may be an advantage for *M. cinxia* because of the short growing season (Kuussaari et al. 2004). Furthermore, fast development decreases the period of time the caterpillar will be vulnerable to its natural enemies (Benrey and Denno 1997; van Nouhuys and Lei 2004).

Another advantage of feeding on plants with a high concentration of iridoid glycosides may be chemical defense against predators and parasitoids. Specialized larvae feeding on iridoid-producing plants are able to sequester these iridoids and become distasteful or noxious to their natural enemies (Bowers 1980, 1981, 1990; Bowers and Puttick 1986; Gardner and Stermitz 1988; L'Empereur and Stermitz 1990; Stermitz et al. 1994; Camara 1997; Nieminen et al. 2003).

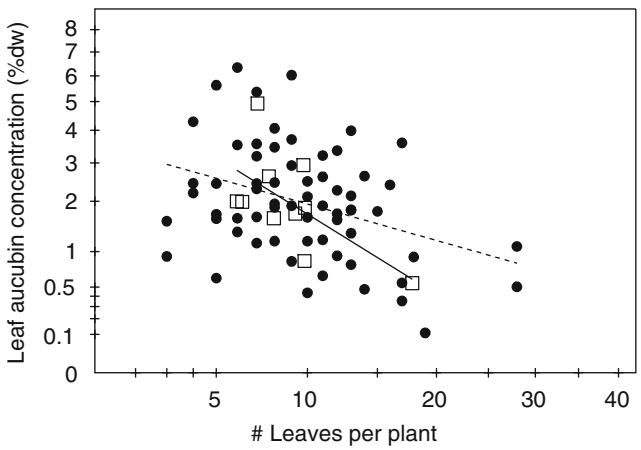

Fig. 3 Regression of phenotypic values (*black dots*, *dotted line*) and genotype means (*open squares*, *solid line*) of leaf aucubin concentration on the number of leaves per *Plantago lanceolata* plant in experiment 3

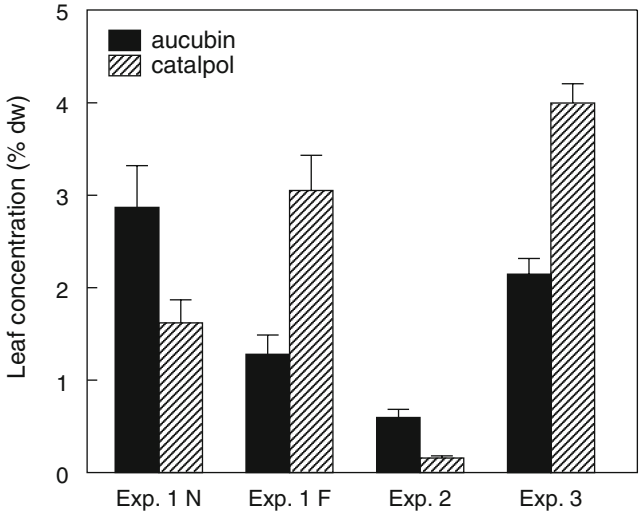

Fig. 4 Mean aucubin and catalpol concentration of *Plantago lanceolata* plants used in the three experiments. *Black bars* indicate the mean aucubin concentration, *hatched bars* the mean catalpol concentration. Exp. 1 N: small cages, Finland (Dutch genotypes); exp. 1 F: small cages, Finland (Finnish plants); exp. 2: large cage, the Netherlands; exp. 3: field site, Finland. See text for a detailed description of the different experiments

The fact that we did not observe an association of oviposition choice with IGs in experiment 2 (large cages NL) could be because of the overall low concentration of IGs in these plants. Perhaps, the female *M. cinxia* is not able to discriminate at such low absolute amounts of IGs (on average lower than 1% of the dry weight). This would suggest a threshold concentration below which these secondary metabolites are no longer used as a suitable cue for oviposition decisions although the small sample size of six females also could have contributed to the lack of a significant relationship.

The results of the field experiment (exp. 3) differed from the results of the cage studies. Perhaps, this is not surprising. In the field, *M.* cinxia and other checkerspots visit many possible plants without ovipositing, and the ranking of plants can depend on time and context (Schöps and Hanski 2001; Singer 2004). Thus, the plants used in the field should probably be considered a more absolute choice than in the cages where the butterflies may have selected plants that they would reject in other circumstances. Notably, the size of the plant (mainly the number of leaves per plant) had a positive effect on the oviposition preference of the females in the field, while in the cage experiment there was no association (exp. 1) or a significantly negative association (exp. 2) between these parameters. As mentioned, the higher oviposition rates of *M. cinxia* on larger plants in the field are in agreement with observations in natural populations by Nieminen et al. (2003). This is not unexpected given the limited mobility of early instar gregarious caterpillars (Kuussaari et al. 2004).

A further possible explanation for the stronger impact of plant size on oviposition in the field than in cage experiments is the different range of plant sizes between the field and cage studies. In the field, the difference in leaf number between the smallest and largest plant was 78; in the cage study in Finland, the mean difference in leaf number between the plants between which *M. cinxia* could choose was only 3.3. Because of this smaller variation in plant size in the cage experiments, the oviposition choice of the female in the cages could be based to a greater extent on chemical rather than visual stimuli. In addition, the use of visual cues may manifest more clearly in the field experiment where butterflies may encounter plants at a distance, whereas in the cages they are constrained in close proximity to each plant.

Future studies are needed to disentangle the relative importance of plant size and IG concentrations of individual plants for oviposition in the field. In the cage experiments, IGs were measured for each individual plant. Associations between oviposition and traits including IGs could be studied at the individual plant level. However, in the field experiment (exp. 3), IG concentrations were measured for a subset of plants only. Based on these measurements, genotype mean IG concentrations were used to assess associations between oviposition and IGs. This resulted in a loss of power to detect associations and reduced our ability do distinguish effects of morphological traits from IGs on oviposition.

In summary, our results show that ovipositing *M. cinxia* discriminate between host plants based at least on factors associated with plant chemistry and size. For females in the field experiment, the size of the plant was a positive stimulus, whereas in the cages, where plants were more

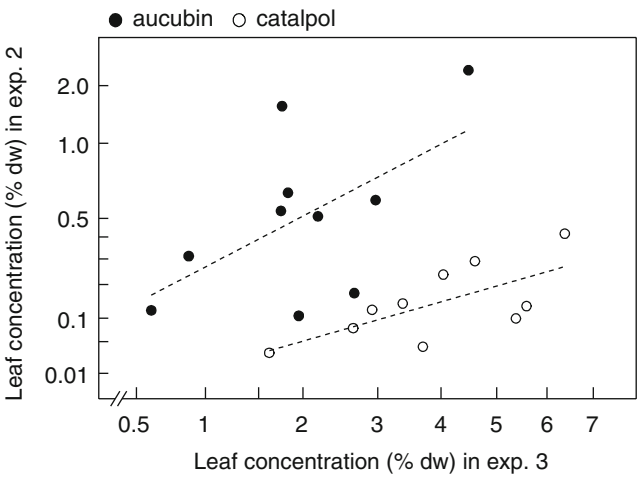

Fig. 5 Average leaf concentrations of aucubin (*black dots*) and catalpol (*open dots*) of ten *Plantago lanceolata* genotypes used in experiment 2 vs. the average IG concentrations of the same genotypes in experiment 3. See text for a detailed description of the experiments

similar in size and appearance, chemical stimuli were apparently more important. In particular, oviposition tended to increase with constitutive concentration of aucubin.

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References

ADLER, L. S., SCHMITT, J., and BOWERS, M. D. 1995. Genetic-variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). *Oecologia* 101:75–85.

AGRAWAL, A. A. 2000. Plant defense: signals in insect eggs. *Trends Ecol. Evol.* 15:357.

BADENES, F., SHELTON, A., and NAULT, B. 2004. Evaluating trap crops for diamondback moth, *Plutella xylostella* (Lepidoptera: Plutellidae). *J. Econ. Entomol.* 97:1365–1372.

BENREY, B., and DENNO, R. F. 1997. The slow-growth-high-mortality hypothesis: a test using the cabbage butterfly. *Ecology* 78:987–999.

BERNAYS, E. A., and CHAPMAN, R. F. 1994. Host-Plant Selection by Phytophagous Insects. Chapman and Hall, New York.

BOWERS, M. D. 1980. Unpalatability as a defense strategy of *Euphydryas phaeton* (Lepidoptera: Nymphalidae). *Evolution* 34:586–600.

BOWERS, M. D. 1981. Unpalatability as a defense strategy of western checkerspot butterflies (*Euphydryas*, Nymphalidae). *Evolution* 35:367–375.

BOWERS, M. D. 1983. The role of iridoid glycosides in host-plant specificity of checkerspot butterflies. *J. Chem. Ecol.* 9:475–793.

BOWERS, M. D. 1990. Recycling plant natural products for insect defense, pp. 353–386, in D. L. Evans, and J. O. Schmidt (eds.). Insect Defenses: Adaptive Mechanisms and Strategies of Prey and PredatorsState University of New York Press, Albany.

BOWERS, M. D. 1991. Iridoid glycosides, pp. 297–325, in G. A. Rosenthal, and M. R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant MetabolitesAcademic, San Diego.

BOWERS, M. D., and PUTTICK, G. M. 1986. Fate of ingested iridoid glycosides in Lepidopteran herbivores. *J. Chem. Ecol.* 12:169–178.

BOWERS, M. D., and STAMP, N. E. 1992. Chemical variation within and between individuals of *Plantago lanceolata* (Plantaginaceae). *J. Chem. Ecol.* 18:985–995.

BOWERS, M. D., and STAMP, N. E. 1993. Effects of plant-age, genotype, and herbivory on *Plantago* performance and chemistry. *Ecology* 74:1778–1791.

CAMARA, M. D. 1997. Predator responses to sequestered plant toxins in buckeye caterpillars: are tritrophic interactions locally variable? *J. Chem. Ecol.* 23:2093–2106.

COLAZZA, S., FUCARINO, A., PERI, E., SALERNO, G., GONTI, E., and BIN, F. 2004. Insect oviposition induces volatile emission in herbaceous plants that attracts egg parasitoids. *J. Exp. Biol.* 207:47–53.

COURTNEY, S. P. 1982. Coevolution of pierid butterflies, and their cruciferous foodplants. IV. Crucifer apparency and *Anthocharis cardamines* oviposition. *Oecologia* 52:258–265.

DARROW, K., and BOWERS, M. D. 1999. Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. *J. Chem. Ecol.* 25:1427–1440.

EL-NAGGAR, L. J., and BEAL, J. L. 1980. Iridoids: a review. *J. Nat. Prod.* 43:649–707.

FEENY, P. P., STADLER, E., AHMAN, I., and CARTER, M. 1989. Effects of plant odor on oviposition by the black swallowtail butterfly, *Papilio polyxenes* (Lepidoptera: Papilionidae). *J. Insect Behav.* 2:803–827.

FUCHS, A., and BOWERS, M. D. 2004. Patterns of iridoid glycoside production and induction in *Plantago lanceolata* and the importance of plant age. *J. Chem. Ecol.* 30:1723–1741.

GARDNER, D. R., and STERMITZ, F. R. 1988. Host plant utilization and iridoid glycoside sequestration by *Euphydryas anicia* (Lepidoptera, Nymphalidae). *J. Chem. Ecol.* 14:2147–2168.

HARVEY, J. A., VAN, NOUHUYS, S., and BIERE, A. 2005. Effects of quantitative variation in allelochemicals in *Plantago lanceolata* on development of a generalist and a specialist herbivore and their endoparasitoids. *J. Chem. Ecol.* 31:287–302.

HEBERT, P. D. N. 1983. Egg dispersal patterns and adult feeding behavior in the Lepidoptera. *Can. Entomol*. 115:1477–1481.

HEISSWOLF, A., OBERMAIER, E., and POETHKE, H. J. 2005. Selection of large host plants for oviposition by a monophagous leaf beetle: nutritional quality or enemy-free space? *Ecol. Entomol.* 30:299–306.

HILKER, M., STEIN, C., SCHRÖDER, R., VARAMA, M., and MUMM, R. 2005. Insect egg deposition induced defence response in *Pinus sylvestris*. *J. Exp. Biol.* 208:1849–1854.

JANZ, N., and NYLIN, S. 1997. The role of female search behavior in determining host plant range in plant feeding insects: a test of the information processing hypothesis. *Proc. R. Soc. B: Biol. Sci*. 264:701–707.

JENSEN, S. R. 1991. Plant iridoids, their biosynthesis and distribution in angiosperms, pp. 133–158, in J. B. Harborne, and F. A. Tomas-Barberan (eds.). Ecological Chemistry and Biochemistry of Plant TerpenoidsClarendon, Oxford.

KUUSSAARI, M., SINGER, M., and HANSKI, I. 2000. Local specialization and landscape-level influence on host use in an herbivorous insect. *Ecology* 81:2177–2187.

KUUSSAARI, M., VAN, NOUHUYS, S., HELLMANN, J., and SINGER, M. 2004. Larval biology of checkerspots, pp. 138–160, in P. R. Ehrlich, and I. Hanski (eds.). On the Wings of Checkerspots: A Model System for Population BiologyUniversity Press, Oxford.

L'EMPEREUR, K. M., and STERMITZ, F. R. 1990. Iridoid glycoside content of *Euphydryas anicia* (Lepidoptera: Nymphalidae) and its major host plant, *Besseya plantaginea* (Scophulariaceae), at high plains Colorado site. *J. Chem. Ecol.* 16:187–197.

LASTRA, J. A. S., BARRIOS, L. E. G., ROJAS, J. C., and RIVERA, H. P. 2006. Host selection behavior of *Leptophobia aripa* (Lepidoptera: Pieridae). *Fla. Entomol.* 89:127–134.

MARAK, H. B., BIERE, A., and VAN, DAMME, J. M. M. 2000. Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. *J. Evol. Biol.* 13:985–996.

MARAK, H. B., BIERE, A., and VAN, DAMME, J. M. M. 2002. Systemic, genotype-specific induction of two herbivore-deterrent iridoid glycosides in *Plantago lanceolata* L. in response to fungal infection by *Diaporthe adunca* (Rob.) Niessel. *J. Chem. Ecol.* 28:2429–2448.

MAYHEW, P. J. 1997. Adaptive patterns of host-plant selection by phytophagous insects. *Oikos* 79:417–428.

MURPHY, D. D., WAHLBERG, N., HANSKI, I., and EHRLICH, P. R. 2004. Introducing checkerspots: taxonomy and ecology, pp. 17–33, in P. R. Ehrlich, and I. Hanski (eds.). On the Wings of Checkerspots: A Model System for Population BiologyOxford University Press, Oxford.

NIEMINEN, M., SINGER, M., FORTELIUS, W., SCHÖPS, K., and HANSKI, I. 2001. Experimental confirmation that inbreeding depression increases extinction risk in butterfly populations. *Am. Nat.* 157:237–244.

NIEMINEN, M., S, uomi, J., VAN, NOUHUYS, S., SAURI, P., and RIEKKOLA, M. L. 2003. Effect of iridoid glycoside content on oviposition host plant choice and parasitism in a specialist herbivore. *J. Chem. Ecol.* 29:823–844.

OLMSTEAD, R. G., KIM, K. J., JANSEN, R. K., and WAGSTAFF, S. J. 2000. The phylogeny of the Asteridae *sensu latu* based on chloroplast *ndh*F gene sequences. *Mol. Phylogenet. Evol.* 16:96–112.

PEÑUELAS, J., SARDANS, J., STEFANESCU, C., PARELLA, T., and FILLELLA, I. 2006. *Lonicera implexa* leaves bearing naturally laid eggs of the specialist herbivore *Euphydryas aurinia* have dramatically greater concentrations of iridoid glycosides than other leaves. *J. Chem. Ecol.* 32:1925–1933.

PEREYRA, P. C., and BOWERS, M. D. 1988. Iridoid glycosides as oviposition stimulants for the buckeye butterfly, *Junonia coenia* (Nymphalidae). *J. Chem. Ecol.* 14:917–928.

PRICE, P. W. 1991. The plant vigour hypothesis and herbivore attack. *Oikos* 62:244–492.

RAUSHER, M. D. 1978. Search image for leaf shape in a butterfly. *Science* 200:1071–1073.

REUDLER, TALSMA, J. H., TORRI, K., and VAN, NOUHUYS, S. 2008. Host plant use by the Heath fritillary butterfly, *Melitaea athalia*: plant habitat, species and chemistry. *Arthropod–Plant Interact* 2:63–75.

ROFF, D. A. 1992. The Evolution of Life Histories. Chapman and Hall, New York.

SAASTAMOINEN, M. 2007. Life-history, genotypic and environmental correlates of clutch size in the Glanville fritillary butterfly. *Ecol. Entomol.* 32:235–242.

SAASTAMOINEN, M., VAN, NOUHUYS, S., NIEMINEN, M., O'HARA, B., and SUOMI, J. 2007. Development and survival of a specialist herbivore, *Melitaea cinxia*, on host plants producing high and low concentrations of iridoid glycosides. *Ann. Zool. Fenn.* 44:70–80.

SAGAR, G. R., and HARPER, J. L. 1964. Biological flora of the British isles. *Plantago major* L., *Plantago media* L. and *Plantago lanceolata* L. *J. Ecol.* 52:189–221.

SCHÖPS, K., and HANSKI, I. 2001. Population level correlation between pre-alighting and post-alighting host plant preference in the Glanville fritillary butterfly. *Ecol. Entomol.* 26:517–524.

SINGER, M. C. 1986. The definition and measurement of oviposition preference in plant-feeding insects, pp. 65–94, in J. R. Miller, and M. A. Miller (eds.). Insect–Plant InteractionsSpringer, New York.

SINGER, M. C. 2004. Measurement, correlates, and importance of oviposition preference in the life of checkerspots, pp. 112–137, in P. R. Ehrlich, and I. Hanski (eds.). On the Wings of Checkerspots: A Model System for Population BiologyOxford University Press, Oxford.

STAMP, N. E. 1980. Egg deposition patterns in butterflies: why do some species cluster their eggs rather than deposit them singly? *Am. Nat.* 115:367–380.

STAMP, N. E., and BOWERS, M. D. 2000. Do enemies of herbivores influence plant growth and chemistry? Evidence from a seminatural experiment. *J. Chem. Ecol.* 26:2367–2386.

STERMITZ, F. R., KADER, M. S. A., FODERARO, T. A., and POMEROY, M. 1994. Iridoid glycosides from some butterflies and their larval food plants. *Phytochemistry* 37:997–999.

VAN, NOUHUYS, S., and LEI, G. C. 2004. Parasitoid and host metapopulation dynamics: the influences of temperature mediated phenological asynchrony. *J. Anim. Ecol.* 73:526–535.

VISSER, J. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

WAHLBERG, N. 2001. The phylogenetics and biochemistry of host-plant specialization in Melitaeine butterflies (Lepidoptera: Nymphalidae). *Evolution* 55:522–537.

WAHLBERG, N., EHRLICH, P. R., BOGGS, C. L., and HANSKI, I. 2004. Bay checkerspot and Glanville fritillary compared with other species, pp. 219–244, in P. R. Ehrlich, and I. Hanski (eds.). On the Wings of Checkerspots: A Model System for Population BiologyOxford University Press, Oxford.

WU, L., and ANTONOVICS, J. 1975. Experimental ecological genetics in *Plantago*. I. Induction of roots and shoots on leaves for large scale vegetative propagation and metal tolerance testing in *P. lanceolata*. *New Phytol.* 75:277–282.

and subtemperate regions, mostly for consumption as leafy vegetable and herb. Because of the pungent flavor, it is traditionally used to flavor beans and other South American dishes. Its extract or EO has medicinal (Boiteau 1986; Kishore et al. 1996; Lall and Meyer 1999; Noumi and Yomi 2001), acaricidal, and insecticidal properties (Malik and Mujtaba-Naqvi 1984; Su 1991; Chiasson et al. 2004a,b). However, investigations of its antifungal properties are rare (Dubey et al. 1983).

The chemical composition and yield of epazote EO shows wide variation in composition, which may be related to the herbal source, chemotype of the plant species, and/or the analytical methods used (Guenther 1952; Bauer and Brasil 1973; Onocha et al. 1999; Tapondjou et al. 2002; Gupta et al. 2002; Huang and Kong 2002; Pino et al. 2003; Cavalli et al. 2004; Kasali et al. 2006). Although there are a few reports regarding the fungicidal properties of epazote extracts, fungicidal properties have not been linked to specific EO component(s). Use of EO to protect agricultural commodities requires consistency of results, which can only be obtained if the EO is standardized against the most active component of the oil. Identification of such components helps in understanding the EO mode of action and can lead to discovery of new antifungal compounds.

In this study, we report the chemical composition and antifungal activity of Brazilian epazote EO and identify the major constituents responsible for this activity.

Methods and Materials

Plant Material and Hydrodistillation Epazote leaves were harvested from shrubs in Viçosa, Minas Gerais, Brazil, and mixed with water (1:5 *w*/*v*) for hydrodistillation. The distillate was extracted twice with dichloromethane (1:1 *v*/*v*). The organic phase was collected and dried with anhydrous sodium sulfate; dichloromethane was evaporated in a rotatory evaporator at 30°C under reduced pressure and weighed.

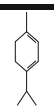
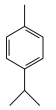
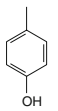
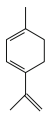
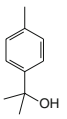
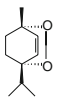
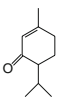
Identification of EO Components The compounds in the crude EO and its fungitoxic component were tentatively identified by gas chromatography (GC) using Kováts retention indices (RIs) and gas chromatography combined with mass spectrometry (GC-MS). The peaks were first identified by GC-MS library system based on similarity indices (SI). Also, RIs were obtained for all the major GC peaks. The final identification was based on the best SI and RI fits (Adams 1995).

Data were obtained on two instruments. For RIs, data were obtained on a gas chromatograph with a flame ionization detector (Shimadzu, Kyoto, Japan, model GC 17 A), an auto sampler, and workstation. Mass spectral data were obtained on a gas chromatograph-mass spectrometer (Shimadzu, Kyoto, Japan, model QP 5000 and software program-Classs-5000, version.1.2) fitted with an auto sampler, workstation, and a database (Wiley 229) with about 350,000 entries. Fused capillary columns (30 m× 0.25 mm; film thickness of 0.25 μm) coated with the DB-5 stationary phase were purchased from Supelco (Bellefonte, PA, USA). In all cases, the GC oven temperature was programmed from 60°C (1 min hold) to 240°C (9 min hold) at a rate of 3°C/min. One microliter of the sample dissolved in hexane (10%) was injected by the split mode (10:1) with the split vent being closed for 30 s. Nitrogen and He were the carrier gases for GC and GC-MS, respectively, at flow rates of 1.33 ml/min. The mass spectrometer was scanned from *m*/*z* 40 to 350 in the electron impact mode (70 eV). To obtain representative data, the mass spectra over the entire GC peaks (~50 scans) of interest were grouped and subtracted from the grouped mass spectra of the region closest (before or after) to where no compound eluted (~50 scans). Only compounds with similarity indexes of 90% (SI=90) were considered as positive identifications.

For RI data, a mixture of linear saturated hydrocarbons was co-injected and the mixture analyzed by GC. RIs were calculated by using the following formula: $KI=100y+100(z-y)\times t_{R(x)}-t_{R(y)}/t_{R(z)}-t_{R(y)}$, where y and z are the carbon numbers in the hydrocarbons eluting before and after a GC peak of interest, respectively; $t_{R(x)}$ is the retention time of the acetate; $t_{R(y)}$ and $t_{R(z)}$ are the retention times of the hydrocarbons eluting before and after a GC peak of interest, respectively.

Antifungal Activity Antifungal activity of the EO was tested with the poison food assay on potato-dextrose agar (PDA; Dhingra and Sinclair 1995). To obtain a uniform dispersal, the EO was dissolved in methanol (1:1 *v*/*v*), and the mixture was added to cool molten PDA to obtain EO concentrations of 0.1% and 0.05%. The active EO fraction was tested at 0.1%, 0.05%, and 0.03% concentrations. Ten milliliter of the medium was poured into 9-cm culture plates. The controls consisted of PDA containing an equivalent amount of methanol. *Aspergillus flavus*, *Aspergillus glaucus*, and *Aspergillus ochraceous* (stored grain and feed deteriorating fungi); *F. semitectum*, *Colletotrichum gloeosporioides*, and *Aspegillus niger* (postharvest rot of tropical fruits and vegetables); *Colletotrichum musae* (banana fruit anthracnose); and *Fusarium oxysporum* were used as test fungi. The medium in each plate was spot-seeded with the conidia of either of the test fungus (Dhingra and Sinclair 1995). Colony diameter was measured at either at fifth or sixth day after incubation at 25°C. All tests were performed in triplicate. Percent growth inhibition was calculated by dividing radial growth in the treatment plates by growth in the control plates and multiplying by 100. The data were

Table 1 Percent composition of the crude essential oil of *C. ambrosioides*

Peak no	Kováts retention index	Compound	Structure	%
1	1018	α-Terpinene		0.9
2	1026	*p*-Cymene		2.0
3	1033	Benzyl alcohol		0.3
4	1075	*p*-Cresol		0.3
5	1113	*p*-Mentha-1,3,8-triene		0.2
6	1191	*p*-Cimen-8-ol		0.6
7	1197	α-Terpineol		0.5
8	1248	(*Z*)-Ascaridole		61.4
9	1260	Piperitone		0.9
10	1297	Carvacrol		3.9
11	1307	(*E*)-Ascaridole		18.6
12	1342	(*E*)-Piperitol acetate		0.5
13	1363	(*Z*)-Carvyl acetate		0.2
		Other unidentified compounds		10.6

Tentative identifications of components based on Kováts retention indices and gas chromatography-mass spectrometry

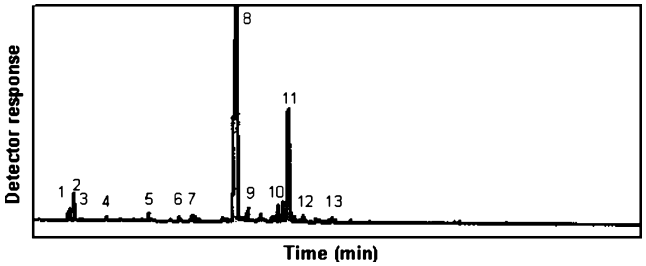


Fig. 1 Reconstructed gas chromatogram of crude *C. ambrosioides* essential oil. Chromatography conditions: oven temperature programmed from 60°C (1 min hold) to 240°C (9 min hold) at 3°C/min; He carrier gas (1.33 mL/min), temperatures of injector, transfer line, and detector 240°C, 240°C, and 250°C, respectively, 70 eV; scanning range (*m*/*z*), 40–350 with a fused silica capillary column coated with the DB-5 stationary phase

analyzed by analysis of variance and the means compared by the Tukey test (P=0.05).

Identification of Fungitoxic Components Preparative thin layer chromatography (TLC) bioautography (Rahalison et al. 1991) was used to separate the major antifungal fraction of the EO. A 200-mg aliquot of the crude EO was applied onto each of the six preparative silica gel TLC plates (20× 20 cm, 1-mm thick, 60GF, Merck) and developed in dichloromethane:ethyl acetate (9:1 *v*/*v*). After evaporation of the solvent at room temperature, cool molten PDA containing 100 mg/l streptomycin sulfate and conidia of the respective fungus (10^3/ml) was spread over each plate and incubated for 6 days at 30°C in a moisture-saturated plastic box. The plate area where the fungus did not grow was delineated, measured, scrapped, and extracted twice by shaking for 2 h with 200 ml of dichloromethane. The mixture was filtered, the filtrate was dehydrated with anhydrous sodium sulfate, and the dichloromethane removed by rotary evaporation. The residue was weighed, collected in vials, and stored at 4°C until use.

Results

Hydrodistillation of the epazote leaves yielded 0.3% EO on a fresh weight basis. Through GC-MS and GC analysis using RI, 13 components were tentatively identified (see Table 1) and represented 90.4% of the crude EO. These included (*Z*)-ascaridole (61.4%), (*E*)-ascaridole (18.6%), carvacol (3.9%), and *p*-cymene (2%), constituting 85.9% of the EO, while the remaining 4.5% was made up by the other nine components having relative concentrations of less than 1% (Fig. 1, Table 1).

The crude EO completely inhibited the mycelial growth of all eight of the test fungi at a concentration of 0.3% (Fig. 2). At a concentration of 0.1%, the growth inhibition

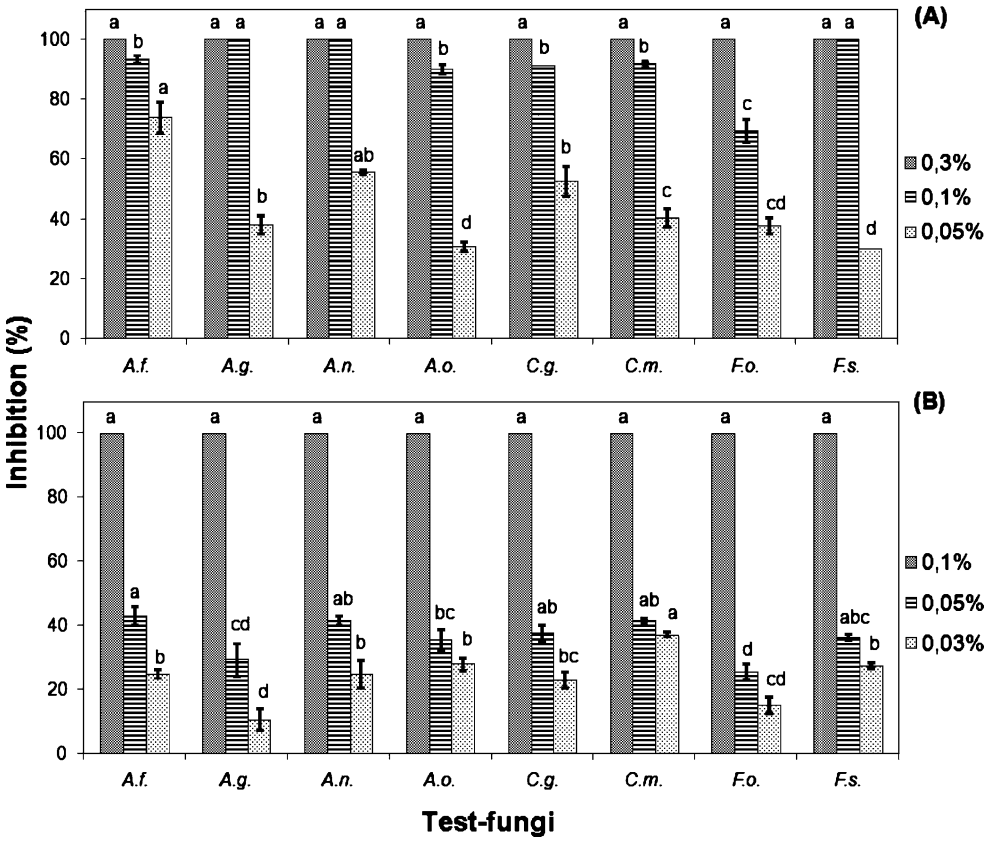


Fig. 2 Percent radial growth inhibited by **a** crude essential oil of *C. ambrosioides* essential oil after 6-day incubation at concentration of 0.05%, 0.1%, or 0.3%, and **b** by the purified fraction at a concentration of 0.1%, 0.05%, and 0.03% after 5-day incubation at 25°C. Mean of three replications ±SD within each treatment. For each concentration, the histograms of different fungi, headed by the same letter, do not differ at P=0.05. *A.f. Aspergillus flavus*, *A. g. A. glaucus*, *A.n. A. niger*, *A.o. A. ochraceous*, *C.g. C. gloesporioides*, *C.m. C. musae*, *F.o. F. oxysporum*, and *F.s. F. semitectum*

of fungi varied from 90% to 100%, except *F. oxysporum* whose growth was inhibited by about 70%.

Fractionation of crude EO to isolate and identify the most active antifungal constituent yielded only one band without fungal growth on the autobiographic TLC (R_f= 0.89, longitudinal width 5 cm). The GC and GC-MS analysis of this fraction revealed the presence of only *Z*-ascaridole (44.4%), *E*-ascaridole (30.2%), and *p*-cymene (25.4%). This fraction at 0.1% completely inhibited growth of all test fungi, but at 0.05% the inhibition of *A. flavus*, *A. niger*, and *Colletotrichum gloesporioides* was lower, and that of other fungi was similar to the crude EO at the same concentration.

Discussion

The yield of crude oil was within the range of 0.06% reported from Nigeria (Onocha et al. 1999) to 0.8% from Cameroon (Tapondjou et al. 2002). Yields between 0.2% and 0.3% have been reported (Bauer and Brasil 1973; Muhayimana Uhayimana et al. 1998; Gupta et al. 2002), but higher yields (1–2%) have also been noted (Guenther 1952). The yield of plant EO is affected by several factors such as genotype and environment (Guenther 1952; Wink 1993).

This composition of the epazote EO is substantially different from that reported from southern Brazil plants (Bauer and Brasil 1973), where limonene (29.6%), mycrene (19%), and β-pinene (3.6%) constituted the major portion of the EO. These compounds were not detected in the present study. Moreover, the results from the two studies may not be comparable, since in the previous study, GC with a thermal conductivity detector was used to determine relative concentrations of the compounds without specifying the identification procedure. In addition, 30.6% of the components could not be identified. In this study, we identified 90.4% of the volatile oil by using a widely employed methodology for EO identification (Adams 1995).

The chemical composition of the epazote EO was quite different from that reported in other studies. Ascaridoles (*Z* and *E*) were the major components (80%) in the present study, while *p*-cymene (2%) and α-terpinene (0.9%) were minor components. On the other hand, *p*-cymene and α-terpinene were the major components in EO, with respective concentrations of 15.5% and 56% from Nigeria (Onocha et al. 1999), 19.5% and 63.6% from India (Gupta et al. 2002), and 50% and 37.6% from Cameroon, (Tapondjou et al. 2002), while (*Z*)- and (*E*)-ascaridole were the minor components. Cavalli et al. (2004) reported α-terpinene (9.7%), *p*-cymene (16.2%), and ascaridole (41.8%) in a commercial EO available in France. Thus, in all cases, the ascaridoles, considered to be quality indicators of *C. ambrosioides* EO (Guenther 1952), were much lower than those obtained in Brazil.

Dubey et al. (1983) reported a minimum concentration of 0.1% for complete inhibition of *Rhizoctonia solani*. However, even at 0.05%, the growth of the many important postharvest fungi was inhibited by over 50% in this study, with that of *A. flavus* being inhibited by 70%. The higher sensitivity of *A. flavus* to epazote EO is of particular interest, since this fungus produces potentially carcinogenic alfatoxins in grain and feeds colonized by it. This EO might be a good candidate for use with such commodities.

Although higher concentrations are required to inhibit the growth of other postharvest deteriorating fungi, under certain circumstances, crude EO might be useful for retarding fungal growth. The toxicity of EO to *A. flavus* in this study is much higher than that reported for the dichloromethane extract, where fungal growth was inhibited by 13% at a 0.05% concentration (Arispuro et al. 1997), and *Tilletia indica* by 40% (Castaneda et al. 2001). However, the chemical profile of that extract was not reported.

These data, along with the fact that the percentage of ascaridoles was only slightly lower in comparison to the crude EO (74.6% vs. 80%), suggest that they are the major fungitoxic components. However, total fungitoxcity may involve other minor components that have additive or synergistic effects. The high proportion of *p*-cymene in the active fraction, with no increase in the fungitoxicity, suggests that it may not be involved.

Despite little evidence, insecticidal activity of epazote EO has been attributed to ascaridoles (Pollack et al. 1990; Tapondjou et al. 2002). There is no study that reports the antifungal component(s) of epazote EO. This study shows, for the first time, the wide spectrum antifungal activity of this EO and indicates that ascaridoles are the major antifungal components, which may be used as markers for standardizing the product for use in postharvest protection of agricultural commodities. Identification of fungicidal properties of ascaridole opens the possibility of developing new fungicides by using it as the lead compound.

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References

ADAMS, R. P. 1995. Identification of essential oil components by gas chromatography/mass spectroscopy. Allured Publishing Corporation, Carol Stream.

ARISPURO, I. V., BERNAL, S. A., TELLEZ, M. A. M., and NIEBLAS, M. O. 1997. Effect of plant extracts on the growth and aflatoxin production of *Aspergillus flavus* and *Aspergillus parasiticus*. *Rev. Mex. Fitopatol.* 15:91–95.

ATANDA, O. O., AKPAN, I., and OLUWAFEMI, F. 2007. The potential of some spice essential oils in the control of *A. parasiticus* CFR 223 and aflatoxin production. *Food Control* 18:601–607.

BAUER, L., and BRASIL, S. G. A. 1973. Essential oils of *Chenopodium ambrosioides* and *Schinus terebinthifolius* from Rio Grande do Sul. *Rev. Bras. Farm.* 54:240–242.

BOITEAU, P. 1986. Médecine Traditionnelle et Pharmacopée: Précis de Matière Médicale Malgache. Agence de Coopération Culturelle et Technique, Paris.

CASTANEDA, G. R., TELLEZ, M. A. M., COHEN, S. V., MANILLA, G. A., ARISPURO, I. C. V., SANZ, P. M., and YUFERA, E. P. 2001. In vitro inhibition of mycelial growth of *Tilletia indica* by extracts of native plants from Sonora, Mexico. *Rev. Mex. Fitopatol.* 19:214–217.

CAVALLI, J. F., TOMI, F., BERNARDINI, A. F., and CASANOVA, J. 2004. Combined analysis of the essential oil of *Chenopodium ambrosioides* by GC, GC-MS and 13C-NMR spectroscopy: quantitative determination of ascaridole, a heat-sensitive compound. *Phytochem. Anal*. 15:275–279.

CHIASSON, H., BOSTANIAN, N. J., and VINCENT, C. 2004a. Acaricidal properties of a Chenopodium-based botanical. *J. Econ. Entomol.* 97:1373–1377.

CHIASSON, H., VINCENT, C., and BOSTANIAN, N. J. 2004b. Insecticidal properties of a *Chenopodium*-based botanical. *J. Econ. Entomol.* 97:1378–1383.

DHINGRA, O. D., and SINCLAIR, J. B. 1995. Basic methods in plant pathology. CRC, Boca Raton.

DUBEY, N. K., KISHORE, N., SRIVASTAVA, O. P., DIKSHIT, A., and SINGH, S. K. 1983. Fungitoxicity of some higher plants against *Rhizoctonia solani*. *Plant Soil* 72:91–94.

DUKE, S. O., BAERSON, S. R., DAYAN, F. E., RIMANDO, A. M., SCHEFFLER, B. E., TELLEZ, M. R., WEDGE, D. E., SCHRADER, K. K., AKEY, D. H., ARTHUR, F. H., DE LUCCA, A. J., GIBSON, D. M., HARRISON Jr, H. F., PETERSON, J. K., GEALY, D. R., TWORKOSKI, T., WILSON, C. L., and BRAD MORRIS, J. 2003. United States Department of Agriculture-Agricultural Research Service research on natural products for pest management. *Pest Manag. Sci.* 59:708–717.

FERHOUT, H., BOHATIER, J., GUILLOT, J., and CHALCHAT, J. C. 1999. Antifungal activity of selected essential oils, cinnamaldehyde and carvacrol against *Malassezia furfur* and *Candida albicans*. *J. Essent. Oil Res.* 11:119–129.

GUENTHER, E. 1952. The essential oils, vol VI. Van Nostrand, New York.

GUPTA, D., CHARLES, R., MEHTA, V. K., GARG, S. N., and KUMAR, S. 2002. Chemical examination of the essential oil of *Chenopodium ambrosioides* L. from the southern hills of India. *J. Essent. Oil Res.* 14:93–94.

HUANG, X. F., and KONG, L. Y. 2002. Study on chemical constituents of volatile oil from *Chenopodium ambrosioides* L. *J. Chin. Pharm. Univ.* 33:256–257.

JESUS FARIA, T., FERREIRA, R. S., YASSUMOTO, L., SOUZA, J. R. P., ISHIKAWA, N. K., and MELO BARBOSA, A. 2006. Antifungal activity of essential oil isolated from *Ocimum gratissimum* L. (eugenol chemotype) against phytopathogenic fungi. *Braz. Arch. Biol. Technol.* 49:867–871.

KASALI, A. A., EKUNDAYO, O., PAUL, C., KONIG, W. A., ESHILOKUN, A. O., and IGE, B. 2006. 1,2,3,4-Diepoxy-p-menthane and 1,4-epoxy-p-menth-2-ene: Rare monoterpenoids from the essential oil of *Chenopodium ambrosioides* L. var *ambrosioides* leaves. *J. Essent. Oil Res.* 18:13–15.

KISHORE, N., CHANSOURIA, J. P. N., and DUBEY, N. K. 1996. Antidermatophytic action of the essential oil of *Chenopodium ambrosioides* and an ointment prepared from it. *Phytother. Res.* 10:453–455.

LALL, N., and MEYER, J. J. M. 1999. In vitro inhibition of drug-resistant and drug-sensitive strains of *Mycobacterium tuberculosis* by ethnobotanically selected South African plants. *J. Ethnopharmacol.* 66:347–354.

MALIK, M. M., and MUJTABA-NAQVI, S. H. 1984. Screening of some indigenous plants as repellants or antifeedants for stored grain insects. *J. Stored Prod. Res.* 20:41–44.

MARI, M., and GUIZZARDI, M. 1998. The postharvest phase: Emerging technologies for the control of fungal diseases. *Phytoparasitica* 26:59–66.

MUHAYIMANA UHAYIMANA, A., CHALCHAT, J. C., and GARRY, R. P. 1998. Chemical composition of essential oils of *Chenopodium ambrosioides* L. from Rwanda. *J. Essent. Oil Res.* 10:690–692.

NOUMI, E., and YOMI, A. 2001. Medicinal plants used for intestinal diseases in Mbalmayo Region, Central Province, Cameroon. *Fitoterapia* 72:246–254.

ONOCHA, P. A., EKUNDAYO, O., ERAMO, T., and LAAKSO, I. 1999. Essential oil constituents of *Chenopodium ambrosioides* L. leaves from Nigeria. *J. Essent. Oil Res.* 11:220–222.

PINO, J. A., MARBOT, R., and REAL, I. M. 2003. Essential Oil of *Chenopodium ambrosioides* L. from Cuba. *J. Essent. Oil Res.* 15:213–214.

POLLACK, Y., SEGAL, R., and GOLENSER, J. 1990. The effect of ascaridole on the in vitro development of *Plasmodium falciparum*. *Parasitol. Res.* 76:570–572.

PRADEEP, A. G., LOKESH, S., and RAI, V. R. 2003. Efficacy of some essential oils on seed mycoflora and seedling quality of some crop species saved by farmers. *Adv. Plant Sci.* 16:53–58.

RAHALISON, L., HAMBURGER, M., HOSTETTMANN, K., MONOD, M., and FRENK, E. A. 1991. Bioautographic agar overlay method for the detection of antifungal compounds from higher plants. *Phytochem. Anal.* 2:199–203.

SABULAL, B., DAN, M., PRADEEP, N. S., VALSAMMA, R. K., and GEORGE, V. 2006. Composition and antimicrobial activity of essential oil from the fruits of *Amomum cannicarpum*. *Acta. Pharm.* 56:473–480.

SRIDHAR, S. R., RAJAGOPAL, R. V., RAJAVEL, R., MASILAMANI, S., and NARASIMHAN, S. 2003. Antifungal activity of some essential oils. *J. Agric. Food Chem.* 51:7596–7599.

SU, H. C. F. 1991. Toxicity and repellency of *Chenopodium* oil to four species of stored-product insects. *J. Entomol. Sci.* 26:178–182.

SZCZERBANIK, M., JOBLING, J., MORRIS, S., and HOLFORD, P. 2007. Essential oil vapours control some common postharvest fungal pathogens. *Aust. J. Exp. Agric.* 47:103–109.

TAPONDJOU, L. A., ADLER, C., BOUDA, H., and FONTEM, D. A. 2002. Efficacy of powder and essential oil from *Chenopodium ambrosioides* leaves as post-harvest grain protectants against six-stored product beetles. *J. Stored Prod. Res.* 38:395–402.

TRIPATHI, P., and DUBEY, N. K. 2004. Exploitation of natural products as an alternative strategy to control postharvest fungal rotting of fruit and vegetables. *Postharvest Biol. Technol.* 32:235–245.

WINK, M. 1993. Production and application of phytochemicals from agricultural perspective, pp. 171–213, in T. A. VAN BEEK, and H. BRETELER (eds.). Phytochemistry and Agriculture, Vol. 34. Clarendon, Oxford.

YANG, J. K., CHOI, M. S., SEO, W. T., RINKER, D. L., HAN, S. W., and CHEONG, G. W. 2007. Chemical composition and antimicrobial activity of *Chamaecyparis obtusa* leaf essential oil. *Fitoterapia* 78:149–152.

et al. 2007a, b), pollinators (Caissard et al. 2004), herbivores, viruses, bacteria, and fungi (Panizzi et al. 1993; Pasqua et al. 2002; Lahlou and Berrada 2003; Giordani et al. 2004). Plant terpenes may also be sensitive to abiotic factors such as ultraviolet B radiation (Zavala and Ravetta 2002), severe conditions of water stress (Delfine et al. 2005), high temperatures and light intensity (Flesh et al. 1992; Wassner and Ravetta 2005), and ozone (Kainulainen et al. 2000). The numerous factors affecting terpene production represent a significant obstacle for developing a model capable of predicting the quantities of plant secondary metabolites, such as terpenes, under differing environmental conditions. Hence, the construction of such models is still one of the most challenging issues related to plant secondary metabolism.

Numerous studies have covered the impact of climatic conditions on terpene content in the Mediterranean area, whereas only a few advances have been made in understanding the effect of other abiotic factors such as soil type on terpene accumulation within plant tissues. Studies focused on elucidating soil contributions to terpene accumulation in plants mostly have been performed by considering different fertilizing doses (Muzika et al. 1989; McCullough and Kulman 1991; Heyworth et al. 1998). Other studies have dealt with terpene content variation in different geographical areas (*Rosmarinus officinalis*—Diab et al. 2002; *Pinus halepensis*—Ghanmi et al. 2005), but little is known about the effect of the type of natural soil, such as calcareous and siliceous soils, on terpene production by plants.

The Mediterranean region and particularly the Provence-Alpes-Côtes-d'Azur (PACA) region (southern France) features predominantly calcareous and siliceous soils (also called alkaline and acid soils, respectively), which are geographically well separated. These soils could affect terpene production since they are known to differ in pH, which may affect nutrient availability. These soils also vary in temperature (Gorenflot 1998); species needing warmer and low acidity soils prefer calcareous soils (Gorenflot 1998).

A few studies, all performed in the Mediterranean area, have investigated terpene content of plants growing in calcareous and siliceous soils. These reported that species growing in these two soil types show: (i) qualitative differences in terpene production (Robles and Garzino 2000; Flamini et al. 2004), suggesting the existence of different chemotypes and (ii) quantitative differences (Robles and Garzino 1998), indicating that properties of these soils affect plant terpene production.

The main goals of the present study were: (i) to explore the chemical diversity of monoterpenes and sesquiterpenes stored in leaves of woody Mediterranean species, *P. halepensis*, *R. officinalis*, and *Cistus albidus*, on calcareous and siliceous soils. In contrast to isoprene (hemiterpene with five carbons), monoterpenes and sesquiterpenes may be stored in leaves before being volatilized into the atmosphere; (ii) to assess quantitative differences in terpene production among plants growing in calcareous and siliceous soils; and (iii) to investigate whether nitrogen (N) and extractable phosphorus (P_E) from these soils affect leaf terpene content. This was of particular interest since leaf terpene emissions vary when they grow in calcareous or siliceous soils owing to different nutrient concentrations in these soils (Ormeño et al. 2007c). In addition to leaf terpene content, mean annual radial growth of each species was calculated allowing us to verify any preference for calcareous soils.

Methods and Materials

Species, Sites, and Sampling Three Mediterranean species that prefer calcareous rather than siliceous soils were chosen: the evergreen tree *P. halepensis* Mill. (Pinales, Pinaceae), the sclerophyllous shrub *R. officinalis* L. (Lamiales, Lamiaceae), and the semideciduous shrub *C. albidus* L. (Malvales, Cistaceae). All are terpene-accumulating species that store terpenes in specialized structures. *C. albidus* and *R. officinalis* store terpenes in glands (Gulz et al. 1996; Bottega and Corsi 2000), while in *P. halepensis*, they are contained in resin or secretory ducts, typical of conifers species. These species store different terpene mixtures: *C. albidus* stores sesquiterpenes alone, *P. halepensis* needles contain both monoterpenes and sesquiterpenes, and leaves of *R. officinalis* mainly posses monoterpenes (Ormeño et al. 2007a).

This study took place in southeastern France, in PACA region, from 19 to 24 March 2004 and between 1100 and 1530 hours (solar time). All sites were placed in open garrigue habitats, since terpene content of the selected species vary quantitatively between closed and open habitats (with and without a canopy structure, respectively; Ormeño 2006). The study area consisted of three open calcareous sites (C1, C2, C3; 43°28′ N–5°26′ E; 43°29′ N–5°18′ E; 43°15′ N–5°37′ E, respectively) and three open siliceous sites (S1, S2, S3; 43° 13′ N–6°10′ E; 43°12′ N–6°9′ E; 43°30′ N–6°39′ E, respectively) per species. Since *R. officinalis* was absent from one siliceous site (S3), a supplementary siliceous site was chosen (S4; 43°20′ N–6°30′ E), where only this species was sampled. All sites were located at similar altitudes (between 150–300 m) with negligible slopes. Geographical limits between these soils are known to be clearly marked in the study area. Distance between calcareous and siliceous sites is approximately ~150–200 km but climatic conditions are similar. Thus, mean precipitation (±standard error (SE)) and mean temperatures (±SE) recorded for the last 5 years in March were 30.1±4.4 mm and 11.1±0.17°C for calcareous sites and 37.7±6.7 mm and 12.1±0.3°C for siliceous sites (Meteo France Data). During the sampling period, similar temperatures (mean temperature, 23.5°C), relative air humidity (<30%; psychrometer, Jules Richard), and horizontal

wind speed (<7 m s^{-1}; Wind Speed Meter, WSC, 888H, Huger®) were recorded, and no precipitation occurred. Moreover, during sampling hours, atmospheric O_3 concentrations were not significantly different (Student's test, P=0.35) in calcareous (hourly average=80.9 and maximum=100 ppb) and in siliceous sites (hourly average=90.5 and maximum=103 ppb; AIRMARAIX Data).

To study physical and chemical properties of soils from calcareous and siliceous sites, four 200 g soil samples from the A1 horizon were collected at each site and then analyzed in different laboratories (Laboratoire de Chimie et Environnement, Laboratoire de Chimie et Agriculture). Properties analyzed following standard methods (Table 1) were pH, total organic carbon (TOC), nitrogen, total and extractable phosphorus, Mg^{2+}, Ca^{2+}, K^{+}, and texture.

Sampling was carried out on mature individuals of similar age (see below mean annual radial growth measurement). Because light conditions (Flesh et al. 1992) or stress factors such as herbivores or pathogens (Keeling and Bohlmann 2006) may alter or induce terpene production, only sun exposed individuals that showed apparent healthy leaves and that were free of pests were sampled. Health of leaves was checked visually (Llusià and Peñuelas 2000; Peñuelas and Llusià 2002). Four individuals per site were studied. For each plant, a single branch containing current and mature leaves was used to extract terpenes.

Terpene Extraction and Analysis Sampled leaves collected per plant were stored in a portable refrigerator at +4°C until being stored at −20°C in the laboratory within a period not longer than 2 h. Prior to terpene extraction, leaves were lyophilized for 48 h in a lyophilizer/freeze drier (Liovac-GT-2E Sterys®), in order to express results as leaf dry mass (DM; in sampled leaves, water content was on average 50% of total leaf mass). Lyophilization did not induce terpene losses (Ormeño et al. 2007a). Leaves were mechanically ground with a grinder to obtain powder. As previously tested in the laboratory, this mechanical procedure is preferred over the manual procedure often reported, in which a pestle is used to grind leaves in a chilled mortar. The latter procedure results in leaf terpene concentrations that are on average 37±0.6% lower in comparison to those obtained if the mechanical grinding technique is employed. This probably occurs because finer powder leads to a high contact between the leaf surface and the solvent. The extraction method consisted of dissolving 1 g of leaf DM in cyclohexane, for 20 min, under constant shaking at room temperature (Ormeño et al. 2007c). Well-filled, tightly closed glass vials wrapped in aluminum foil were used to avoid exposure to light and oxygen (Guenther 1949). A nonisoprenoid volatile internal standard (Undecane) was added to quantify measurements. Extracts were immediately analyzed or stored at −20°C until analysis (within 24–72 h).

Analyses were performed with a gas chromatography (Hewlett Packard GC6890®) coupled to a mass selective detector (MSD; HP 5973N). The system is described in Ormeño et al. (2007a). Briefly, the system was fitted with a dual column. One of them, the HP-5MS capillary column, in constant flow mode, was connected directly to MSD for qualitative analysis. The other, HP5 capillary column, was connected directly to the flame ionization detector for quantitative analyses. Sampled volumes (2 μl) were injected

Table 1 Soil properties of calcareous and siliceous soils

Soil properties	Calcareous soils			Siliceous soils				Mean of the calcareous sites	Mean of the siliceous sites
	Cal1	Cal2	Cal3	Sil1	Sil2	Sil3	Sil4		
pH (H_2O)	7.22 (0.05)	7.47 (0.05)	7.32 (0.11)	5.82 (0.17)	6.02 (0.22)	6.19 (0.20)	5.66 (0.56)	**7.34 (0.09)**	5.92 (0.15)
TOC (%)	5.91 (0.78)^{b}	3.09 (0.17)^{a}	6.15 (0.44)^{b}	3.92 (0.57)	3.27 (0.41)	2.01 (0.48)	2.95 (0.35)	**5.05 (0.50)**	3.04 (0.27)
N (%)	1.02 (0.28)^{b}	0.36 (0.04)^{a}	0.84 (0.15)^{b}	0.24 (0.04)^{bc}	0.21 (0.01)^{ab}	0.09 (0.02)^{a}	0.29 (0.03)^{c}	**0.74 (0.13)**	0.21 (0.02)
C/N	6.39 (1.02)	8.72 (0.55)	12.77 (4.11)	16.25 (1.36)^{b}	15.37 (1.45)^{ab}	20.78 (1.50)^{b}	10.12 (1.05)^{a}	**9.30 (1.51)**	15.63 (1.11)
P (g kg^{-1})	1.19 (0.13)^{b}	0.71 (0.06)^{a}	1.49 (0.12)^{b}	0.82 (0.04)^{b}	0.85 (0.02)^{b}	0.19 (0.02)^{a}	1.23 (0.09)^{c}	**1.129 (0.11)**	0.78 (0.04)
P_E (mg kg^{-1})	48.50 (4.92)	33.25 (4.11)	52.25 (9.94)	30.00 (0.14)	29.50 (2.36)	28.00 (1.91)	44.00 (8.13)	**44.67 (4.34)**	32.87 (2.58)
Ca^{2+}(g kg^{-1})	10.13 (0.74)^{b}	7.61 (0.41)^{a}	10.00 (0.72)^{b}	1.66 (0.19)	1.35 (0.13)	1.27 (0.09)	1.49 (0.45)	**9.33 (0.46)**	1442.80 (86.70)
Mg^{2+}(g kg^{-1})	0.10 (0.04)	0.12 (0.02)	0.09 (0.04)	0.14 (0.021)^{b}	0.02 (0.004)^{a}	0.11 (0.005)^{b}	0.13 (0.017)^{b}	106.02 (21.30)	101.00 (13.74)
K^{+}(g kg^{-1})	0.54 (0.07)	0.54 (0.03)	0.69 (0.07)	0.28 (0.04)^{a}	0.18 (0.02)^{a}	0.29 (0.03)^{a}	0.48 (0.03)^{b}	**590.20 (39.80)**	309.70 (31.20)
Texture	Loam	Silt loam	Silt loam	Loam	Loam	Sandy loam	Sandy loam	Loam–silt loam	Loam–sandy loam

Values are mean±SE (in parentheses). N=4 per site and N=12 if all calcareous and siliceous sites are lumped together, respectively. Intracalcareous and intrasiliceous differences tested through post hoc Tukey test are denoted by different letters (a<b<c). Letters are not shown when no significant differences were detected. Significant intersoil differences (P<0.05) tested through Student's test, appear in bold type
TOC Total organic carbon, *N* nitrogen, *P* total phosphorus, P_E extractable phosphorus

through an automatic injector (ALS 7683). Helium (99.995%) was used as carrier gas. The oven temperature was initially set at 50°C and then increased to 160°C at a rate of 2°C min^{-1}. It then remained constant for 5 min.

Identity of most leaf stored terpenes was established by comparison of the retention time and the mass spectrum of detected compounds with those of authentic reference samples (Aldrich–Firmenich). Terpene identity was further confirmed by comparing the experimental Kovats index of each compound with that of bibliography (Jennings and Shibamoto 1980; Adams 1989; Table 2). When terpene standards were not available (shyobunone, γ-cadinene, and α-eudesmol), their identity was tentatively achieved using this second approach. To determine terpene concentrations, the integrated area of each peak was multiplied by the appropriate response factor and divided by the sample volume. Response factors were determined from high-concentration standards.

Mean Annual Radial Growth Measurements Mean annual radial growth (mm $year^{-1}$) was measured by dividing the radius of the main stem at the collar level of each sampled plant by their age. For shrub species, age and radius were calculated at the main branch of each plant, which was cut off in order to collect cross sections (N=4, for each species). Meticulous sanding of cross-sections was necessary in order to evidence ring limits, since ring boundaries were difficult to detect. For each cross-section, rings were counted along two radii. The radius of a shrub plant was measured with a vernier calliper (graduated from 0–17 cm, 2/100 mm precision). For *P. halepensis*, age and radius were calculated from cores collected as low as possible. Age was calculated by following classical dendrometric methods (Schweingruber 1988). Mean age was calculated by counting rings on cross-sections of cores with pith.

Data Analyses Statistical analyses of variance by one-way analysis of variance (ANOVA), followed by post hoc Tukey tests, were used to detect differences in: (i) leaf terpene content of each species; and (ii) soil properties, within calcareous and siliceous sites (intracalcareous and intrasiliceous differences). Before applying the ANOVA test, both normality and homocedasticity of data were checked. Then, Student's t test was applied to test differences in: (i) foliar terpene content; (ii) soil properties; (iii) plant age; and (iv) plant annual radial growth, between calcareous and siliceous sites (intersoil differences). Quantitative differences in leaf terpene content between calcareous and siliceous soils were tested separately: (i) total monoterpenes and sesquiterpenes, and (ii) each major and minor compound. Main compounds were considered as major or minor when they contributed to at least 15% or 3% of total foliar terpene content, respectively. To study whether soil nutrients selected (N and P_E) were correlated to terpene content, linear and nonlinear regression analysis were performed. All results presented throughout the text represent the mean±SE. Statistical analyses were carried out with Statistical Graphics Plus®, version 4.1.

Results and Discussion

Calcareous and Siliceous Soil Properties Significant heterogeneity was found between soils of the same type (ANOVA, $P<0.05$, Table 1). For calcareous soils: (1) Cal2 appeared to be the poorest soil in terms of TOC, N, P, and Ca^{2+} (Table 1, Tukey test) and (2) Cal1 showed a loam texture, such as some siliceous soils (Sil1, Sil2). For siliceous soils: (1) S4 had the highest N and P concentrations, and (2) Sil2 contained the lowest Mg^{2+} and K^+ concentrations.

The analyses of intersoil differences indicated that pH and most nutrients concentrations (N, total and P_E, Ca^{2+}, and K^+) except Mg^{2+}, were significantly greater in calcareous than in siliceous soils ($P<0.05$, Student's t test, Table 1). Calcareous soils consisted of loamy and silty loam textures, whereas siliceous soils were loamy and sandy loam. However, some calcareous and siliceous soils showed similar textures as described above (Table 1). These results suggest that these calcareous and siliceous soils differ mainly in their pH and nutrient status and to a lesser degree in textures.

The low overall fertility found for siliceous soils has been described in other studies performed in the Mediterranean area (Canadell and Vila 1992; Castells and Peñuelas 2003). However, in high alkaline calcareous soils in the Mediterranean region (pH~8.5), lower concentrations of extractable phosphorus have been reported compared to siliceous soils (Sardans et al. 2004). This is attributed to excessive basic conditions that cause excessive calcium in soil solution that can precipitate with P, thus decreasing P availability (Haynes 1982). Differences in pH conditions may be due to the existence of different parent rocks. Consequently, some heterogeneity may appear between calcareous and siliceous soils.

Growth of Study Species in Calcareous and Siliceous Soils All study species showed similar ages in calcareous and siliceous soils (Student's test, $P>0.05$). Their mean age (years± SE) was 7.5±0.5 and 9.4±0.7 for *R. officinalis*, 44.0±3.7 and 55.1±3.3 for *P. halepensis*, and 6.0±0.7 and 6.4±1.2 for *C. albidus* in calcareous and siliceous soils, respectively.

Mean annual growth rate was higher in calcareous than in siliceous soils for all species (Student's t test, $P<0.05$). This was 0.73±0.03 mm $year^{-1}$ and 0.61±0.05 mm $year^{-1}$ for *R. officinalis*, 0.52±0.27 mm $year^{-1}$ and 0.38±0.13 mm $year^{-1}$ for *P. halepensis*, and 3.15±0.06 mm $year^{-1}$ and 2.0± 0.04 mm $year^{-1}$ for *C. albidus* in calcareous and siliceous soils, respectively. This was the expected result not only

Table 2 Terpene composition of leaves of *Rosmarinus officinalis*, *Pinus halepensis* and *Cistus albidus,* in calcareous (Cal) and siliceous (Sil) soils

		Pinus halepensis				*Rosmarinus officinalis*				*Cistus albidus*			
		Concentration (mg g_{DM}^{-1})		Concentration (%)		Concentration (mg g_{DM}^{-1})		Concentration (%)		Concentration (mg g_{DM}^{-1})		Concentration (%)	
KI	Terpene Type	Cal	Sil	Cal	Sil	Cal	Sil	Cal	Sil	Cal	Sil	Cal	Sil
	MONOTERPENES												
937	α-thujene	0.16 (0.05)	0.05 (0.01)	1.7	0.6	X	X	X	X	X	X	X	X
941	α-pinene	1.75 (0.17)	1.49 (0.10)	18.0	17.7	**1.53 (0.23)**	**2.05 (0.25)**	14.2	21.4	X	X	X	X
955	Camphene	X	X	X	X	0.66 (0.10)	0.57 (0.08)	6.2	5.9	X	X	X	X
968	Sabinene	**0.57 (0.08)**	**0.26 (0.04)**	5.9	3.1	X	X	X	X	X	X	X	X
970	β-pinene	0.16 (0.02)	0.12 (0.01)	1.7	1.4	**0.42 (0.07)**	**0.28 (0.03)**	3.9	2.93	X	X	X	X
983	Myrcene	0.61 (0.20)	0.79 (0.23)	6.3	9.3	**0.80 (0.22)**	**0.37 (0.10)**	7.4	3.8	X	X	X	X
996	δ3-carene	**0.32 (0.05)**	**0.20 (0.04)**	3.5	2.4	X	X	X	X	X	X	X	X
998	α-phellandrene	X	X	X	X	tc	tc	0.9	0.25	X	X	X	X
1003	α-terpinene	tc	tc	0.3	0.1	0.07 (0.02)	tc	0.6	0.1	X	X	X	X
1012	*p*-cymene	X	X	X	X	0.17 (0.03)	0.11 (0.02)	1.6	1.1	X	X	X	X
1013	Limonene	0.13 (0.01)	0.10 (0.00)	1.4	1.2	**0.44 (0.06)**	**0.31 (0.05)**	4.1	3.2	X	X	X	X
1015	1,8-cineol	X	X	X	X	1.82 (0.32)	1.74 (0.24)	17.0	18.1	X	X	X	X
1023	*Trans*-β-ocimene	0.15 (0.02)	0.08 (0.01)	1.6	1.0	X	X	X	X	X	X	X	X
1041	γ-terpinene	tc	tc	0.6	0.3	tc	tc	0.4	0.6	X	X	X	X
1068	δ-terpinene	**0.62 (0.08)**	**0.32 (0.04)**	6.6	3.8	tc	tc	0.1	0.2	X	X	X	X
1081	Linalool	X	X	X	X	tc	tc	0.6	0.6	X	X	X	X
1121	Camphor	tc	tc	<0.1	<0.1	**3.18 (0.42)**	**2.23 (0.54)**	29.6	23.1	X	X	X	X
1144	Borneol	X	X	X	X	0.15 (0.03)	0.38 (0.08)	3.6	3.99	X	X	X	X
1159	4-terpinol	X	X	X	X	tc	tc	0.3	0.3	X	X	X	X
1174	α-terpineol	X	X	X	X	0.14 (0.02)	0.15 (0.02)	1.3	1.6	X	X	X	X
1209	Verbenone	X	X	X	X	0.33 (0.04)	0.31 (0.06)	3.0	3.2	X	X	X	X
1274	Bornyl acetate	X	X	X	X	0.19 (0.04)	0.15 (0.02)	1.4	2.0	X	X	X	X
	SESQUITERPENES												
1345	α-cubebene	X	X	X	X	X	X	X	X	tc	X	0.3	X
1371	α-copaene	tc	tc	0.7	0.8	X	X	X	X	tc	tc	0.3	0.2
1382	β-bourbonene	X	X	X	X	X	X	X	X	0.030 (0.006)	0.043 (0.004)	4.1	3.2
1389	β-cubebene	X	X	< 0.1	< 0.1	X	X	X	X	0.022 (0.009)	< 0.1	2.9	< 0.1
1415	β-caryophyllene	4.05 (0.23)	3.85 (0.15)	41.2	45.7	**0.27 (0.06)**	**0.55 (0.10)**	2.5	5.7	0.037 (0.009)	0.046 (0.019)	5.0	7.7
1415	α-gurjunene	X	X	X	X	X	X	X	X	tc	tc	0.9	0.6
1450	α-humulene	0.71 (0.04)	0.68 (0.03)	7.2	8.1	0.03 (0.01)	0.10 (0.03)	0.25	1.0	tc	tc	0.5	0.5
1459	Alloaromadendrene	X	X	X	X	X	X	X	X	**0.053 (0.011)**	**0.125 (0.014)**	7.0	9.5
1483	Germacrene D	X	X	X	X	X	X	X	X	0.073 (0.015)	0.093 (0.009)	9.9	7.8
1485	Ar-curcumene	X	X	X	X	X	X	X	X	0.060 (0.011)	0.092 (0.011)	8.3	5.5
1492	Shyobunone[a]	X	X	X	X	X	X	X	X	0.036 (0.007)	0.045 (0.007)	4.9	4.9
1497	α-zingiberene	X	X	X	X	X	X	X	X	0.118 (0.020)	0.098 (0.001)	15.1	10.7
1501	α-muurolene	0.05 (0.01)	0.10 (0.04)	0.5	1.3	X	X	X	X	tc	tc	0.2	0.4
1513	γ-cadinene[a]	X	X	X	X	X	X	X	X	0.064 (0.012)	0.078 (0.014)	8.8	8.5

Table 2 (continued)

		Pinus halepensis			*Rosmarinus officinalis*			*Cistus albidus*			
		Concentration (mg g_{DM}^{-1})		Concentration (%)	Concentration (mg g_{DM}^{-1})		Concentration (%)	Concentration (mg g_{DM}^{-1})		Concentration (%)	
KI	Terpene Type	Cal	Sil	Cal	Cal	Sil	Cal	Cal	Sil	Cal	Sil
1513	Unknown1 (shyobunol?)	X	X	X	X	X	X	0.049 (0.013)	0.065 (0.008)	6.7	7.1
1523	δ-cadinene	tc	tc	0.1	X	X	X	**0.060 (0.011)**	**0.105 (0.009)**	8.3	8.9
1580	Caryophyllene oxide	0.11 (0.00)	0.12 (0.01)	1.0	0.08 (0.00)	0.07 (0.01)	0.7	0.010 (0.003)	0.020 (0.010)	1.4	2.9
1600	Guaiol	0.14 (0.01)	0.10 (0.01)	1.5	X	X	X	X	X	X	X
1608	Unknown2	X	X	X	X	X	X	0.017 (0.003)	0.022 (0.005)	2.4	2.4
1649	α-eudesmol[a]	X	X	X	X	X	X	0.015 (0.003)	0.020 (0.003)	2.0	1.9
1685	α-bisabolol	X	X	X	X	X	X	0.082 (0.017)	0.102 (0.017)	10.4	11.1
1688	Unknown3	X	X	X	X	X	X	0.061 (0.016)	0.055 (0.009)	7.1	6.0

Values are mean±SE (in parentheses; n=12). Significant statistical differences (P<0.05) through Student's test are applied to concentrations expressed in mg g_{DM}^{-1} and are denoted with bold type. Statistical differences have only been tested with regards to major compounds.
X Non detected compound (concentration lower than the detection limit, 0.085 ng), *tc* trace concentrations (lower than 0.28 ng), *KI* Kovats index
[a] Tentatively identified compound

because calcareous soils showed a higher level of fertility but also because the naturally occurring selected species prefer calcareous soils (*C. albidus*—Passama 1970; *R. officinalis*, *P. halepensis*—Trabaud 2001).

Terpene Content Diversity of Study Species in Calcareous and Siliceous Soils All species appeared to contain numerous terpenes in leaves. For *P. halepensis*, 20 terpenes were identified (three of them in trace amounts; Table 2). Most of them were monoterpenes, but similar concentrations of sesquiterpenes were found (4.05±0.37 and 5.03±0.25 mg g_{DM}^{-1}, respectively; Fig. 1). β-caryophyllene and α-pinene were the most abundant compounds in both types of soils. β-caryophyllene represented 41±2% and 46±2% of total content for plants growing in calcareous and siliceous soils, respectively, and α-pinene accounted for 18±2% of total content in both types of soils (Table 2). Terpene constituents in needles of *P. halepensis* vary in other Mediterranean areas: Algeria, β-caryophyllene>α-humulene (Dob et al. 2005) and Spain, α-pinene>myrcene (Llusià and Peñuelas 2000), indicating that, in our study, *P. halepensis* showed a different chemotype. Important concentrations (higher than 0.25 mg g_{DM}^{-1}) of α-humulene, myrcene, δ-terpinene, sabinene, and δ3-carene also were found in needles of *P. halepensis* (Table 2). These minor terpenes contributed to 29±5% and 27±5% of total content for plants growing in calcareous and siliceous soils, respectively.

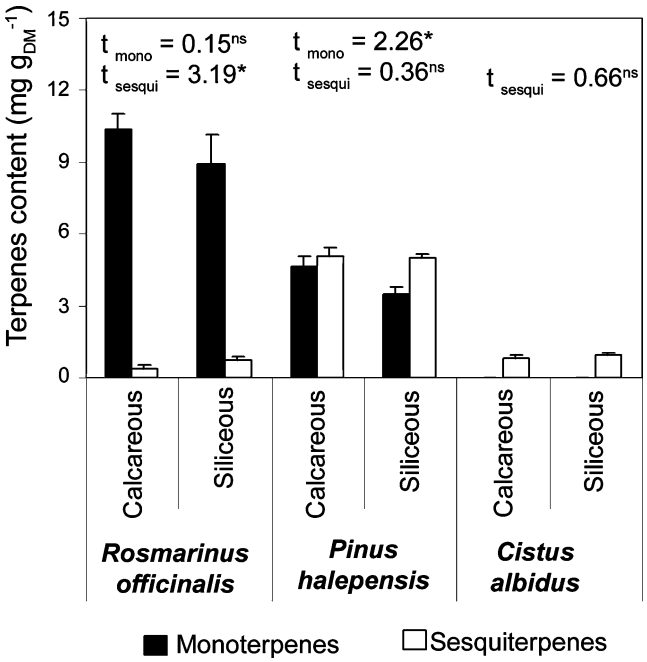

Fig. 1 Mean monoterpene and sesquiterpene concentration in leaves of *R. officinalis*, *P. halepensis*, and *C. albidus* in calcareous and siliceous soils (*bars* indicate SE; N=12). Statistical differences through Student's t test are also shown. t_{mono}, t_{sesqui}—Student's test value for monoterpene and sesquiterpene content, respectively. *P< 0.05; **P<0.01. *ns* Not significant at 95% of confidence

Twenty-one terpenes (six of them in trace concentrations) were identified in leaves of *R. officinalis* (Table 2). Most of them belonged to the monoterpene group. Mean monoterpene concentration was 9.64±0.93 mg g_{DM}^{-1} for both calcareous and siliceous soils. The major compound in leaves of *R. officinalis* was camphor, followed by α-pinene and eucalyptol (Table 2). These compounds showed similar relative amounts (percentage in respect to total content) in calcareous and siliceous soils. Camphor represented 30±5% and 23±6%, α-pinene accounted for 14±1% and 21±3%, and eucalyptol constituted 17±2% and 18±2% of total content in plants from calcareous and siliceous sites, respectively. These findings contrast with those of Peñuelas and Llusià (1997), where α-pinene was the major compound. This difference is probably due to *R. officinalis*, which generally exhibits numerous chemotypes, such as camphor, α-pinene, or eucalyptol chemotypes (Elamrani et al. 2000). High concentrations of myrcene, camphene, limonene, β-pinene, borneol, and verbenone were also extracted. These compounds represented 32±4% of total content in *R. officinalis*, for both calcareous and siliceous soils, and are, thus, minor major compounds. *R. officinalis* contained low concentrations of sesquiterpenes (0.55±0.12 mg g_{DM}^{-1}), which were mostly represented by β-caryophyllene (Table 2).

For *C. albidus* leaves, 21 terpenes were identified (five of them in trace concentration; Table 2). All of them were sesquiterpenes. Leaf terpene concentration (0.85±0.12 mg g_{DM}^{-1}) was tenfold lower than that of *P. halepensis* and *R. officinalis*. α-Zingiberene was the main compound (Table 2), in agreement with results of Robles and Garzino (1998), but unlike Llusià and Peñuelas (2000) who did not report this compound in extracts of this species. This compound represented 15% and 10% of total content in plants growing in calcareous and siliceous soils, respectively. Alloaromadendrene, D-germacrene, ar-curcumene, δ-cadinene, γ-cadinene, β-caryophyllene, and α-bisabolol were present in leaves in similar concentrations (<0.1 mg g_{DM}^{-1} both soils; Table 2). These minor compounds represented 49±8% and 50±7% of total content in calcareous and siliceous soils, respectively.

No qualitative differences in terpene diversity were observed between soil types and major compounds appeared in similar percentages in species located in both soils, indicating that sampled plants did not belong to different chemotypes; different chemotypes are recognized by the presence of distinct secondary metabolites, such as terpenes, and their relative concentrations (e.g., *P. halepensis*, Petrakis et al. 2000; *R. officinalis*, Lahlou and Berrada 2003).

These findings provide evidence that terpene diversity is species specific as expected since terpene mixtures are mainly genetically defined (Loreto 2002). Moreover, the terpene diversity (also called the terpene mixture) of all species studied appeared to be remarkable. Why plants allocate resources for synthesizing a wide variety of terpenes, is a question often debated by ecologists. The screening hypoth-

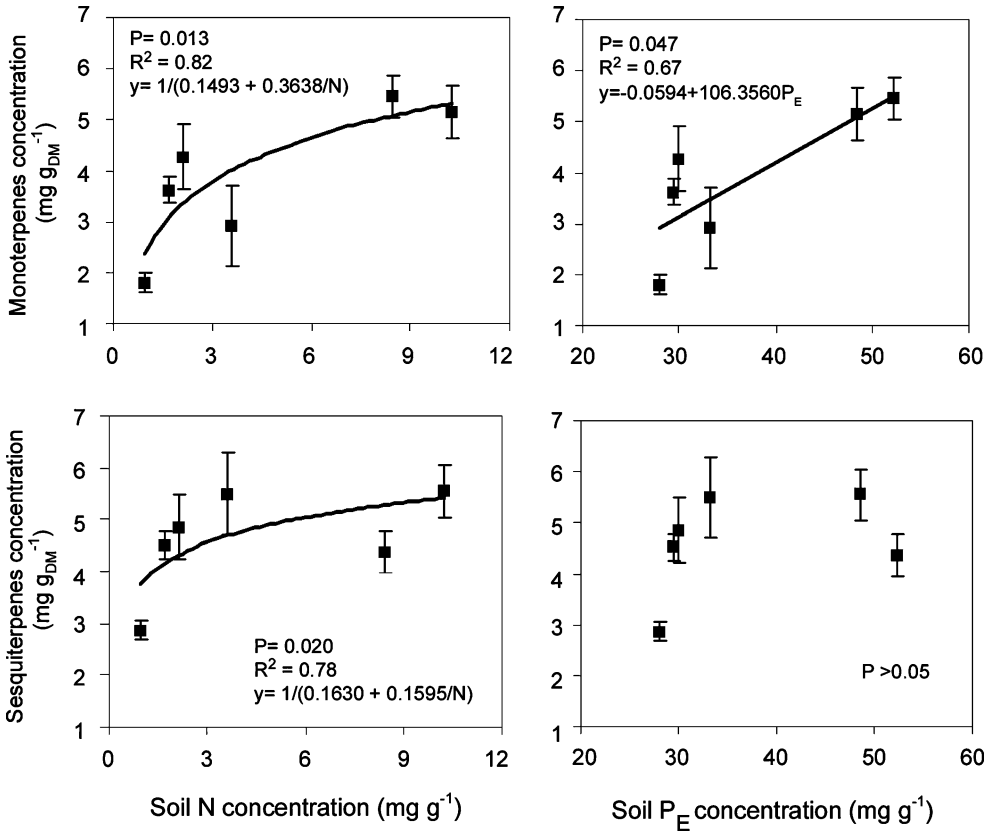

Fig. 2 Relationship between monoterpene and sesquiterpene concentration of *P. halepensis* and soil nitrogen (*N*; *left graphics*) and extractable phosphorus (P_E) concentration (*right graphics*). *Bars* shown are SE (*N*=4). Best fitting relationship is shown

esis formulated by Jones and Firn (1991) stresses that terpene mixtures would have ecological advantages over single substances, since most secondary metabolites do not have functions because their concentrations in plant tissues are too low. Hence, by synthesizing mixtures, the likelihood that some compounds are functional or active is improved, since the chemical diversity increases the chances of synthesizing an appropriate defensive product. This hypothesis is based "on a simple and single proposition: potent biological activity is a rare property for any molecule to possess" (Firn and Jones 2003).

Soil Type Effect on Terpene Content: Importance of Nutrients No previous study, except for *C. albidus*, has analyzed terpene content of the selected species in calcareous and siliceous soils (Robles and Garzino 1998). In that case, content was examined during the dormancy period. In the present study, it was tested when plants grew either in calcareous or siliceous soils and under nutrient differences (N and P_E).

For *P. halepensis*, total monoterpenes were higher when growing in calcareous soils (Student's *t* test, $P<0.05$; Fig. 1). Sabinene, δ3-carene, and δ-terpinene were more highly concentrated when *P. halepensis* grew in calcareous soils (Student's *t* test, $P<0.05$; Table 2). Total monoterpenes were significantly and positively correlated to both soil N and soil P_E ($P<0.05$; Fig. 2), indicating that high monoterpene content in calcareous soils is partly explained by the high N and P_E of these soils. This is consistent with previous findings (Ormeño et al. 2007c), where it was noted that higher concentrations of these nutrients in soil produce larger terpene emissions of *P. halepensis*. Unlike monoterpenes, total sesquiterpenes (i) were not significantly different when *P. halepensis* grew in the different soils (Student's *t* test, $P>0.05$; Fig. 1) and (ii) were not significantly correlated to soil P_E ($P>0.05$; Fig. 2). However, as for monoterpenes, overall sesquiterpene content was significantly and positively correlated to soil N concentration ($P<0.05$; Fig. 2).

P. halepensis showed high terpene content when growing in soils that contained high N and P_E presumably because increasing nutrient concentrations (mainly N) favor resin duct formation (Bjorkman et al. 1991; Kainulainen et al. 1996; Schindler et al. 1998), and in turn allow the storage of high terpene concentrations in needles of conifer species (e.g., *P. sylvestris* L., Bjorkman et al. 1998). In light of those results, it has been suggested that under an environment characterized by chronic increasing nitrogen deposition (Lu et al. 2007), *P. halepensis* might respond by allocating carbon resources to the synthesis of terpene metabolites, without detrimental effects on growth. Consequently, *P. halepensis* fitness might benefit from this scenario, since constitutive terpenes may act as defenses that allow plant species to cope with natural enemies (pathogens, virus, bacteria, fungi, herbivores; Rizvi and Rizvi 1992).

No consensus has been achieved to date regarding the way soil nutrients influence leaf terpene storage in woody species. Under varying nitrogen, terpene concentrations in mature needles of *P. sylvestris* have shown variable responses: enhancement (Kainulainen et al. 2000), reduction (Kainulainen et al. 1996), or no change (Heyworth et al. 1998). Leaf terpenes of *Eucalyptus* species are also variable: increase under nursery high fertilizer (Close et al. 2004) and remain invariable in field conditions (Close et al. 2004; King et al. 2004), when nutrients are supplied. Barnola and Cedeño (2000) showed high concentrations of most terpenes produced by *P. caribea* Morelet, in soils poor in P and N.

Total monoterpenes of *R. officinalis* were homogeneous within calcareous and siliceous soils (ANOVA, $P>0.05$; Table 3). They were not influenced by soil type (Student's *t* test, $P>0.05$; Fig. 1), although some major compounds were more highly concentrated in leaves growing in calcareous soils (β-pinene, myrcene, limonene and camphor, β-caryophyllene) or in siliceous soils (α-pinene; Student's *t* test, $P<0.05$; Table 2). In contrast, total sesquiterpene content (Fig. 1) was higher when *R. officinalis* grew in siliceous soils (Student's *t* test, $P<0.05$). The higher sesquiterpene concentration was due

Table 3 Terpene content of plants growing on calcareous and siliceous soils

		Calcareous sites				Siliceous sites			
Species		C1	C2	C3	*F*	S1	S2	S3/S4	*F*
Pinus halepensis	Monoterpenes	5.1 (0.5) ab	2.9 (0.8) a	5.4 (0.4) b	5.6*	4.3 (0.6) b	3.6 (0.3) ab	2.4 (0.2) a	5.1*
	Sesquiterpenes	5.5 (0.3)	5.5 (0.1)	4.4 (0.5)	2.1 ns	4.8 (0.3)	4.5 (0.4)	5.5 (0.1)	3.3 ns
Rosmarinus Officinalis	Monoterpenes	9.9 (1.5)	10.3 (0.9)	10.9 (1.6)	0.13 ns	6.7 (1.6)	10.6 (2.5)	9.3 (2.1)	0.8 ns
	Sesquiterpenes	0.4 (0.1) ab	0.1 (0.0) a	0.7 (0.2) b	7.65*	0.5 (0.2)	0.9 (0.3)	0.8 (0.1)	0.5 ns
Cistus albidus	Sesquiterpenes	0.7 (0.2)	0.4 (0.1)	1.3 (0.2)	3.8 ns	1.2 (0.0)	0.7 (0.1)	0.7 (0.2)	5.9 ns

Values are mean ($mg_{DM} \cdot g^{-1}$)±SE ($N=4$). Intrasoil differences are tested through ANOVAs analysis (*F*—ANOVA value). Results of the comparison are given by letters: values that differ at the 0.05 level are noted with different letters (a<b; i.e., ab=a, ab=b; Tukey test)

*$P<0.05$

ns Non significant

mainly to β-caryophyllene, which had twofold higher concentrations in plants in siliceous soils (Table 2). These results emphasize that monoterpene and sesquiterpene compounds may respond similarly to soil type.

In the case of *C. albidus*, total sesquiterpene content was similar within calcareous and siliceous soils (ANOVA, $P>0.05$; Table 3) and did not vary significantly between calcareous (0.8 mg g_{DM}^{-1}) and siliceous soils (0.9 mg g_{DM}^{-1}; Student's t test, $P>0.05$; Fig. 1). However, alloaromadendrene and δ-cadinene had significantly higher concentrations in leaves when *C. albidus* grew in siliceous soils (Table 2). Together, they contributed 20% on average to the total content. In contrast to results reported here, Robles and Garzino (1998) found high amounts of many terpenes (e.g., alloaromadendrene, β-caryophyllene) in plants growing in calcareous soils and high amounts of some other terpenes (e.g., β-bourbonene) in siliceous soils.

Contrary to *P. halepensis*, terpene content of *R. officinalis* and *C. albidus* showed no significant relationship with either N or P_E ($P>0.05$, $-0.46<r<0.54$), indicating that soil nutrients do not cause changes in terpene content of these species. This diverges from results reported by Ormeño et al. (2007c) who showed a positive effect of these nutrients on some terpene emissions of *R. officinalis* and a negative effect of the same nutrients on some terpene emissions of *C. albidus*. This disparity suggests that terpene content and emissions may respond differently to environmental changes as demonstrated previously by Ormeño et al. (2007a) for both *R. officinalis* and *C. albidus* and by Peñuelas and Llusià (1997) for *R. officinalis*.

It was earlier argued that differing results for *R. officinalis* and *C. albidus* could be due to the fact that calcareous and siliceous soils derive from different parent materials. Possibly, stands located in these soils show different adaptive differentiation (Canadell and Vila 1992) that lead to differences in secondary metabolism.

It was suggested also that leaf content of some terpenes of *C. albidus* and *R. officinalis* increased in siliceous soils because plants have been submitted to a greater stress in those soils. In the study area, the preference for calcareous soils is accentuated in the case of *C. albidus* and in *R. officinalis* to a lesser degree (Albert and Jahandiez 1985), compared to *P. halepensis*. This hypothesis is supported by previous results reported for *Quercus coccifera* L. by Ormeño et al. (2007a) under controlled conditions and during the same season. In that study, terpenes synthesized by potted seedlings of *Q. coccifera*, which is almost absent from siliceous soils in southern France, were strongly favored in siliceous soils. The stress generated in siliceous soils for species that prefer calcareous soils is probably due to soil acidity (Gorenflot 1998).

It is well known that plants produce a bouquet of volatile terpenes that mediate interactions between plants and other organisms (D'Alessandro and Turlings 2006), thus playing a central role in mediating interactions at different trophic levels, between plants and herbivores, between herbivores and their natural enemies, between plants and microorganisms, and also among plants themselves (Baldwin et al. 2002; Dicke et al. 2003). The chemical production of these compounds depends on both abiotic (humidity, temperature, light, soil nutrients) and biotic factors (plant cultivar, growth stage, and attacking pathogen and herbivore species; Vuorinen et al. 2007) and can provide information on plant physiological status and the stresses they are being subjected to (D'Alessandro and Turlings 2006).

Hence, the observed quantitative variation in terpene production of *R. officinalis* and *C. albidus* as a response to soil-induced stress might mediate interaction among plants and arthropods, leading to an ecological advantage. High leaf concentration of some terpenes when plants grow in siliceous soils might protect them against biotic attack and, thus, produce a fitness benefit. Thus, it would not simply account for a response to a soil-induced stress that entails extra synthesis costs for producing a high terpene concentrations without functional advantage. This could account for a strategy that allows species to grow in soils where their growth is not optimal.

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References

ADAMS, R. P. 1989. Identification of essential oils by ion trap mass spectroscopy. Harcourt Brace Jovanovivh, San Diego, USA.

ALBERT, A., JAHANDIEZ, E. 1985. Catalogue des plantes vasculaires du Var. Museum d'histoire naturelle de Toulon, Paris.

BALDWIN, I. T., KESSLER, A., and HALITSCHKE, R. 2002. Volatile signaling in plant–plant–herbivore interactions: what is real? *Curr. Opin. Plant Biol.* 5:351–354.

BARNOLA, L. F., and CEDEÑO, A. 2000. Inter-population differences in the essential oils of *Pinus caribaea* needles. *Biochem. Syst. Ecol.* 28:923–931.

BJORKMAN, C., LARSSON, S., and GREF, R. 1991. Effects of nitrogen-fertilization on pine needle chemistry and sawfly performance. *Oecologia* 86:202–209.

BJORKMAN, C., KYTO, M., LARSSON, S., and NIEMELA, P. 1998. Different responses of two carbon-based defences in Scots pine needles to nitrogen fertilization. *Ecoscience* 5:502–507.

BOTTEGA, S., and CORSI, G. 2000. Structure, secretion and possible functions of calyx glandular hairs of *Rosmarinus officinalis* L. (Lamiaceae). *Bot. J. Linn. Soc.* 132:325–335.

CAISSARD, J. C., MEEKIJJIRONENROJ, A., BAUDINO, S., and ANSTETT, M. C. 2004. Localization of production and emission of pollinator attractant on whole leaves of *Chamaerops humilis* (Arecaceae). *Am. J. Bot.* 91:1190–1199.

CANADELL, J., and VILA, M. 1992. Variation in tissue element concentrations in *Quercus ilex* L over a range of different soils. *Vegetatio* 100:273–282.

CASTELLS, E., and PEÑUELAS, J. 2003. Is there a feedback between N availability in siliceous and calcareous soils and *Cistus albidus* leaf chemical composition? *Oecologia* 136:183–192.

CLOSE, D. C., MCARTHUR, C., PIETRZYKOWSKI, E., FITZGERALD, H., and PATERSON, S. 2004. Evaluating effects of nursery and post-planting nutrient regimes on leaf chemistry and browsing of eucalypt seedlings in plantations. *For. Ecol. Manag.* 200:101–112.

D'ALESSANDRO, M., and TURLINGS, T. C. J. 2006. Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. *Analyst* 131:24–32.

DELFINE, S., LORETO, F., PINELLI, P., TOGNETTI, R., and ALVINO, A. 2005. Isoprenoids content and photosynthetic limitations in rosemary and spearmint plants under water stress. *Agr. Ecosyst. Environ.* 106:243–252.

DIAB, Y., AUEZOVA, L., CHEBIB, H., CHALCHAT, J. C., and FIGUEREDO, G. 2002. Chemical composition of Lebanese rosemary (*Rosmarinus officinalis* L.) essential oil as a function of the geographical region and the harvest time. *J. Essent. Oil. Res.* 14:449–452.

DICKE, M., VANPOECKE, R. M. P., and DEBOER, J. G. 2003. Inducible indirect defence of plants: from mechanisms to ecological functions. *Basic Appl. Ecol.* 4:27–42.

DOB, T., BERRAMDANE, T., and CHELGOUM, C. 2005. Chemical composition of essential oil of *Pinus halepensis* Miller growing in Algeria. *C. R. Chimie.* 8:1939–1945.

ELAMRANI, A., ZRIRA, S., BENJILALI, B., and BERRADA, M. 2000. A study of Moroccan rosemary oils. *J. Essent. Oil. Res.* 12:487–495.

FIRN, R. D., and JONES, C. G. 2003. Natural products—a simple model to explain chemical diversity. *Nat. Prod. Rep.* 20:382–391.

FLAMINI, G., CIONI, P. L., MORELLI, I., MACCIONI, S., and BALDINI, R. 2004. Phytochemical typologies in some populations of *Myrtus communis* L. on caprione promontory (East Liguria, Italy). *Food Chem.* 85:599–604.

FLESH, V., JACQUES, M., COSSON, L., TENG, B. P., PETIARD, V., and BALZ, J. P. 1992. Relative importance of growth and light level on terpene content of *Gingo biloba*. *Phytochemistry* 31:1941–1945.

GHANMI, M., ELABID, A., CHAOUCH, A., AAFI, A., ABERCHANE, M., ELALAMI, A., and FARAH, A. 2005. The yield and the chemical composition of turpentine of the maritime Pine (*Pinus pinaster*) and the Aleppo Pine (*Pinus halepensis*) of Morocco. *Acta Bot. Gallica* 152:3–10.

GIORDANI, R., REGLI, P., KALOUSTIAN, J., MIKAIL, C., ABOU, L., and PORTUGAL, H. 2004. Antifungal effect of various essential oils against *Candida albicans*. Potentiation of antifungal action of amphotericin B by essential oil from *Thymus vulgaris*. *Phytother. Res.* 18:990–995.

GORENFLOT, R. 1998. Biologie végétale. Plantes supérieures: appareil végétatif. Masson, Paris.

GUENTHER, E. 1949. The essential oil. Van Nostrand, New York.

GULZ, P. G., HERRMANN, T., and HANGST, K. 1996. Leaf trichomes in the genus *Cistus*. *Flora* 191:85–104.

HAMILTON, J. G., ZANGERL, A. R., DELUCIA, E. H., and BERENBAUM, M. R. 2001. The carbon–nutrient balance hypothesis: its rise and fall. *Ecol. Lett.* 4:86–95.

HAYNES, R. 1982. Effects of liming on phosphate availability in acid soils. *Plant Soil* 68:289–308.

HEMMING, J. D. C., and LINDROTH, R. L. 1999. Effects of light and nutrient availability on aspen: growth, phytochemistry and insect performance. *J. Chem. Ecol.* 25:1687–1714.

HEYWORTH, C. J., IASON, G. R., TEMPERTON, V., JARVIS, P. G., and DUNCAN, A. J. 1998. The effect of elevated CO_2 concentration and nutrient supply on carbon-based plant secondary metabolites in *Pinus sylvestris* L. *Oecologia* 115:344–350.

JENNINGS, W., and SHIBAMOTO, T. 1980. Quantitative analysis of flavour and fragrance volatiles by glass capillary gas chromatography. Academic, New York.

JONES, C. G., and FIRN, R. D. 1991. On the evolution of plant secondary chemical diversity. *Philos. Trans. R. Soc. B. Philos.* 333:273–280.

KAINULAINEN, P., HOLOPAINEN, J., PALOMAKI, V., and HOLOPAINEN, T. 1996. Effects of nitrogen fertilization on secondary chemistry and ectomycorrhizal state of Scots pine seedlings and on growth of grey pine aphid. *J. Chem. Ecol.* 22:617–636.

KAINULAINEN, P., UTRIAINEN, J., HOLOPAINEN, J. K., OKSANEN, J., and HOLOPAINEN, T. 2000. Influence of elevated ozone and limited nitrogen availability on conifer seedlings in an open-air fumigation system: effects on growth, nutrient content, mycorrhiza, needle ultrastructure, starch and secondary compounds. *Glob. Chang Biol.* 6:345–355.

KEELING, C. I., and BOHLMANN, J. 2006. Genes, enzymes and chemicals of terpenoid diversity in the constitutive and induced defence of conifers against insects and pathogens. *New Phytol.* 170:657–675.

KING, D. J., GLEADOW, R. M., and WOODROW, I. E. 2004. Terpene deployment in *Eucalyptus polybractea*: relationships with leaf structure, environmental stresses, and growth. *Funct. Plant Biol.* 31:451–460.

LAHLOU, M., and BERRADA, R. 2003. Composition and niticidal activity of essential oils of three chemotypes of *Rosmarinus officinalis* L. acclimatized in Morocco. *Flavour Fragr. J.* 18:124–127.

LLUSIÀ, J., and PEÑUELAS, J. 2000. Seasonal patterns of terpene content and emission from seven Mediterranean woody species in field conditions. *Am. J. Bot.* 87:133–140.

LORETO, F. 2002. Distribution of isoprenoid emitters in the *Quercus* genus around the world: chemo-taxonomical implications and evolutionary considerations based on the ecological function of the trait. *Perspect. Plant Ecol.* 5:185–192.

LU, C., TIAN, H., and HUANG, Y. 2007. Ecological effects of increased nitrogen deposition in terrestrial ecosystem. *J. Plant Ecol.* 31:205–218.

MCCULLOUGH, D. G., and KULMAN, H. M. 1991. Effects of nitrogen-fertilization on young jack pine (*Pinus-Banksiana*) and on its suitability as a host for jack pine budworm (*Choristoneura-Pinus-Pinus*) (Lepidoptera, Tortricidae). *Can. J. For. Res.* 21:1447–1458.

MUZIKA, R. M., PREGITZER, K. S., and HANOVER, J. W. 1989. Changes in terpene production following nitrogen-fertilization of grand fir (*Abies-Grandis* (Dougl) Lindl) seedlings. *Oecologia* 80:485–489.

ORMEÑO, E. 2006. Strategies d'émission de composés organiques volatils (COV) par quatre espèces végétales méditerranéenes. Ph.D. dissertation, Aix Marseille I, Marseille.

ORMEÑO, E., FERNANDEZ, C., and MÉVY, J. P. 2007a. Plant coexistence alters terpene emission and content of Mediterranean species. *Phytochemistry* 68:840–852.

ORMEÑO, E., BOUSQUET-MELOU, A., MÉVY, J. P., GREFF, S., ROBLES, C., BONIN, G., and FERNANDEZ, C. 2007b. Effect of intraspecific competition and substrate type on terpene emissions of some Mediterranean species. *J. Chem. Ecol.* 33:277–286.

ORMEÑO, E., FERNANDEZ, C., BOUSQUET-MELOU, A., GREFF, S., MORIN, E., ROBLES, C., VILA, B., and BONIN, G. 2007c. Monoterpene and sesquiterpene emissions of three Mediterranean species through calcareous and siliceous soils in natural conditions. *Atmos. Environ.* 41:629–639.

PANIZZI, L., FLAMINI, G., CIONI, P. L., and MORELLI, I. 1993. Composition and antimicrobial properties of essential oils of 4 Mediterranean Lamiaceae. *J. Ethnopharmacol.* 39:167–170.

PASQUA, G., MONACELLI, B., MANFREDINI, C., LORETO, F., and PEREZ, G. 2002. The role of isoprenoid accumulation and oxidation in sealing wounded needles of Mediterranean pines. *Plant Sci.* 163:355–359.

PASSAMA, L. 1970. Composition minérale de diverses espèces calcicoles et calcifuges de la région méditerranéene française. *Oecol. Plant.* 5:225–246.

PEÑUELAS, J., and LLUSIÀ, J. 1997. Effects of carbon dioxide, water supply, and seasonality on terpene content and emission by *Rosmarinus officinalis*. *J. Chem. Ecol.* 23:979–993.

PEÑUELAS, J., and LLUSIÀ, J. 2002. Linking photorespiration, monoterpenes and thermotolerance in *Quercus*. *New Phytol.* 155:227–237.

PETRAKIS, P. V., ROUSSIS, V., and ORTIZ, A. 2000. Monoterpenoid diversity in relation to morphology of *Pinus brutia* and *Pinus halepensis* in an east Mediterranean area (Attiki, Greece): implications for pine evolution. *Edinburgh. J. Bot.* 57:349–375.

RIZVI, S. J. H., and RIZVI, V. 1992. Allelopathy. Basic and applied aspects. Chapman & Hall, London.

ROBLES, C., and GARZINO, S. 1998. Essential oil composition of *Cistus albidus* leaves. *Phytochemistry* 48:1341–1345.

ROBLES, C., and GARZINO, S. 2000. Infraspecific variability in the essential oil composition of *Cistus monspeliensis* leaves. *Phytochemistry* 53:71–75.

SARDANS, J., RODA, F., and PENUELAS, J. 2004. Phosphorus limitation and competitive capacities of *Pinus halepensis* and *Quercus ilex* subsp rotundifolia on different soils. *Plant Ecol.* 174:305–317.

SCHINDLER, T., KOTZIAS, D., SPARTÀ, C., and VERSINO, B. 1998. Comparison of monoterpene volatilization and leaf oil composition of conifers. *Naturwissenschaften* 76:475–476.

SCHWEINGRUBER, F. H. 1988. Tree rings. basics and applications of dendrochronology. Reidel, Dordrecht.

SEIGLER, D. S. 1998. Plant secondary metabolism. Kluwer, Dordrecht.

TRABAUD, L. 2001. Relation entre les teneurs en bio-éléments chez divers végétaux méditerranéens. *Ann. For. Sci.* 58:555–567.

VUORINEN, L., NERG, A.-M., SYRJÄLÄ, L., PELTONEN, P., and HOLOPAINEN, J. K. 2007. *Epirrita autumnata* induced VOC emission of silver birch differ from emission induced by leaf fungal pathogen. *Arthropod-Plant Interactions* 1:3159–165.

WASSNER, D. E., and RAVETTA, D. A. 2005. Temperature effects on leaf properties, resin content, and composition in Grindelia chiloensis (Asteraceae). *Ind. Crop Prod.* 21:155–163.

ZAVALA, J. A., and RAVETTA, D. A. 2002. The effect of solar UV-B radiation on terpenes and biomass production in *Grindelia chiloensis* (Asteraceae), a woody perennial of Patagonia, Argentina. *Plant Ecol.* 161:185–191.

root elongation, reduces rates of photosynthesis, and inhibits nutrient uptake. At the cellular level, the allelochemical induces lipid peroxidation, affects certain enzymatic activities, and rapidly depolarizes the root cell membrane causing a generalized increase in membrane permeability, thus blocking plant nutrient uptake (Weir et al. 2004). At the same time, ferulic acid may be esterified with cell wall polysaccharides, incorporated into the lignin structure, or form bridges that connect lignin with wall polysaccharides, thus making cell walls rigid and restricting cell growth (Iiyama et al. 1990; Sánchez et al. 1996; Lam et al. 2001).

More recently, dos Santos et al. (2004) reported that ferulic acid reduction of soybean growth might be due to premature lignification of root tissues associated with increases in enzyme activities of the phenylpropanoid pathway, such as phenylalanine ammonia lyase (PAL) and POD. The aim of the present report was to investigate what the mode of action of ferulic acid is on the lignification process. For this, light and electron microscopy studies and determinations of CAD and POD activities, H_2O_2 level, and lignin content and composition were carried out after treatment of soybean roots with ferulic acid and inhibitors of the phenylpropanoid pathway.

Methods and Materials

General Procedures Soybean (*Glycine max* L. Merrill) seeds, surface-sterilized with 2% sodium hypochlorite for 5 min and rinsed extensively with deionized water, were dark-germinated (at 25°C) on two sheets of moistened filter paper. Twenty-five 3-d-old seedlings of uniform size were supported on an adjustable acrylic plate and transferred into a glass container (10×16 cm) filled with 200 ml of half-strength Hoagland's solution (pH 6.0), with or without 1.0 mM ferulic acid. Additional experiments with 0.1 mM of piperonylic acid (PIP) or 2.0 mM of 3,4-(methylenedioxy) cinnamic acid (MDCA) were made as indicated in the figure legends. The container was kept in a growth chamber (25°C, 12/12 hr L/D photoperiod, irradiance of 280 μmol m^{-2} s^{-1}). Roots were measured at the beginning and at the end of experiments (24 or 48 hr). When indicated, the fresh root weight was determined immediately after incubation, and the dry weight was estimated after oven-drying at 80°C until it reached a constant weight. CAD and POD activities and lignin contents were determined after the incubation period of 24 hr, while light and electron microscopy analyzes were carried out after 48 hr of incubation. Ferulic acid, PIP, and MDCA were purchased from Sigma-Aldrich Chemical Co (St. Louis, MO, USA), and all other reagents used were of the purest grade available or chromatographic grade.

Light Microscopy Studies Samples of material for morphological and anatomical studies were fixed in FAA 50 (Johansen 1940). The material was conserved in ethanol 70% (Jensen 1962). The anatomical description was made from the analysis of permanent slides obtained of longitudinal and transversal sections of the roots. In the preparation of permanent slides, plant material was embedded in glycol methacrylate according to the technique described by Gerrits (1991). These slides were stained with toluidine blue O (O'Brien et al. 1964) and mounted in Permount. Additionally, fresh transverse cross-sections of roots were immersed in a freshly prepared solution of phloroglucinol-HCl (Berlyn and Miksche 1976). Micrographs were photographed with an Olympus® photomicroscope. Scales were calculated using a decimal ruler and a micrometer under the same optical conditions used for each case.

Electron Microscopy Studies For scanning electron microscopy, fresh root segments were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2. Tissues were postfixed in a solution containing 1% osmium tetroxide, 0.8% potassium ferrocyanide, and 5 mM calcium chloride in 0.1 M cacodylate buffer, and then in a 1% tannic acid solution. Further, the samples were dehydrated in graded ethanol solutions, critical-point-dried in CO_2, sputter-coated with gold, and examined on a Jeol-JSM-5310® field emission scanning electron microscope. For transmission electron microscopy, fresh root segments were washed in 0.01 M phosphate-buffered saline and fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer. Tissues were postfixed in a solution containing 1% osmium tetroxide and 0.8% potassium ferrocyanide in 0.1 M cacodylate buffer, washed in the same buffer, dehydrated in acetone, and embedded in Spurr resin. Ultrathin sections obtained in a Reichert Ultracut E ultramicrotome were stained with uranyl acetate and lead citrate, and examined in a Zeiss EM900® transmission electron microscope.

Cinnamyl Alcohol Dehydrogenase Assay Cinnamyl alcohol dehydrogenase was extracted from fresh roots (2 g) with 3 ml of an extraction medium containing 40 mM of β-mercaptoethanol and 100 mM potassium phosphate buffer (pH 7.3). The homogenate was centrifuged at 2,200×*g* for 15 min, and the supernatant was used as enzyme preparation (dos Santos et al. 2006). CAD was assayed chromatographically by the reaction of reduction of sinapaldehyde to sinapyl alcohol. The assay was carried out, at 30°C, in 1.0 ml of reaction mixture containing 200 μl of crude enzyme preparation (≤0.35 mg of protein), 104 nmol NADPH, and 150 nmol Tris–HCl buffer (pH 8.0). At the start, 50 nmol of sinapaldehyde were added, and the reaction was stopped after 3 min of incubation by adding 50 μl of 5 N HCl. Parallel controls with sinapaldehyde added in the reaction mixture (without NADPH) were made. All samples were filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., NV, USA) and analyzed (20 μl) with a Shimadzu® Liquid Chromatograph (Tokyo,

Japan) equipped with an LC-10AD pump, a Rheodyne® injector, an SPD-10A UV detector, a CBM-101 Communications Bus Module, and a Class-CR10 workstation system. A reversed-phase Shimpack® CLC-ODS (M) column (150× 4.6 mm, 5 μm) was used at room temperature together with the same type of pre-column (10×4.6 mm). The mobile phase was methanol/acetic acid 4% in water (20:80, *v*/*v*) with a flow rate of 1.2 ml min^{-1} for an isocratic run of 20 min. Absorption was detected at 345 nm. Data collection and integration were performed with Class-CR10 software (Shimadzu®, Tokyo, Japan). Sinapyl alcohol was identified by comparing its retention time with standard values. CAD activity was expressed as nanomole sinapaldehyde consumed per minute per milligram protein. Protein was determined spectrophotometrically at 595 nm (Bradford 1976), with bovine serum albumin as a standard.

Peroxidase Assay Root (0.8 g) was homogenized in Eppendorf tubes with 0.12 ml of cold extraction medium containing 0.886 M sodium potassium buffer pH 7.0, 1.0 mM EDTA, 1.0 mM sodium metabisulfite, 9.96 mM sodium borate, 5% PVP-40 (polyvinylpyrrolidone), 0.5% β-mercaptoethanol, 10% glycerol, 2% ascorbic acid, and 4% polyethylene glycol (Pereira et al. 2001). The homogenates were centrifuged (21,900 ×*g*, 30 min, 4°C), and the supernatant was used as soluble POD extract. For cell wall-bound POD isolation, fresh roots (5 g) were macerated with 67 mM phosphate buffer (50 ml, pH 7.0) containing 0.5 g PVP (dos Santos et al. 2004). The extract was centrifuged (2,200 ×*g*, 5 min, 4°C). The pellet was washed with deionized water until no soluble POD activity was detected in the supernatant. The pellet was incubated in 10 ml of 1 M NaCl (prepared in 50 mM phosphate buffer, pH 7.0) for 1 hr. The homogenate was centrifuged (2,200 ×*g*, 5 min, 4°C) and the supernatant obtained. Cold acetone (30 ml) was slowly added in the supernatant under constant stirring. After centrifugation (10,000 ×*g*, 30 min, 4°C), the pellet was resuspended with 0.15 ml of 1 M NaCl and considered as cell wall-(ionically)-bound POD.

For polyacrylamide gel electrophoresis (PAGE), samples (50 μl) of the enzyme extract were applied in 12% gel prepared in 0.375 M Tris–HCl pH 8.8 buffer. Electrophoresis was performed for 5 hr at 200 V. The running buffer used was 0.1 M Tris–glycine pH 8.3 (Pereira et al. 2001). This gel was incubated at 37°C for 15 min with 50 ml of 1 M sodium citrate buffer, pH 4.7, adjusted with acetic acid, 50 ml of methanol, and 0.05 g of benzidine. Then, 5 ml of 30% H_2O_2 were added to the staining mixture and the preparation was maintained at 37°C until the time of isozyme detection (Mangolin et al. 1994).

H_2O_2 Quantification Fresh roots (2 g) were homogenized in 3 ml of 50 mM phosphate buffer, pH 6.8 (Hsu and Kao 2007). The homogenate was centrifuged at 2,200 ×*g* for 20 min. Further, 1.5 ml of extracted solution, mixed with 0.5 ml of 0.1% titanium chloride in 20% (*v*/*v*) H_2SO_4, was then centrifuged at 2,200 ×*g* for 15 min. Absorbance was measured at 410 nm, and H_2O_2 was quantified with a calibration curve of known standard concentrations. Whereas the blank consisted of a reaction mixture without tissue extract, its absorbance was subtracted from the mixture with H_2O_2 extract. Results were expressed as nanomole H_2O_2 per gram fresh weight.

Quantification of Lignin Content and Composition After the incubation period, dry roots (0.3 g) were homogenized in 50 mM potassium phosphate buffer (7 ml, pH 7.0) with a mortar and pestle and transferred into a centrifuge tube (Ferrarese et al. 2002). The pellet was centrifuged (1,400 ×*g*, 4 min) and washed by successive stirring and centrifugation as follows: twice with phosphate buffer pH 7.0 (7 ml); 3× with 1% (*v*/*v*) Triton® X-100 in pH 7.0 buffer (7 ml); 2×with 1 M NaCl in pH 7.0 buffer (7 ml); 2×with distilled water (7 ml); and 2×with acetone (5 ml). The pellet was dried in an oven (60°C, 24 hr) and cooled in a vacuum desiccator. The dry matter was defined as a protein-free cell wall fraction. Further, all dry protein-free tissue was placed into a screw-cap centrifuge tube containing the reaction mixture (1.2 ml of thioglycolic acid plus 6 ml of 2 M HCl) and heated (95°C, 4 hr). After cooling at room temperature, the sample was centrifuged (1,400 ×*g*, 5 min), and the supernatant was discarded. The pellet contained the complex lignin–thioglycolic acid (LTGA). The pellet was washed three times with distilled water (7 ml) and the LTGA extracted by shaking (30°C, 18 hr, 115 oscillations per minute) in 0.5 M NaOH (6 ml). After centrifugation (1,400 ×*g*, 5 min), the supernatant was stored. The pellet was washed again with 0.5 M NaOH (3 ml) and mixed with the supernatant obtained earlier. The combined alkali extracts were acidified with concentrated HCl (1.8 ml). After precipitation (0°C, 4 hr), LTGA was recovered by centrifugation (1,400 ×*g*, 5 min) and washed two times with distilled water (7 ml). The pellet was dried at 60°C, dissolved in 0.5 M NaOH, and diluted to yield an appropriate absorbance for spectrophotometric determination at 280 nm. Lignin was expressed as milligram LTGA per gram dry weight.

Alkaline cupric oxidation was used to determine lignin monomer composition (Chen and McClure 2000). Protein-free cell wall fraction (25 mg) was sealed in a Pyrex® ampule containing 1 ml of 2 M NaOH plus 0.2 g of CuO and heated to 170°C for 2 hr, while shaking the sample occasionally during the reaction. After oxidation, the sample was cooled at room temperature, acidified to pH 2 with 2 M HCl, and extracted twice with anhydrous ethyl ether. The organic extracts were combined, dried, and resuspended in methanol/acetic acid 4% in water (20:80, *v*/*v*). All samples were filtered through a 0.45-μm disposable syringe filter (Hamilton® Co., NV, USA) and analyzed by high-performance liquid chroma-

tography (HPLC), as described earlier. The mobile phase was methanol/acetic acid 4% in water (20:80, *v*/*v*), with a flow rate of 0.8 ml min^{-1} for an isocratic run of 20 min. Quantification of *p*-hydroxybenzaldehyde, vanillin, and syringaldehyde was performed at 290 nm by corresponding standards. Results were expressed as microgram monomer per milligram cell wall.

Additionally, cell wall-bound ferulic acid was extracted after alkaline hydrolysis (de Ascensao and Dubery 2003). Dry roots (0.2 g) were homogenized in 4 ml of 50% methanol with a mortar and pestle and transferred into a centrifuge tube, heated to 80°C for 1.5 hr. Supernatant was discarded after centrifugation (2,200 ×*g*, 10 min). The pellet was washed twice with 2 ml of 50% methanol and dried in an oven (60°C, 24 hr). Dry material (0.1 g) was resuspended in 10 ml of 0.5 M NaOH and heated to 96°C for 2 hr. The sample was acidified to pH 2 with HCl, centrifuged (2,200 ×*g*, 10 min), and the supernatant was extracted twice with 10 ml of anhydrous ethyl ether. The organic extracts were combined, dried, and resuspended in methanol/acetic acid 4% in water (30/70, *v*/*v*). All samples were filtered through a 0.45-µm disposable syringe filter (Hamilton® Co., NV, USA) and analyzed by HPLC, as described earlier. The mobile phase was methanol/water (30:70, *v*/*v*) with a flow rate of 0.8 ml min^{-1} for an isocratic run of 20 min. Cell wall-released ferulic acid was identified at 330 nm by co-injection with authentic standard.

Statistical Design The experimental design was completely randomized, and each plot was represented by one glass container with 25 seedlings. Data are expressed as the mean of three to five independent experiments ±S.E. The one-way variance analysis to test the significance of the observed differences was performed by Sisvar® package (Version 4.6, UFLA, Brazil). The difference among parameters was evaluated by the Scott–Knott test, and P values <0.05 were considered as statistically significant.

Results

Effects of Ferulic Acid on Root Growth As may be judged visually, differences in the roots were apparent (Fig. 1). Primary root elongation of treated seedlings was inhibited by the compound. Roots became brown, thicker, and less flexible. After 24 and 48 hr, the allelochemical prompted significant decreases in the root lengths when compared to the control conditions (Table 1).

Light and Electron Microscopy Analyses Subsequent experiments were carried out to evaluate anatomical changes in roots by light microscopy (Table 1). The effects of ferulic acid on diameter (root and vascular cylinder) measures of medial and basal sections were examined after root treatments. After 24 hr, the vascular cylinder diameters increased in both medial (40.9%) and basal (14.3%) sections in comparison to controls. Significant effects of ferulic acid were evident at 48 hr; the root diameter decreased (11.3%) while the vascular cylinder diameter increased (19%) in the medial section. Cross sections of the medial region in untreated roots showed a normal pattern of growth (Fig. 2A,C). Results of the two histochemical methods used to verify lignification gave results that agreed closely. When the ferulic acid-exposed roots (Fig. 2B) were subjected to phloroglucinol-HCl reagent, an intense coloration indicating lignified cell walls developed when compared to control (Fig. 2A). Moreover, the number of differentiated xylem elements formed was greater in ferulic acid-treated roots than in control roots. In ferulic acid-treated roots stained with toluidine blue O (Fig. 2D), the metaxylem also showed lignification when compared to control (Fig. 2C). In addition, longitudinal sections of the transition zone in treated roots indicated initiation of lateral roots and lignification of the primary xylem (Fig. 2E,F). Similar sections revealed a smaller distance between the quiescent center and the differentiated cells in ferulic acid-treated roots when compared to the untreated roots (Fig. 2G,H).

Transmission electron microscopy observations of ferulic acid-treated root caps showed a reduced number of starch granules in comparison to control (Fig. 3A,B). Micrographs of the quiescent center in treated roots revealed that the cells were smaller and compressed, and contained irregular and enlarged nucleoli and many lipid globules in comparison to the untreated roots (Fig. 3C,D). Finally, scanning electron microscopy photomicrographs showed disintegration of the root cap in the ferulic acid treated roots in comparison to control (Fig. 3E,F).

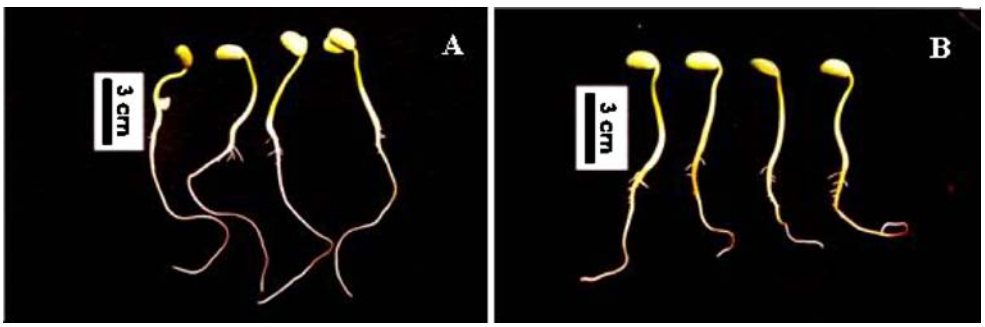

Fig. 1 Effects of ferulic acid on soybean root length. Control (A) and treated (B) roots with 1.0 mM ferulic acid after 48 hr

Table 1 Anatomical characteristics of ferulic acid treated soybean roots after 24 and 48 hr

			Medial region		Basal region	
		Root length (cm)	Root diameter (μm)	Cylinder diameter (μm)	Root diameter (μm)	Cylinder diameter (μm)
24 hr	C	3.3±0.21	5.8±0.37	2.2±0.07	7.7±0.50	2.8±0.10
	T	2.2±0.09[a]	7.5±0.59	3.1±0.06[a]	8.2±0.22	3.2±0.09*
	%	−33.3	ns	+40.9	ns	+14.3
48 hr	C	7.0±0.14	7.1±0.05	2.1±0.02	8.0±0.32	3.0±0.11
	T	2.7±0.16[a]	6.3±0.44[a]	2.5±0.07[a]	7.2±0.26	2.9±0.11
	%	−61.4	−11.3	+19.0	ns	ns

The symbol % represents inhibition (−) or activation (+) of statistically significant means after treatment in comparison to control.
C control, *T* treatment, *ns* not significant.
[a] Means (N=4±SE) significantly ($P \leq 0.05$) smaller than the control experiment (Scott–Knott's test)

Effects of Ferulic Acid on CAD and POD Activities and H_2O_2 Content Ferulic acid-affected CAD activity was significantly different from control (Fig. 4). The allelochemical decreased the enzymatic activity by 42.8% at 1.0 mM treatment. In addition to this earlier finding, electrophoretic patterns of POD isozymes were determined in soybean roots after allelochemical treatment (Fig. 5). PAGE zimograms of POD in ferulic acid treated roots revealed that only the anionic form PODa5 increased compared to the control condition. Moreover, the allelochemical decreased the H_2O_2 content 30% when compared to control (Fig. 6).

Effects of Ferulic Acid on Lignin Content and Composition Lignin content in soybean roots increased following 1.0 mM ferulic acid treatment by about 46% at 24 hr (Figs. 7 and 8). Figure 7 reveals that PIP, a potent quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H), reduced lignin content of soybean roots 37.6% compared to control. Similar to ferulic acid, treatment of roots with allelochemical plus PIP increased lignin content 26.5% compared to the control condition. Experiments with MDCA, a competitive inhibitor of 4-coumarate:CoA ligase (4CL), are seen in Fig. 8. Lignin content was not affected by MDCA, alone or jointly with ferulic acid (FA plus MDCA), in comparison with the control experiment. The analysis of alkaline cupric oxidation products (Fig. 9) revealed that lignin monomer content (*p*-hydroxybenzaldehyde+guaiacyl+syringyl; H+G+S) increased 3.8-fold compared to that in untreated roots. The allelochemical mainly increased the guaiacyl (G) monomer. When subjected to alkaline hydrolysis, cell walls of allelochemical-exposed roots released significant amount of ferulic acid identified chromatographically (Fig. 10).

Discussion

Root growth is characterized by high metabolic rates, and for this reason roots are highly susceptible to environmental stresses, such as allelochemicals in soils (Cruz-Ortega et al. 1998). The experimental conditions used in this work were chosen because the net uptake of ferulic acid by the root system is high (Shann and Blum 1987a), and lignification begins during the early stages of seedling growth (dos Santos et al. 2004; Passardi et al. 2005). At the final stages of xylem cell differentiation, lignin is deposited within the carbohydrate matrix of the cell wall by infilling of interlamellar voids and, at the same time, by the formation of chemical bonds with the non-cellulosic carbohydrates (Donaldson 2001). An important fact revealed in the present work is that the reduction of soybean root length (Table 1) by ferulic acid is associated with an increase in the lignin contents (Figs. 7 and 8).

Structural changes of root cells have been associated with root growth inhibition induced by stress (Cruz-Ortega et al. 1998). The increase in root diameter, especially of vessel cylinder, may be attributed to the early differentiation of vessel tissues verified by the lignification of metaxylem (Fig. 2D). As reported earlier, cell walls may be lignified when stressed or when they differentiate to xylem (Christensen et al. 1998). Anatomical observations here showed that the diameter of the vascular cylinder in the medial and basal regions was enhanced after 24 and 48 hr of treatments, compared with controls (Table 1). Ferulic acid-treated roots showed a greater number of differentiated xylem elements and lignification of cell walls (Fig. 2B), induction of lateral roots (Fig. 2F) with lignification of the metaxylem (Fig. 2D), and an increase of the central cylinder (Fig. 2D), all consistent with increased lignin production (Figs. 7 and 8). Furthermore, a smaller distance between the quiescent center and the first differentiated cells together with clustered cells in the longitudinal section of treated roots (Fig. 2H) indicate premature cessation of root growth. There is evidence to corroborate these results (Chon et al. 2002). For example, coumarin-treated alfalfa (*Medicago sativa*) roots revealed a significant increase in diameter due to an expanding of the vascular cylinder and cortex cell layers. Moreover, this allelochemical inhibited root elongation and cell division, indicating that the thickness of roots was enlarged due to inhibition of root longitudinal

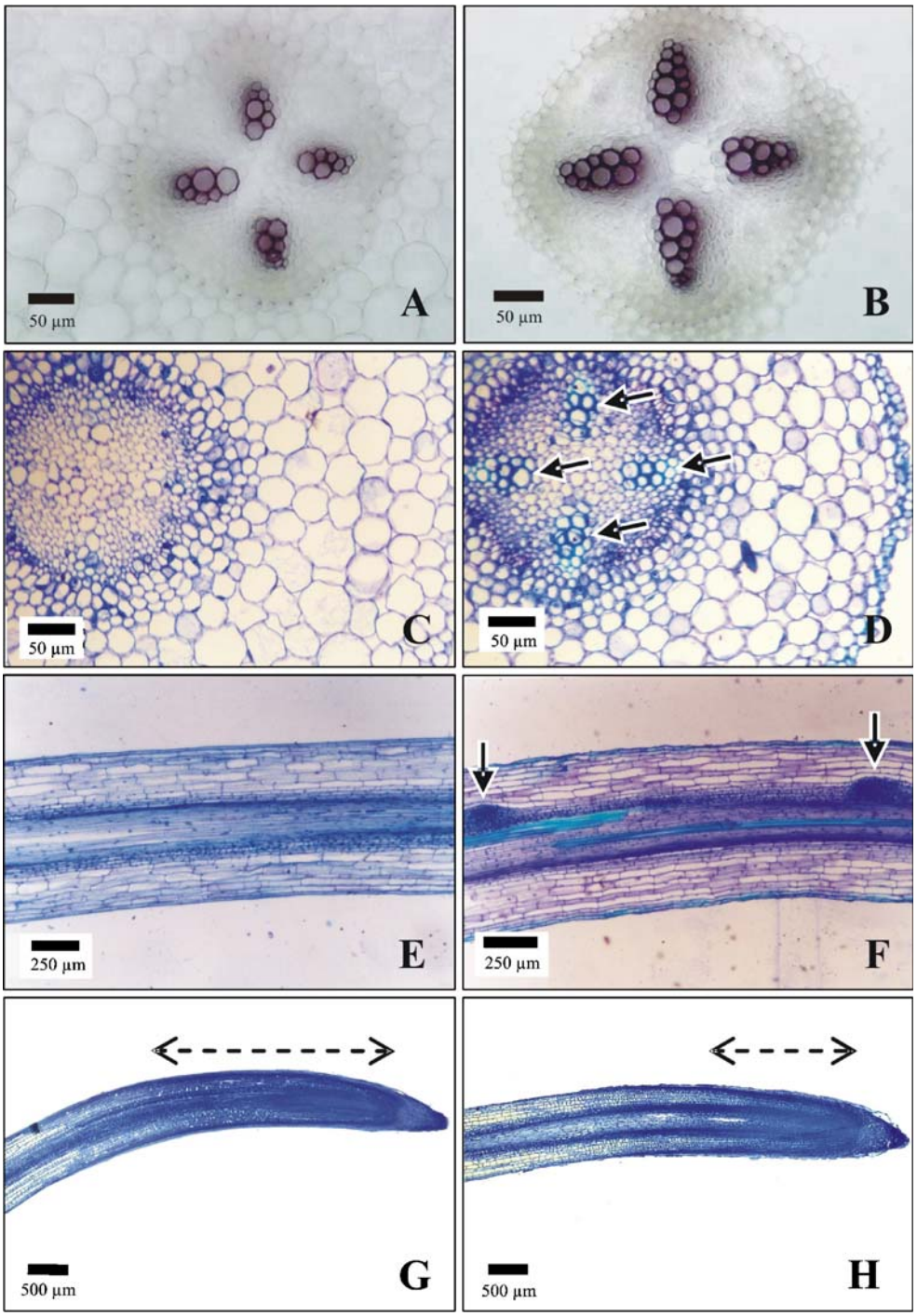

Fig. 2 Light microscopy photomicrographs of control (A, C, E, G) and treated (B, D, F, H) soybean roots with 1.0 mM ferulic acid for 48 hr. A and B: phloroglucinol-HCl staining method indicating lignified cell walls in cross sections of the medial zone. C and D: toluidine blue O staining method in cross sections of the medial zone (*arrows* indicate lignification of metaxylem). E and F: longitudinal sections of the central cylinder in the basal region (*arrows* indicate induction of lateral roots and lignification). G and H: longitudinal sections (*dotted lines* indicate the distance between the quiescent center and first differentiated cells)

growth. The water-soluble extract of alfalfa leaves, which contains mainly ferulic acid among other allelochemicals, showed similar effects (Chon et al. 2002).

At the ultrastructural level, treatment with ferulic acid adversely affected soybean root cells. Electron micrographs showed a reduction in the number of starch granules (Fig. 3B), an increase in lipid globules, and compression of cells with enlarged nucleoli (Fig. 3D) in ferulic acid treated roots. Disintegration of the root cap in treated roots (Fig. 3F) suggests death of cells after direct contact with the allelochemical. In simplest terms, ferulic acid inhibited the root growth and led to cellular ultrastructural abnormalities. Studies have shown that allelochemicals can affect the cellular structure of growing roots. White mustard (*Sinapis alba*) radicle treated with gramine and hordenine showed vacuolation, disorganization of organelles, and damage to cells walls (Liu and Lovett 1993). Root cell ultrastructure of cucumber exposed to 2-benzoxazolinone presented cytoplasmatic vacuolation and

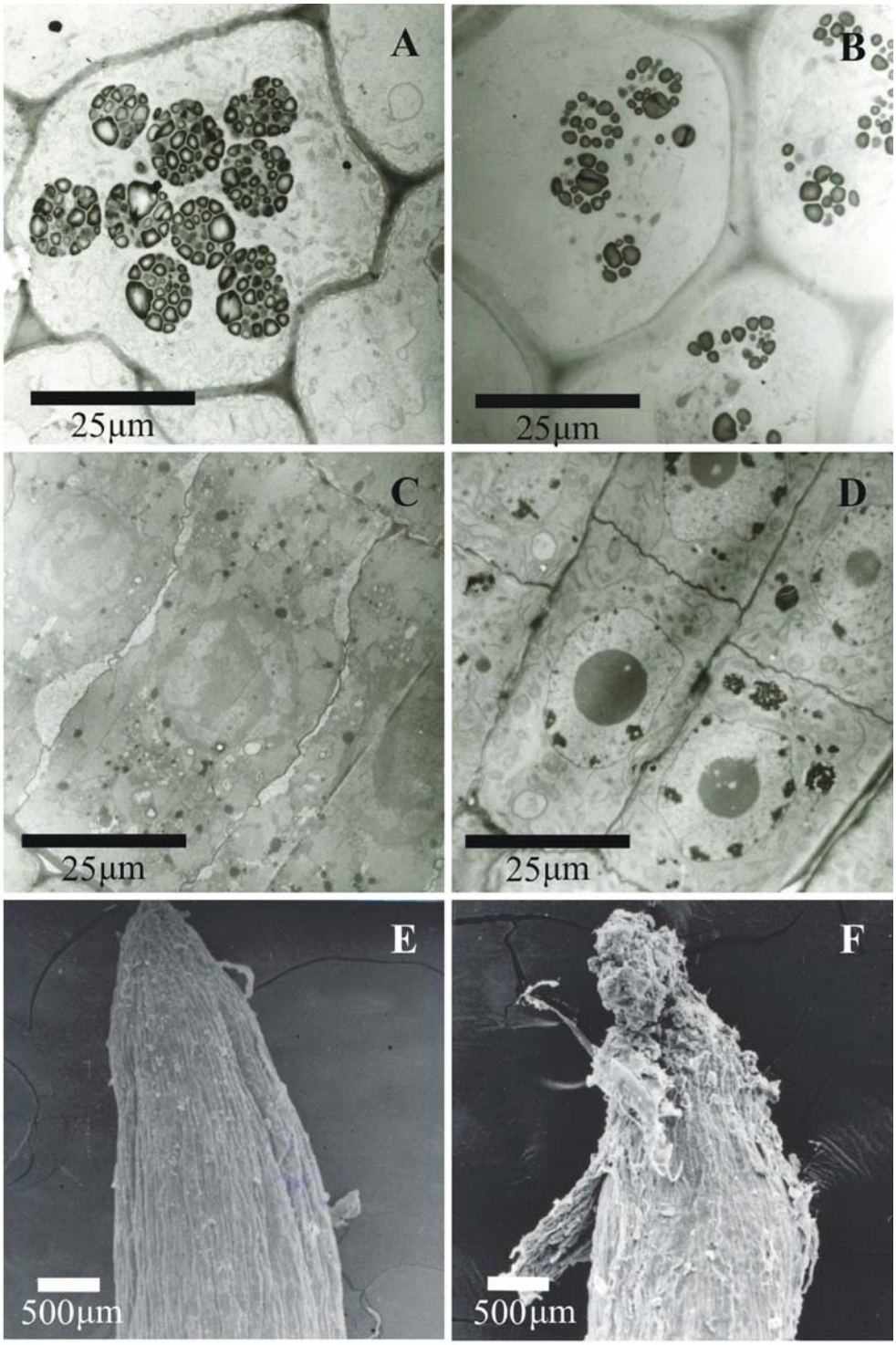

Fig. 3 Electron microscopy photomicrographs of control (A, C, E) and treated (B, D, F) soybean roots with 1.0 mM ferulic acid for 48 hr. A and B: transmission electron microscopy of root cap showing starch granules. C and D: transmission electron microscopy of quiescent center cells showing compression with enlarged nucleoli and lipid globules in treated roots. E and F: scanning electron microscopy of root cap showing epidermic tissue disintegrated in treated roots

reduced number of starch granules (Burgos et al. 2004). Mustard (*Brassica juncea*) seedlings treated with benzoic acid showed cellular disorganization (Kaur et al. 2005). According to these authors, inhibition of root growth was due to reduced lipid catabolism, protein synthesis, and adverse effects on cell division and mineral uptake. In agreement, ferulic acid inhibits protein synthesis, affects membrane permeability, and decreases uptake of nutrients (Politycka 1996; Baziramakenga et al. 1997). Moreover, it reduces lipid mobilization followed by accumulation of unsaturated fatty acids in germinating canola (*Brassica napus*) seeds (Baleroni et al. 2000). It also increases the contents of saturated and unsaturated fatty acids, xylose, fructose, and sucrose in soybean root (Ferrarese et al. 2001). Thus, the cellular structure changes reported here appear to be, at least partially, associated with changes in the lipid and carbohydrate metabolism (Ho 1988).

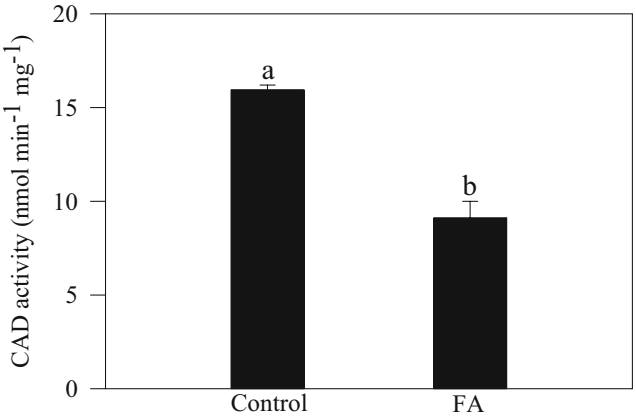

Fig. 4 Effects of ferulic acid (*FA*) on CAD activity in soybean roots. Mean±SE values (*N*=4) followed by the different letter are significantly different according to the Scott–Knott test ($P \leq 0.05$)

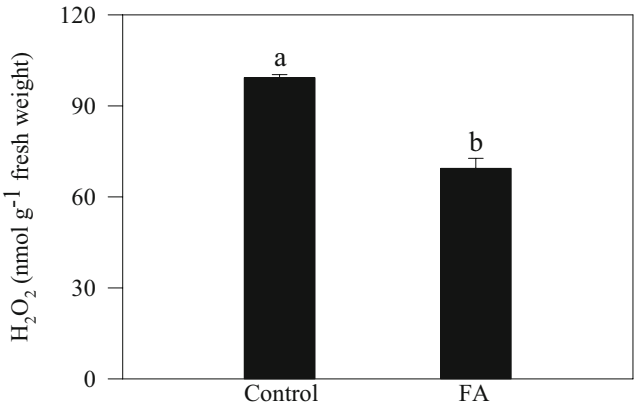

Fig. 6 Effects of ferulic acid (*FA*) on H_2O_2 content in soybean roots. Mean±SE values (*N*=3) followed by the different letter are significantly different according to the Scott–Knott test ($P \leq 0.05$)

There is evidence that the effects of ferulic acid on soybean are related to premature lignification of roots, since enzyme (PAL and POD) activities of the phenylpropanoid pathway increased jointly with lignin production (dos Santos et al. 2004). Direct incorporation of exogenous ferulic acid, independent of phenylpropanoid metabolism, was initially hypothesized. This possibility should not be discounted. The radiotracer [U-ring-^{14}C]ferulic acid] was found in residues of lignin isolated from cucumber seedlings treated with the allelochemical (Shann and Blum 1987b). To test the hypothesis that exogenous ferulic acid induces the lignification process, CAD activity was determined in treated soybean roots. Surprisingly, this showed that CAD activity decreases in ferulic acid treated roots (Fig. 4) despite lignin production (Figs. 7 and 8). Increase in CAD activity would be anticipated, since it is considered to be a lignification marker (Boerjan et al. 2003). Increased lignification under reduced CAD activity might, at least in principle, strengthen the hypothesis of direct incorporation of exogenous ferulic acid into lignin polymer. However, plants are able to circumvent the block in CAD activity by shipping substrates, the cinnamaldehydes, to the cell wall for polymerization (Boerjan et al. 2003). This indicates that the impact of CAD on lignin biosynthesis may not be critical. Cross-coupling of hydroxycinnamyl aldehydes into lignin compensates for the reduced availability of monolignols in CAD-deficient plants (Kim et al. 2000; Li et al. 2001).

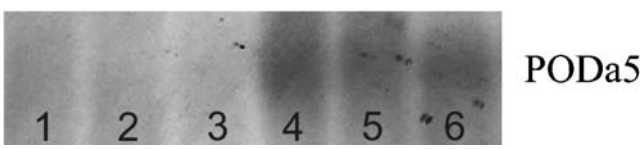

Fig. 5 PAGE isozyme patterns of POD in soybean roots. 4 to 6, ferulic acid-treated roots showing increase of the anionic isoform of soluble enzyme (*PODa5*) in comparison to control (*1* to *3*). POD activity was stained using H_2O_2 as the substrate

Ferulic acid increased the activity of anionic isoform PODa5 in treated roots (Fig. 5). Anionic isoperoxidases are often held to be those most directly involved in lignification of xylem cells (Wallace and Fry 1999; Passardi et al. 2005). POD is able to dehydrogenate monolignols, to induce lignification after addition of H_2O_2 in tissue sections, and to reveal specific colocalization of isoforms in lignifying tissues (Ros Barceló et al. 2004; Passardi et al. 2005). H_2O_2, produced by the pH-dependent POD and NADPH oxidase complex, is a necessary substrate for the cell wall's lignifying process catalyzed by POD, thus causing a rapid cross-linking of cell-wall polymers (Wojtaszek 1997). Ferulic acid exposure reduced the H_2O_2 content of roots (Fig. 6). It is feasible that these facts may, in part, explain the increase in anionic isoform PODa5 in treated roots (Fig. 5), consistent with increased lignin production (Figs. 7 and 8).

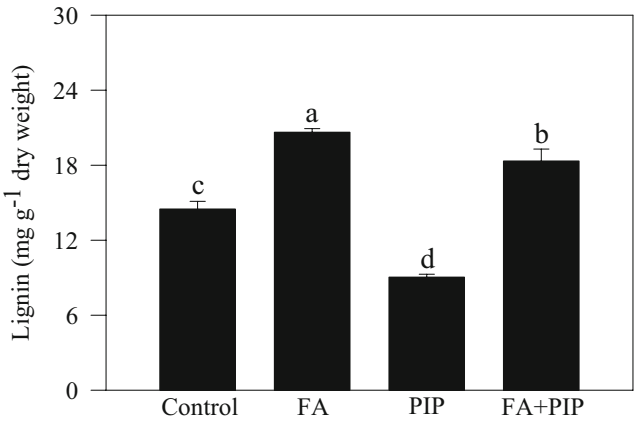

Fig. 7 Changes in the lignin contents of soybean roots untreated (*Control*) or treated with 1.0 mM ferulic acid (*FA*), 0.1 mM piperonylic acid (*PIP*) and 1.0 mM ferulic acid plus 0.1 mM piperonylic acid (*FA+PIP*) for 24 hr. Mean±SE valus (*N*=5) followed by different letters are significantly different according to the Scott–Knott test ($P<0.05$)

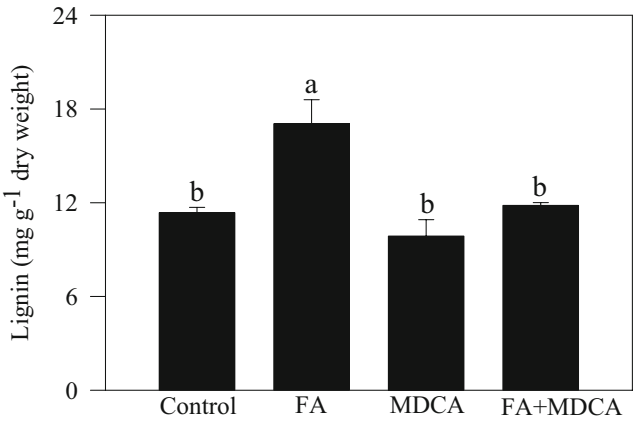

Fig. 8 Changes in the lignin contents of soybean roots untreated (*Control*) or treated with 1.0 mM ferulic acid (*FA*), 2.0 mM methylene dioxocinnamic acid (*MDCA*) and 1.0 mM ferulic acid plus 2.0 mM methylene dioxocinnamic acid (*FA+MDCA*) for 24 hr. Mean±SE values (N=5) followed by same letter are not significantly different according to the Scott–Knott test (P<0.05)

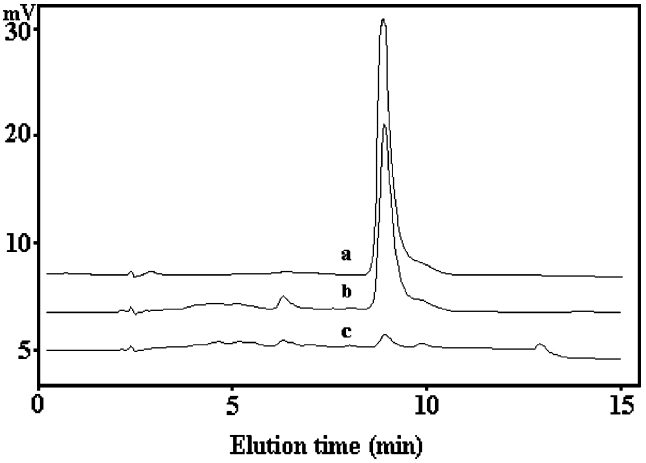

Fig. 10 HPLC elution profiles of ferulic acid standard (*a*, retention time=8.83 min), cell wall-released ferulic acid in the treated roots (*b*, retention time=8.87 min), and cell wall-released ferulic acid in the control roots (*c*, retention time=8.85 min)

Hamada et al. (2003) demonstrated that exogenously supplied ferulic acid was converted to feruloyl and then to coniferyl and sinapyl alcohols, in poplar (*Populus alba*) callus. Since feruloyl CoA is an intermediate of phenylpropanoid metabolism (Fig. 10), a possible entry of free ferulic acid into the pathway, by the 4CL reaction, must be considered. To elucidate, subsequent experiments were made by growing roots with two inhibitors of the pathway enzymes: PIP, a quasi-irreversible inhibitor of cinnamate 4-hydroxylase (C4H), and MDCA, a competitive inhibitor 4-coumarate:CoA ligase (4CL). Figure 7 shows that roots grown under ferulic acid treatment produce more lignin, while PIP-treated roots synthesize less lignin, compared to standard conditions. This is in agreement with the fact that PIP is an effective inhibitor of C4H, and acts before the entry point of ferulic acid in the pathway (Schoch et al. 2002). When applied jointly with PIP, ferulic acid prompted an increase in lignin content, suggesting its entrance into pathway by the 4CL reaction. To strengthen this assumption, roots were incubated with MDCA, an inhibitor of 4CL (Schoch et al. 2002). Lignin content did not change after MDCA or MDCA plus ferulic acid treatments, compared to controls (Fig. 8), thus indicating that the access of exogenous allelochemical has been blocked at this metabolic point.

Since exogenous ferulic acid may be incorporated into lignin structure by means of the phenylpropanoid pathway, changes in the lignin monomer content should not be discounted. In order to ascertain lignin composition, cell walls isolated from ferulic acid exposed roots were subjected to alkaline cupric oxidation. Results shown herein reveal a striking feature. The lignin monomer contents increased in treated roots (Fig. 9). Root lignin from ferulic acid exposed plants was mainly composed of guaiacyl (G) unit in a 18:72:10 (H/G/S) ratio, compared to control plants (40:50:10 ratio). It is well-known that in dicotyledonous angiosperms, lignin is derived from three monomer types; *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S) units, derived from *p*-coumaryl, coniferyl, and sinapyl alcohol, respectively (Boerjan et al. 2003). One of the important precursors of *p*-coumaryl alcohol in the phenylpropanoid pathway is ferulic acid (Fig. 11). Moreover, lignin polymerization is regulated by the cell through the supply of available monomers (Boerjan et al. 2003). In robinia (*Robinia pseudoacacia*), labeled ferulic acid was incorporated into guaiacyl (G) and syringyl (S) lignin, and these incorporations increased as cell-wall lignification proceeded (Yamauchi and Fukushima 2004). Based on results reported herein (Figs. 6, 7, 8, 9 and 10), it is

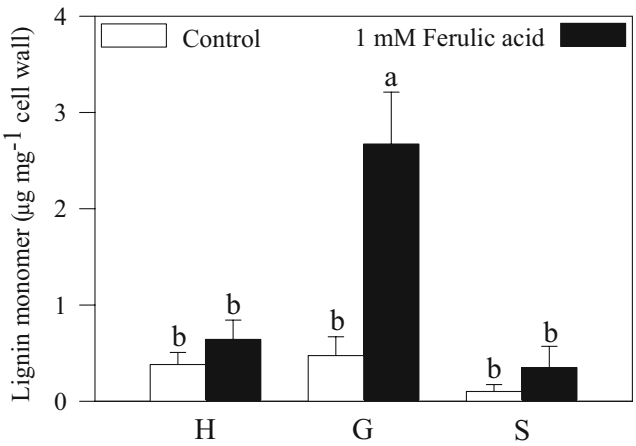

Fig. 9 Effects of ferulic acid on lignin monomer composition. *H*, *p*-hydroxybenzaldehyde; *G*, guaiacyl, and *S*, syringyl monomers. Mean± SE values (N=3) followed by the different letters are significantly different according to the Scott–Knott test (P≤0.05)

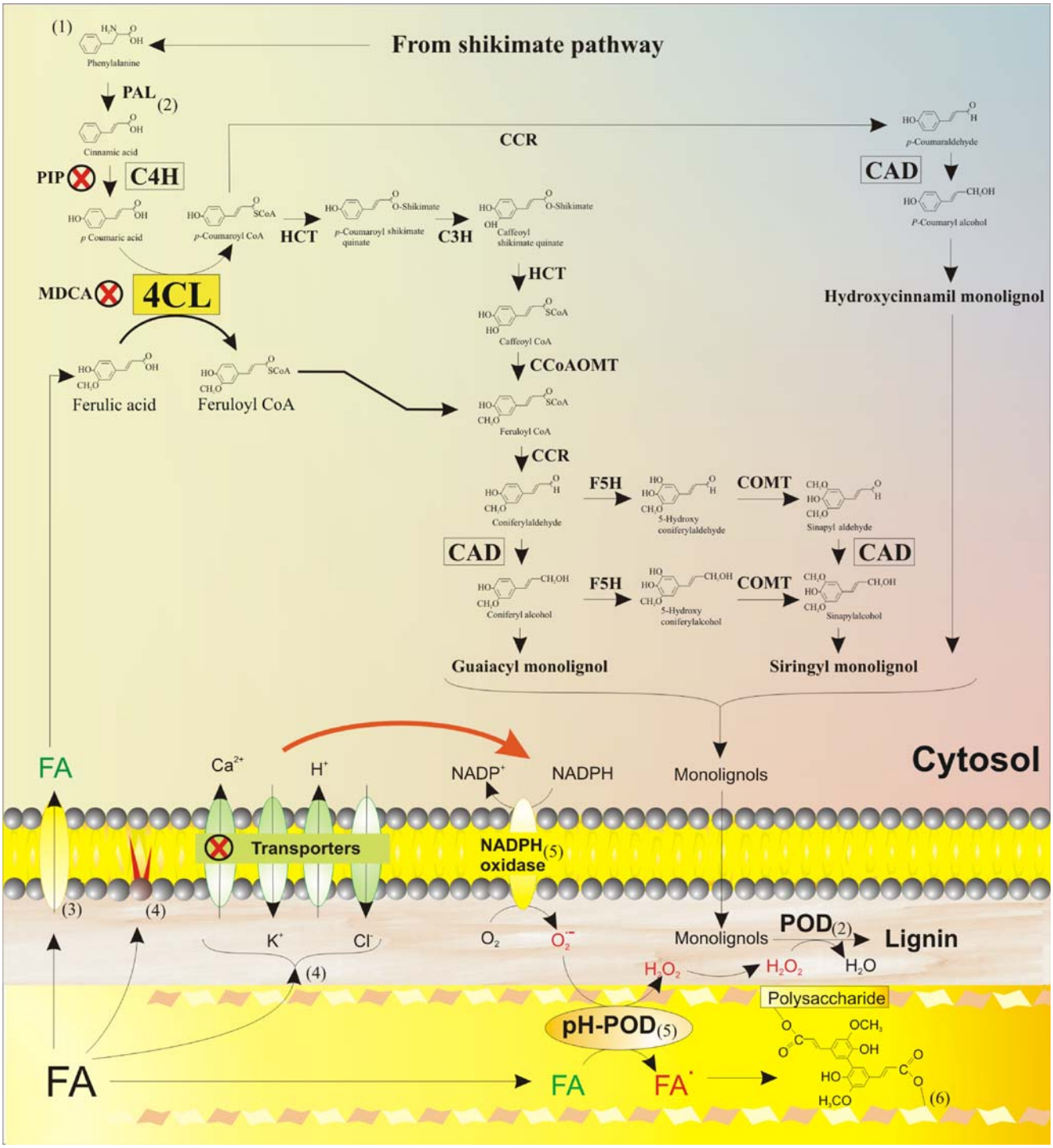

Fig. 11 Proposed mode of action for ferulic acid on lignification of soybean roots. *PAL* phenylalanine ammonia lyase, *C4H* cinnamate 4-hydroxylase, *4CL* 4-coumarate:CoA ligase, *HCT* hydroxycinnamoyl-CoA:quinate/shikimate hydroxycinnamoyltransferase, *C3H* *p*-coumarate 3-hydroxylase, *CCoAOMT* caffeoyl-CoA *O*-methyltransferase, *CCR* cinnamoyl-CoA reductase, *F5H* ferulate 5-hydroxylase, *COMT* caffeic acid/5-hydroxy ferulic acid *O*-methyltransferase, *CAD* cinnamyl alcohol dehydrogenase, *POD* peroxidase, *FA* ferulic acid, *PIP* piperonylic acid, *MDCA* 3,4-(methylenedioxy) cinnamic acid. *(1)* Chen et al. (2006), *(2)* dos Santos et al. (2004), *(3)* Shann and Blum (1987a), *(4)* Baziramakenga et al. (1995), *(5)* Wojtaszek (1997), *(6)* Boerjan et al. (2003)

plausible to suggest that exogenous ferulic acid was channeled into the phenylpropanoid pathway, and later on, caused an increase in lignin. Consistent with these findings, cell walls of allelochemical-exposed roots released significant amount of ferulic acid (Fig. 10).

The focus of the present work was to investigate how the mode of action of ferulic acid is related to the lignification process. Light and electron microscopy studies, combined with biochemical assays, suggest a possible mechanism of soybean response to ferulic acid (Fig. 10). Exogenously applied ferulic acid induces premature cessation of soybean root growth, with disintegration of the root cap, and cellular modifications, such as compression of cells in the quiescent center, early lignification of the metaxylem and cell wall, and increase of the vascular cylinder diameter. At the metabolic level, ferulic acid is channeled into the phenylpropanoid pathway and converted to feruloyl CoA by the 4CL reaction. Catalyzed by subsequent enzymatic reactions, feruloyl CoA is then converted to coniferal- and sinapaldehydes. As an endwise enzyme of the pathway, CAD might not be a limiting step. So, either these metabolites are converted into the respective alcohols by CAD or, eventually circumvent the inhibited CAD reaction (Kim et al. 2000; Li et al. 2001; Boerjan et al. 2003) by polymerizing with lignin in the cell wall. Lignin polymerization requires a sufficient supply of H_2O_2, which is produced by the pH-dependent POD and NADPH oxidase complex, after changes in the membrane permeability (Baziramakenga et al. 1995; Wojtaszek 1997). Whether or not this is the actual process, it seems plausible to assume that ferulic acid induced inhibition in root growth of soybean may be due to excessive production of monolignol from exogenously applied ferulic acid. Monolignol polymerization forms a complex network that solidifies the plant cell wall and restricts plant growth.

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References

Baleroni, C. R. S., Ferrarese, M. L. L., Braccini, A. L., Scapim, C. A., and Ferrarese-Filho, O. 2000. Effects of ferulic and *p*-coumaric acids on canola (*Brassica napus* L. cv. Hyola 401) seed germination. *Seed Sci. Technol.* 28:201–207.

Baziramakenga, R., Leroux, G. D., and Simard, R. R. 1995. Effects of benzoic and cinnamic acids on membrane permeability of soybean roots. *J. Chem. Ecol.* 21:1271–1285.

Baziramakenga, R., Leroux, G. D., and Simard, R. R. 1997. Allelopathic effects of phenolic acids on nucleic acid and protein levels in soybean seedlings. *Can. J. Bot.* 75:445–450.

Berlyn, G. P., and Miksche, J. P. 1976. Botanical microtechnique and cytochemistry. The Iowa State University Press, Ames, Iowa.

Boerjan, W., Ralph, J., and Baucher, M. 2003. Lignin biosynthesis. *Annu. Rev. Plant Biol.* 54:519–546.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

Burgos, N. R., Talbert, R. E., Kim, K. S., and Kuk, Y. I. 2004. Growth inhibition and root ultrastructure of cucumber seedlings exposed to allelochemical from rye (*Secale cereale*). *J. Chem. Ecol.* 30:671–689.

Chen, M., and McClure, J. W. 2000. Altered lignin composition in phenylalanine ammonia-lyase-inhibited radish seedlings: implications for seed-derived sinapoyl esters as lignin precursors. *Phytochemistry.* 53:365–370.

Chen, F., Reddy, M. S. S., Temple, S., Jackson, L., Shadle, G., and Dixon, R. A. 2006. Multi-site genetic modulation of monolignol biosynthesis suggests new routes for formation of syringyl lignin and wall-bound ferulic acid in alfalfa (*Medicago sativa* L.). *Plant J.* 48:113–124.

Chon, S.-U., Choi, S.-K., Jung, S., Jang, H.-G., Pyo, B.-S., and Kim, S.-M. 2002. Effects of alfalfa leaf extracts and phenolic allelochemicals on early seedling growth and root morphology of alfalfa and barnyard grass. *Crop Protection.* 21:1077–1082.

Christensen, J. H., Bauw, G., Welinder, K. G., Van Montagu, M., and Boerjan, W. 1998. Purification and characterization of peroxidases correlated with lignification in poplar xylem. *Plant Physiol.* 118:125–135.

Cruz-Ortega, R., Anaya, A. L., Hernández-Bautista, B. E., and Laguna-Hernández, G. 1998. Effects of allelochemical stress produced by *Sicyos deppei* on seedling root ultrastructure of *Phaseolus vulgaris* and *Cucurbita ficifolia*. *J. Chem. Ecol.* 24:2039–2057.

Donaldson, L. A. 2001. Lignification and lignin topochemistry—an ultrastructural view. *Phytochemistry.* 57:859–876.

de Ascensao, A. R. F. D. C., and Dubery, I. A. 2003. Soluble and wall-bound phenolics and phenolics polymers in *Musa acuminata* roots exposed to elicitors from *Fusarium oxysporum* f. sp. *cubense*. *Phytochemistry.* 63:679–686.

dos Santos, W. D., Ferrarese, M. L. L., Finger, A., Teixeira, A. C. N., and Ferrarese-Filho, O. 2004. Lignification and related enzymes in soybean root growth-inhibition by ferulic acid. *J. Chem. Ecol.* 30:1199–1208.

dos Santos, W. D., Ferrarese, M. L. L., and Ferrarese-Filho, O. 2006. High performance liquid chromatography method for the determination of cinnamyl alcohol dehydrogenase in soybean roots. *Plant Physiol. Biochem.* 44:511–515.

Einhellig, F. A. 1995. Characterization of the mechanisms of allelopathy. Modeling and experimental approaches, pppp. 132–141, in H. H. Cheng, Inderjit, and K. M. M. Dakshini (eds.). Allelopathy, Organisms, Processes and ApplicationsAmerican Chemical Society, Washington, DC.

Ferrarese, M. L. L., Souza, N. E., Rodrigues, J. D., and Ferrarese-Filho, O. 2001. Carbohydrate and lipid status in soybean roots influenced by ferulic acid uptake. *Acta Physiol. Plant.* 23:421–427.

Ferrarese, M. L. L., Zottis, A., and Ferrarese-Filho, O. 2002. Protein-free lignin quantification in soybean (*Glycine max*) roots. *Biologia.* 57:541–543.

Gerrits, P. O. 1991. The Application of Glycol Methacrylate in Histotechnology: Some Fundamental Principles. Department of Anatomy and Embryology State University Groningen, Netherlands.

Hamada, K., Ysutsumi, Y., and Nishida, T. 2003. Treatment of poplar callus with ferulic and sinapic acids II. Effects on related monolignol biosynthetic enzyme activities. *J. Wood Sci.* 49:366–370.

HO, L. C. M. 1988. Metabolism and compartmentation of imported sugars in sink organs in relation to sink strength. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 39:355–378.

HSU, Y. T., and KAO, C. H. 2007. Heat shock-mediated H_2O_2 accumulation and protection against CD toxicity in rice seedlings. *Plant Soil.* 300:137–147.

IIYAMA, K., LAM, T. B. T., and STONE, B. A. 1990. Phenolic acid bridges between polysaccharides and lignin in wheat internodes. *Phytochemistry.* 29:733–737.

INDERJIT, and DUKE, S. O. 2003. Ecophysiological aspects of allelopathy. *Planta.* 217:529–539.

JENSEN, W. A. 1962. Botanical histochemistry, principles and practice. W. H. Freeman, San Francisco.

JOHANSEN, D. A. 1940. Plant microtechnique. McGraw-Hill Book Co., New York.

KAUR, H., INDERJIT, and KAUSHIK, S. 2005. Cellular evidence of allelopathic interference of benzoic acid to mustard (*Brassica juncea* L.) seedling growth. *Plant Physiol. Biochem.* 43:77–81.

KIM, H., RALPH, J., YAHIAOUI, N., PEAN, M., and BOUDET, A. M. 2000. Cross-coupling of hydroxycinnamyl aldehydes into lignins. *Organic. Lett.* 2:2197–2200.

LAM, T. B. T., KADOYA, K., and IIYAMA, K. 2001. Bonding of hydroxycinnamic acids to lignin: ferulic and *p*-coumaric acids are predominantly linked at the benzyl position of lignin, not the β-position, in grass cell walls. *Phytochemistry.* 57:987–992.

LI, L., CHENG, X. F., LESHKEVICH, J., UMEZAWA, T., and HARDING, S. A. 2001. The last step of syringyl monolignols biosynthesis in angiosperms is regulated by a novel gene encoding sinapyl alcohol dehydrogenase. *Plant Cell.* 13:1567–1585.

LIU, D. L., and LOVETT, J. V. 1993. Biologically active secondary metabolites of barley. II. Phytotoxicity of barley allelochemicals. *J. Chem. Ecol.* 19:2231–2244.

MANGOLIN, C. A., PRIOLI, A. J., and MACHADO, M. F. P. S. 1994. Isozyme patterns in callus cultures and in plants regenerated from calli of *Cereus peruvianus* (cactaceae). *Biochem. Gen.* 32:237–247.

O'BRIEN, T. P., FEDER, N., and MCCULLY, M. E. 1964. Polychromatic staining of plant cell walls by toluidine blue O. *Protoplasma.* 59:368–373.

PASSARDI, F., COSIO, C., PENEL, C., and DUNAND, C. 2005. Peroxidases have more functions than a Swiss army knife. *Plant Cell. Rep.* 24:255–265.

PEREIRA, A. J., VIDIGAL-FILHO, P. S., LAPENTA, A. S., and MACHADO, M. F. P. S. 2001. Differential esterase expression in leaves of *Manihot esculenta* Crantz infected with *Xanthomonas axonopodis* pv. manihotis. *Biochem. Genet.* 39:289–296.

POLITYCKA, B. 1996. Peroxidase activity and peroxidation lipidic in roots of cucumber seedlings influenced by derivatives of cinnamic and benzoic acids. *Acta Physiol. Plant.* 18:365–370.

ROS BARCELÓ, A., GÓMEZ ROS, L. V., GABALDÓ, N. C., LÓPEZ-SERRANO, M., POMAR, F., CARRIÓN, J. S., and PEDRENO, M. A. 2004. Basic peroxidases: the gateway from lignin evolution? *Phytochem. Rev.* 3:61–78.

SÁNCHEZ, M., PEÑA, M. J., REVILLA, G., and ZARRA, I. 1996. Changes in dehydrodiferulic acids and peroxidase activity against ferulic acid associated with cell walls during growth of *Pinus pinaster hypocotyl*. *Plant Physiol.* 111:941–946.

SCHOCH, G. A., NIKOV, G. N., ALWORTH, W. L., and WERCK-REICHHART, D. 2002. Chemical inactivation of the cinnamate 4-hydroxylase allows for the accumulation of salicylic acid in elicited cells. *Plant Physiol.* 130:1022–1031.

SHANN, J. R., and BLUM, U. 1987a. The uptake of ferulic acid and *p*-hydroxybenzoic acids by *Cucumis sativus*. *Phytochemistry.* 26:2959–2964.

SHANN, J. R., and BLUM, U. 1987b. The utilization of exogenously applied ferulic acid in lignin biosynthesis. *Phytochemistry.* 26:2977–2982.

WALLACE, G., and FRY, S. C. 1999. Action of diverse peroxidases and laccases on six cell wall-related phenolic compounds. *Phytochemistry.* 52:769–773.

WEIR, T. L., PARK, S. W., and VIVANCO, J. M. 2004. Biochemical and physiological mechanisms mediated by allelochemicals. *Curr. Opin. Plant Biol.* 7:472–479.

WOJTASZEK, P. 1997. Oxidative burst: an early plant response to pathogen infection. *Biochem. J.* 322:681–692.

YAMAUCHI, K., and FUKUSHIMA, K. 2004. The regulation from guaiacyl to syringyl lignin in the differentiating xylem of *Robinia pseudacacia*. *C. R. Biologies.* 327:791–797.

(2004). So far, most activated defenses have been discovered in algae. Reported examples in macroalgae include the conversion of halimedatetraacetate to the anti-feeding compound halimedatrial in *Halimeda* spp. (Paul and Van Alstyne 1992), and the wound-activated deacetylation of caulerpenyne in *Caulerpa* spp. (Jung and Pohnert 2001; Jung et al. 2002). Phytoplanktonic diatoms convert polyunsaturated fatty acids to α,β,γ,δ-unsaturated aldehydes as an activated anti-predator defense that negatively affects the reproductive success of copepods (Miralto et al. 1999; Pohnert 2000, 2002). Remarkably, a cell damage-activated conversion of dimethylsulfoniopropionate (DMSP) to acrylic acid and dimethylsulfide (DMS) was found in both macro- and microalgae (Wolfe et al. 1997). In both cases, an ecological relevance as an anti-predator defense was proposed (Van Alstyne et al. 2001; Van Alstyne and Houser 2003).

Many sessile marine invertebrates share numerous ecological features and challenges with plants (e.g., modular body organization, high regenerative capabilities, habitat restriction to exposed sites, structural defenses with low efficiency). Thus, it is not surprising that both groups have evolved similar strategies, including chemical defense. In sessile marine invertebrates, examples of activated chemical defenses have also been reported. In *Tridentata marginata*, tissue damage results in an enzymatic formation of a series of dithiocarbamates, tridentatols A–D, that intensify the chemical protection of the hydroid against fish predators (Lindquist 2002). In sponges of the genus *Aplysina*, isoxazoline alkaloids are cleaved to aeroplysinin-1 and dienone following wounding (Teeyapant and Proksch 1993; Weiss et al. 1996; Ebel et al. 1997; Thoms et al. 2006). Puyana et al. 2003, however, have questioned early studies on activated defenses in the genus *Aplysina*. To our knowledge, these two reactions are the only examples of activated defenses that have been reported in sessile marine invertebrates to date. In a recent review on chemical defense in sponges, the problems and challenges encountered when addressing activated defenses in sponges and the limits of conclusions that can be drawn on ecological relevance of these reactions have been discussed (Thoms and Schupp 2007).

The sponge *Aplysinella rhax* (order Verongida, family Aplysinellidae) is closely related to the sponges of the genus *Aplysina* (order Verongida, family Aplysinidae). The dominant secondary metabolites in *A. rhax* (e.g., psammaplins, aplysinellins, bisaprasin) as well as the isoxazoline alkaloids in *Aplysina* spp., are derived from bromotyrosine, and share pronounced structural similarities (Pham et al. 2000; Shin et al. 2000). Psammaplins, in particular psammaplin A, have gained pharmaceutical interest due to their cytotoxic properties (Kim et al. 1999; Jiang et al. 2004; Newman and Cragg 2004). In past studies on *A. rhax*, psammaplin A was described as one of the dominant compounds in the organic extract of the sponge (Pham et al. 2000; Tabudravu et al. 2002).

In contrast, our experiments have revealed a dominance of psammaplin A sulfate in intact tissue of *A. rhax*, whereas psammaplin A is present in low quantities, only. However, damage to tissue of *A. rhax* results in a pronounced shift in the chemical profile with psammaplin A increasing considerably. In a series of wounding experiments and feeding assays with generalist reef fish and an omnivorous sponge predator, we investigated a possible ecological role of this reaction as an activated chemical defense. For the experimental design and the interpretation of data, we used the following criteria defined by Paul and Van Alstyne (1992) for activated defenses in macroalgae: (1) a less potent stored secondary metabolite is converted into a more potent one. (2) The process is rapid, requiring only seconds to a few minutes. (3) The conversion is catalyzed by one or few enzymes.

Based on our results and other recent observations of potentially enzyme-catalyzed conversions of secondary metabolites in sponges (e.g., a deacetylation of sesterterpenes in the sponge *Luffariella variabilis* (Ettinger-Epstein et al. 2007) and changes in secondary metabolite profiles following wounding in *Suberea* sp. (Schupp et al., unpublished data)), we question whether, in the past, activated defenses may have been overlooked in the phylum Porifera and possibly also in other sessile invertebrate phyla.

Methods and Materials

Sponge Collection A. rhax De Laubenfels, order Verongida, is commonly found in the West–Central Pacific at depths between 5–30 m. Around Guam, the sponge is most abundant on coral patch reefs in Apra Harbor. We collected *A. rhax* at Western Shoals, Apra Harbor, at depths between 15–20 m. The sponges *Hyrtios altum* and *Stylissa massa* were collected in close vicinity. Replicate samples always were taken from sponge colonies at least 5 m apart to minimize collection of clones. We minimized air contact and damage to fresh *A. rhax* by keeping the specimens submerged in seawater during collection and experiments whenever possible and by transporting them in large wide-mouth plastic bottles. While *A. rhax* is usually not overgrown by algae or other invertebrates, we occasionally observed feeding scars on individuals in the field. However, we never observed predators actually feeding on the sponge, and to our knowledge, no predator has yet been reported in the literature.

Preparation of Extracts and Pure Compounds Extractions and isolation of psammaplin A sulfate (**1**) and psammaplin

A (**2**) (Fig. 1) from *A. rhax* were performed according to previously published methods (Pham et al. 2000; Shin et al. 2000). We simplified the protocol by using fresh intact sponge as source material for (**1**) and fresh sponge material that was ground in a mortar for 1 min as source for (**2**). The extraction procedures for both compounds were similar. Sponge tissue was lyophilized, ground to a fine powder, and extracted exhaustively with methanol. The crude extracts were evaporated in vacuo and partitioned between butanol and water. Repetitive partitioning of the water layer with butanol transferred (**1**) and (**2**) entirely into the butanol fraction (both were no longer detectable in the water layer by HPLC). Since large amounts of salt and precipitating sponge pigment were removed by this technique, the butanol fractions appeared better suited for our bioassays than crude extracts. The butanol fraction of extract from intact sponge (in the following referred to as "Ar intact"; Fig. 1) consisted of ~70% wt. of (**1**) and of ~4% wt. of (**2**). The butanol fraction of ground tissue (in the following referred to as "Ar wounded"; see Fig. 1) consisted of ~20% wt. of (**1**) and of ~50%wt. of (**2**).

To obtain pure (**1**) and (**2**), the butanol fractions were subjected to C_{18} reversed-phase vacuum flash chromatography by using gradient mixtures of methanol and water (elution order: 50, 45, 35% methanol). The 50% fraction yielded almost entirely compound (**1**), whereas almost all (**2**) was found in the 35% fraction. Both fractions were further purified by semipreparative and preparative HPLC on a Waters HPLC system coupled to a refraction index detector (column: Alltech Econosil C_{18}, 250×10 mm; eluents (isocratic): 20% aqueous methanol and 100% methanol).

NMR spectra of the purified compounds were recorded in CD_3OD solutions containing Me_4Si as internal standard on a 500-MHz Unity Inova spectrometer. Samples were run in 3-mm tubes at room temperature. Mass spectra were obtained with an Agilent LCMS-ESI-TOF system. Compounds were identified by comparison of our spectral data with literature data (Pham et al. 2000; Shin et al. 2000; Tabudravu et al. 2002).

Wounding Intensity Series To determine correlations between injury to *A. rhax* tissue and changes in its chemical profile, we caused different intensities of wounding to four pieces of identical volume (5 ml) of one sponge individual. Each piece was kept submerged in 15 ml seawater during the experiment. Piece 1 was not further treated; piece 2 was stabbed 60× with a scalpel; piece 3 was ground for 15 s; and piece 4 was ground for 1 min (both pieces, 3 and 4, in a small mortar to keep them submerged in the 15-ml seawater). After a standardized reaction time of 2 min (including treatment), samples were frozen along with the seawater at −80°C. The protocol was repeated with a total of six sponge individuals.

Forceps Experiment To mimic fish bites, we removed small tissue pieces from fresh *A. rhax* with forceps. Two fragments of 50 ml volume were cut from one individual and kept submerged in 150 ml seawater each. Fifty "bite" pieces (10–100 µl volume) were taken randomly from

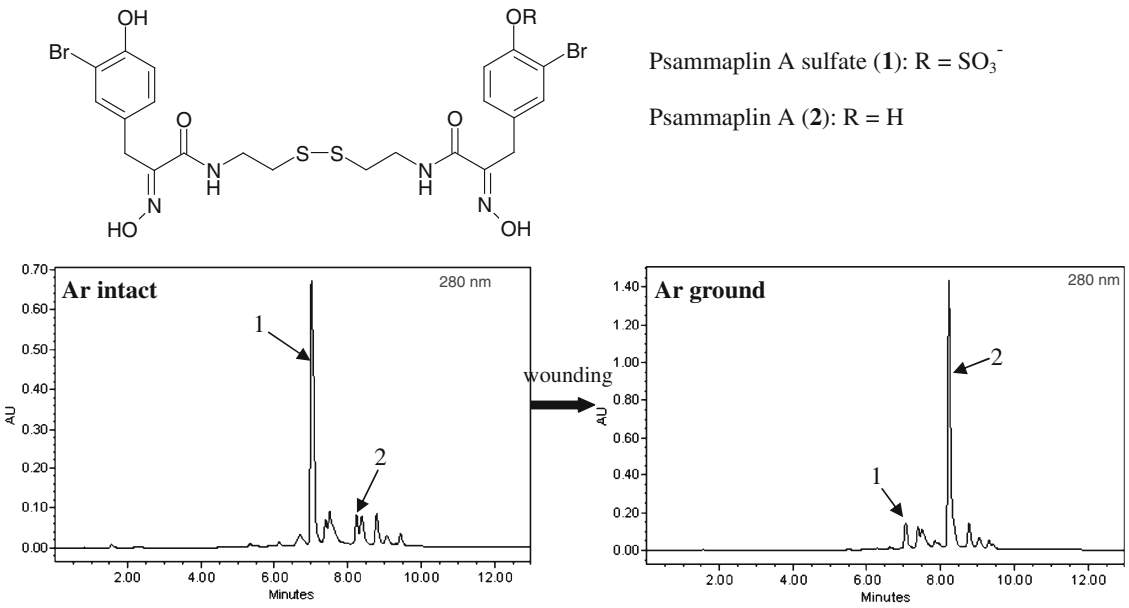

Fig. 1 HPLC chromatograms of the butanol fractions of crude extracts from intact ("Ar intact") and ground ("Ar wounded") *A. rhax* tissue. Tissue wounding resulted in a conversion of psammaplin A sulfate (**1**) to psammaplin A (**2**)

different spots on the sponge surface of one of the pieces and frozen in liquid nitrogen within 2 s. Both 50-ml sponge fragments were frozen at −80°C. The seawater was decanted to remove solid particles and also frozen. The protocol was repeated with a total of four sponge individuals.

Testing for Enzyme-Mediation of the Conversion Reaction Other than grinding of fresh sponge tissue, grinding of lyophilized tissue did not cause any bioconversion, indicating that the conversion enzymes did not function in a water free environment. Two 50-mg subsamples of lyophilized and subsequently ground tissue of *A. rhax* individuals were incubated in 300 µl of distilled water, either at room temperature (25°C) or at 95°C, respectively (2 min incubation time). The suspensions were frozen at −80°C, and the experiment was repeated with tissue powder of three individuals.

Fresh 5-ml pieces of the sponges *A. rhax*, *S. massa*, and *H. altum* were each spiked with 22.8 µmol psammaplin A sulfate dissolved in 75 µl EtOH. Another tissue piece of *A. rhax* was spiked with EtOH only. The tissue pieces were ground vigorously for 4 min and then frozen at −80°C.

Effects of Solvent Exposure In the sponge *Aplysina aerophoba*, reactions similar to those occurring in its activated chemical defense were induced when fresh (wet) tissue contacted organic solvents (Thoms et al. 2006). Preservation of fresh tissue as well as its extraction in organic solvents are common practices in marine chemical ecology. We tested effects of solvent exposure on the metabolite profile in fresh tissue of *A. rhax*.

Five 5-ml pieces of fresh tissue of one *A. rhax* individual were immersed in 10 ml methanol, each. Another piece was frozen immediately at −80°C. In a time series (exposure times 10 min, 30 min, 1 h, 6 h, 24 h), the solvent of each sample was decanted, and the tissue pieces were frozen at −80°C. Solvent samples were dried in a SpeedVac (SPD2010, Thermo Savant). They were re-dissolved in methanol and used to extract the lyophilized and ground sponge pieces as described below. The experiment was repeated with tissue pieces of three sponge individuals.

Chemical Analysis All tissue samples underwent a precisely defined extraction procedure to ensure identical extraction efficiencies. Samples were lyophilized, ground to a fine powder, and homogenized. Fifty milligrams of tissue powder were extracted with 1,500 µl methanol. The samples were vortexed vigorously for 60 s, and after 2 min soaking time, vortexing was repeated for another 30 s. After centrifugation at 13,000 rpm for 2 min, supernatants were analyzed in an HPLC system coupled to a photodiode-array detector (Waters, Milford, Ireland; column: Alltech Rocket Platinum EPS C18, 53×7 mm). Routine detection and quantification of compounds was at 280 nm, but the chromatograms were also inspected with the MaxPlot function of the Waters Empower Pro software that covered wavelengths between 210 and 400 nm.

For quantification, extinction coefficients were determined by repeated analysis of defined amounts of the purified metabolites. Contents in the extracts were calculated based on the detector response at the respective retention times. Seawater samples were lyophilized, redissolved in 50 ml methanol, and an aliquot was analyzed by HPLC as detailed above.

Field Feeding Assay with Generalist Reef Fishes The field assay was conducted based on a method described by Schupp and Paul (1994). In a first set, we tested food treated with psammaplin A sulfate (**1**) against untreated controls. Psammaplin A sulfate (856 mg) dissolved in methanol was added to fish food consisting of 5 g ground catfish pellets, 1.25 g agar, 1.25 g carrageenan, and 80 ml seawater, to match the natural volumetric concentration of (**1**) in *A. rhax* tissue. Controls were prepared by adding the same amount of pure methanol instead. Dissolved extracts and pure compounds always were added to molten food, only after it had cooled down to 60°C in order to avoid compound degeneration due to heat. Mixtures were poured into 1-cm^3 molds containing rubber o-rings. The resulting food cubes were attached with the o-rings and safety pins to ropes. Twenty pairs of ropes, with each rope holding either four treated or four control food cubes, were attached on the reef of Western Shoals, Apra Harbor, Guam. The fish community present was dominated by the species *Abudefduf sexfasciatus*, *A. vaigiensis*, *Amblyglyphidodon curacao*, *Cheilinus fasciatus*, and *Naso vlamingii*. We collected the rope pairs when the fishes had removed half of the cubes. In a second set, we directly compared the repellent activities of psammaplin A sulfate and psammaplin A against generalist reef fish by using the same protocol. In this assay, food treated with natural volumetric concentrations of psammaplin A sulfate was offered together with food treated with an equimolar amount of psammaplin A.

Feeding Assay with a Sponge Predator Feeding experiments were performed with the pufferfish *Canthigaster solandri* based on a laboratory assay described by Schupp et al. (1999). The *A. rhax* extracts "Ar intact" and "Ar wounded" were added in their natural volumetric concentrations to fish food prepared of 2% molten agar and 0.1 g ml^{-1} finely ground catfish food. In a first set, food pieces treated with "Ar intact" and "Ar wounded" were tested separately against controls. Extracts were dissolved in methanol and mixed with the fish food. Control food was prepared similarly, but the same volume of pure methanol was added instead. Treated and control food were poured in

parallel molds backed with fiberglass window screening. When the agar had cooled down, the food was cut into identical strips, each holding a treated and a control food piece (each piece covering 120 squares of the screening). Part of the agar was lyophilized and re-extracted with the same protocol as for extraction of sponge tissue. HPLC comparison with the original extracts revealed no changes in the chemical profile of the extracts after incorporation into the agar.

Food strips were placed in 30-l flow-through tanks and offered to individual pufferfish. Strips were pulled from the tanks, and the number of screening squares where food had been removed was counted when at least 25% of the total food had been eaten. The experiment was terminated after 4 h. In a second set, sponge extracts were compared directly with each other by offering food strips that held two treated food pieces (one with "Ar intact" and the other with "Ar wounded"). Only food strips where no food at all had been removed after 4 h were disregarded in the statistical analysis. A third set of treated food strips was kept in a seawater tank without fish for 4 h. The extract of these strips was compared to extract from strips not exposed to seawater to test for possible diffusion of compounds into the seawater.

Data Analysis Data of the wounding intensity series, the "forceps experiment", and the experiments to test for effects of solvent exposure were analyzed by randomized block analysis of variance. Multiple comparisons were made with the Tukey test (Zar 1999). Levels of significances are given for the treatments. For the field feeding assay, we used Wilcoxon's signed-ranks test for paired comparisons to test for significant differences in the number of cubes eaten. For the feeding assay with the omnivorous sponge predator, we used paired *t*-tests to test for significant differences in the percentage of food squares eaten.

Results

Wounding Intensity Series Mechanical damage to tissue of *A. rhax* resulted in pronounced changes in the concentrations of psammaplin A sulfate (**1**) and psammaplin A (**2**) (Fig. 1). In intact tissue, psammaplin A sulfate (**1**) was present at 52.8±13.4 µmol g^{-1} dry weight (Fig. 2). Upon 1 min grinding, the concentration of (**1**) decreased to 8.2± 3.6 µmol g^{-1}. At the same time, psammaplin A (**2**) concentration increased from 2.1±0.8 to 46.0±8.9 µmol g^{-1} dry weight. Upon gradually increasing the wounding intensity (no wounding<stabbing<grinding for 15 s<grinding for 1 min), (**1**) gradually decreased. This was paralleled by a gradual increase of (**2**) (Fig. 2).

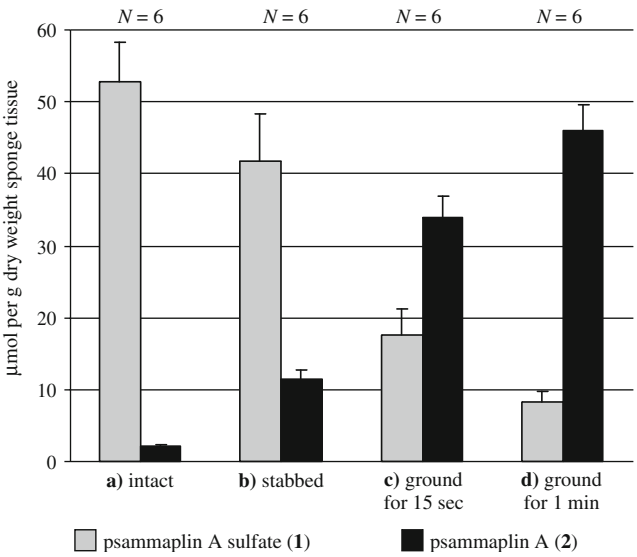

Fig. 2 Changes in psammaplin A sulfate (**1**) and psammaplin A (**2**) concentrations in tissue of *A. rhax* upon different intensities of wounding (overall reaction time was 2 min for all wounding intensities). *Vertical bars* represent SE. Randomized block ANOVA: (**1**) F=36.9, P<0.001; (**2**) F=99.5, P<0.001. Tukey test (significant differences are indicated only): (**1**): $a \neq (c, d)$; $b \neq (c, d)$. (**2**): $a \neq (b, c, d)$; $b \neq (c, d)$; $c \neq d$

Forceps Experiment Tissue pieces that were excised from *A. rhax* with forceps and shock-frozen after 2 s showed a significant decrease in psammaplin A sulfate (**1**) and a concomitant increase in psammaplin A (**2**) when compared to intact sponge tissue (Fig. 3). Similarly, the injured 50-ml sponge fragments (after forceps treatment) showed an increase in psammaplin A concentration, yet to a considerably lesser extent.

Whenever we removed a tissue piece from *A. rhax*, we observed the release of a mucus-like cloud from the wounded sponge area into the ambient seawater. We, therefore, analyzed the seawater and calculated the amounts of (**1**) and (**2**) released per "bite". The amounts, as well as the relative proportions of (**1**) and (**2**) in the seawater came out to be similar to those determined in the removed tissue pieces themselves (Fig. 3).

Testing for Enzyme-Mediation of the Conversion Reaction Following a 2 min incubation of lyophilized and subsequently ground tissue of *A. rhax* in water at room temperature (~25°C), the concentration of psammaplin A sulfate (**1**) decreased from 67.8±2.5 to 1.9±1.6 µmol g^{-1} dry weight sponge tissue. The concentration of psammaplin A (**2**) increased from 2.1±0.4 to 31.1±6.2 µmol g^{-1} dry weight. Upon treatment of another subsample of the same material with hot water (~95°C), instead, the compound concentrations changed to a considerably lesser extent: after a 2-min incubation, psammaplin A sulfate (**1**) amounted for 27.9±7.4 and psammaplin A (**2**) for 4.1±1.1 µmol g^{-1} dry

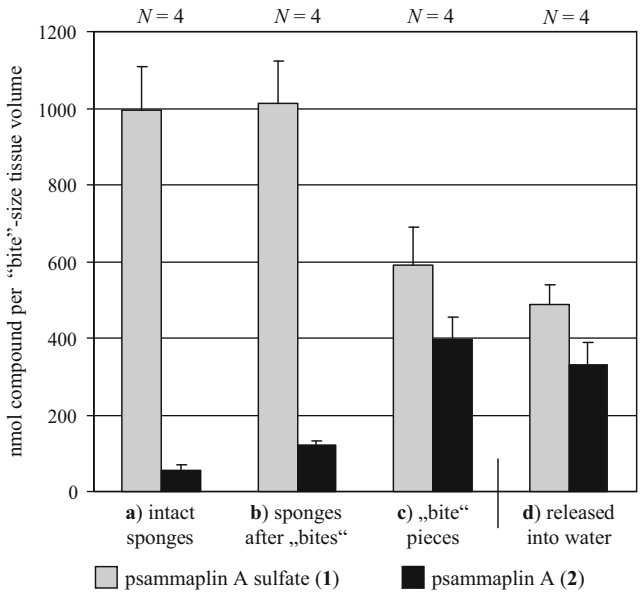

Fig. 3 "Forceps experiment": Contents of psammaplin A sulfate (**1**) and psammaplin A (**2**) in sponge tissue and seawater after mimicked fish bites. "Bite" pieces were shock-frozen within 2 s after removal from the sponges. Concentrations in the sponge tissue samples (*a–c*) are calculated per average "bite" volume (~76 µl). Amounts of compounds released into the water (*d*) are calculated per "bite" caused to the sponge tissue. *Vertical bars* represent SE. Randomized block ANOVA: (**1**) F=12.7, P<0.0025; (**2**) F=15.3, P<0.001. Tukey test (significant differences are indicated, only): (**1**): $a \neq (c, d)$; $b \neq (c, d)$. (**2**): $a \neq (c, d)$; $b \neq (c, d)$

weight. The discrepancy between the total concentrations of psammaplins before and after addition of water (i.e., the apparent loss of compounds) may be explained by the formation of non-UV-detectable side products.

Grinding with additional EtOH-dissolved psammaplin A sulfate (**1**) together with fresh *A. rhax* resulted in an increase of the psammaplin A (**2**) concentration in the sponge tissue to 75.1 µmol g^{-1} dry weight (compared to 50.3 µmol g^{-1} dry weight in sponge tissue ground with EtOH, only). Apparently, artificially added (**1**) was converted to (**2**) when ground together with fresh *A. rhax*. In contrast, no (**2**) was formed when (**1**) was ground together with fresh tissue of the sponges *H. altum* and *S. massa*.

Isolated (**1**) proved stable for at least 6 days when stored at room temperature in seawater or methanol, respectively.

Effects of Solvent Exposure Similar to mechanical damage, solvent exposure had an activating effect on the conversion of psammaplin A sulfate (**1**) to psammaplin A (**2**) in fresh *A. rhax* tissue (Table 1). The concentration of (**1**) dropped quickly—yet much more slowly than upon mechanical damage—within 10 min of exposure, and remained largely constant over the next 24 h. At the same time, (**2**) considerably increased in concentration—likewise mostly within the first 10 min of exposure.

Table 1 Concentration of psammaplin A sulfate (**1**) and psammaplin A (**2**) following methanol exposure of fresh *Aplysinella rhax* tissue

Exposure time	Psammaplin A sulfate (1) µmol g^{-1} dry weight±SE	Psammaplin A (2) µmol g^{-1} dry
a, no exposure	79.7±2.0	6.6±0.6
b, 10 min	28.9±2.8	51.4±1.5
c, 30 min	23.8±3.8	69.4±9.9
d, 1 h	21.2±1.5	76.0±4.7
e, 6 h	25.1±7.9	68.9±7.4
f, 24 h	16.9±1.6	76.1±5.4

N=3 for all time points. Randomized block ANOVA: (**1**) F=48.5, P< 0.001; (**2**) F=41.9, P<0.001. Tukey test (significant differences are indicated, only): (**1**) $a \neq$(b, *c*, *d*, *e*, *f*) and (**2**) $a \neq (b, c, d, e, f)$; $b \neq (d, f)$.

Field Feeding Assay with Generalist Reef Fishes Psammaplin A sulfate (**1**) was strongly deterrent against feeding by generalist reef fish. While control food was readily eaten, food treated with (**1**) was scarcely removed (Fig. 4a). When pairs of ropes, both containing treated food [one with (**1**) and the other with (**2**)] were offered to the fish, they only occasionally took bites; repetitive feeding was rarely observed. Over time, similar amounts of both types of food were removed (Fig. 4b). This indicates a strong repellent effect also by (**2**), but it precludes conclusions on differences in anti-feeding intensity between (**1**) and (**2**), since the occasional choices by the fishes in this assay have to be considered random.

Feeding Assay with a Sponge Predator Both "Ar intact" (butanol fraction of crude extract from intact *A. rhax* tissue) and "Ar wounded" (butanol fraction of crude extract from ground *A. rhax* tissue) had significant repellent effects in

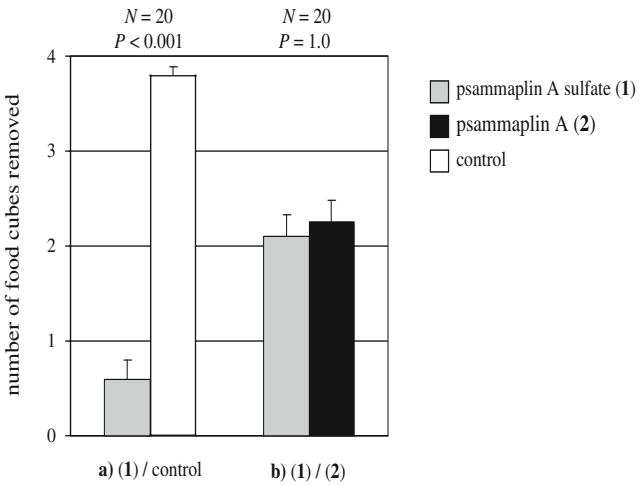

Fig. 4 Results of two field feeding assays testing (*a*) food treated with psammaplin A sulfate against untreated controls and (*b*) food treated with psammaplin A sulfate against food treated with psammaplin A. In each assay, 20 pairs of ropes (each rope holding four food pieces) were offered to generalist reef fish. *Vertical bars* represent SE

choice feeding assays with *C. solandri* when they were tested separately against controls (Fig. 5a and b). When offered in a direct choice without control food, "Ar wounded" proved to be a significantly stronger deterrent than "Ar intact" (Fig. 5c).

Diffusion of compounds from the food strips into the seawater was minimal in the course of the experiment as revealed by HPLC comparison of extracts from strips kept in the seawater tanks (without fish) with food strips not exposed to water. Compound concentrations were >90% compared to the original concentrations after 4 h of seawater exposure.

Discussion

Whereas numerous activated chemical defenses have been documented in terrestrial plants and marine algae (see reviews by Zagrobelny et al. 2004 for terrestrial and Pohnert 2004 for marine-activated defenses), in sessile marine invertebrates, chemical defense is considered largely static. Our study revealed that psammaplin A, a natural product that has been considered constitutive in the marine sponge *A. rhax* (e.g., Shin et al. 2000; Tabudravu et al. 2002), is, in fact, a cleavage product of psammaplin A sulfate. This cleavage reaction is triggered by wounding of the sponge tissue (Figs. 1 and 2).

The conversion of psammaplin A sulfate to psammaplin A occurs within a few seconds following damage of *A. rhax* tissue, as revealed by the "forceps experiment" (Fig. 3). Simulated predator "bites" caused a more than six-fold increase in psammaplin A in the excised tissue compared to undamaged tissue. Psammaplin A was also formed in the remaining damaged sponge, yet to a much lesser extent. Apparently, the conversion reaction was restricted to the wounded tissue area and did not disperse through the sponge. Analogous localized reactions in plants are usually induced by disintegration of compartments that facilitates contact between precursors and enzymes previously stored in separate compartments (Matile 1984; Wittstock and Gershenzon 2002). Storage of secondary metabolites in compartments also has been observed in sponges (Turon et al. 2000; Richelle-Maurer et al. 2003). *Aplysina* sponges, which are closely related to *A. rhax*, contain isoxazoline alkaloids, compounds structurally and biogenetically similar to psammaplin A sulfate (Ciminiello et al. 1994, 1996). These compounds are stored in so-called spherulous cells (Thompson et al. 1983; Turon et al. 2000). By electron microscopy, we found this cell type also to be highly abundant in tissue of *A. rhax* (Thoms et al., unpublished). This suggests that the mechanism that results in the formation of psammaplin A in *A. rhax* is analogous to those that are typically involved in plant activated defenses (i.e., the disruption of compartments to trigger enzymatic compound conversion). We found several further indications that the conversion reaction in *A. rhax* is, indeed, enzyme-mediated. First, the rapidness of the conversion of psammaplin A sulfate, a compound that proved highly stable when isolated from the sponge tissue, indicates enzyme-catalysis. Second, the factor that mediates the conversion appears to be specific to *A. rhax* tissue, since no psammaplin A was formed when psammaplin A sulfate was ground together with tissue of other sponge species. This indicates that *A. rhax* possesses a specific enzyme that catalyzes the cleavage of the sulfate ester group in psammaplin A sulfate. Third, the conversion reaction occurs only in the presence of water. No conversion occurred when lyophilized tissue of *A. rhax* was ground. If, however, water was added to the lyophilized and ground sponge material, psammaplin A was formed at high concentrations. Finally, the conversion reaction was inhibited by heat. In case of a non-enzymatic reaction, the turnover rate should increase with higher reaction temperature. In contrast, the yield of psammaplin A was considerably lower when lyophilized *A. rhax* tissue was treated with hot water compared to water at room temperature. This indicates heat denaturation of the enzyme(s) that catalyzes the cleavage of psammaplin A sulfate in *A. rhax*. In fact, arylsulfatases, enzymes catalyzing reactions such as the cleavage of psammaplin A sulfate in *A. rhax* (i.e., the hydrolysis of a phenolic sulfate ester group) have been described from most organisms, including sponges and marine microorganisms (Mraz and Jatzkewitz 1974; Byun et al. 2004).

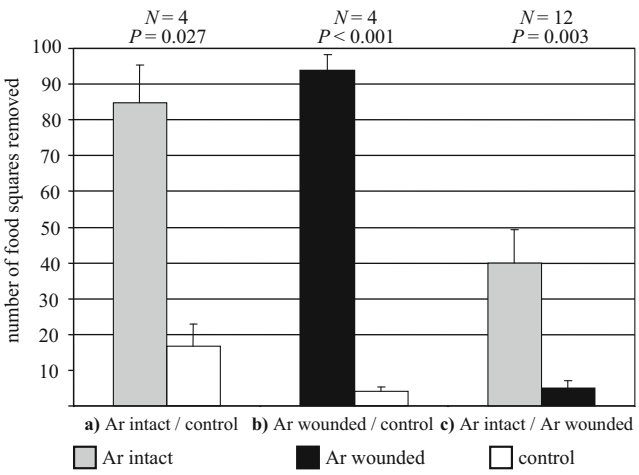

Fig. 5 Results of three choice feeding experiments with the sponge predator *C. solandri*. Fish food was treated with the butanol fractions of extracts from intact ("Ar intact") and ground ("Ar wounded") *A. rhax* tissue. The two fractions were tested separately against controls (*a*+*b*) and in a direct comparison against each other (*c*). *Vertical bars* represent SE

To test for ecological relevance of the observed wound-activated conversion in *A. rhax* as a chemical defense mechanism, we conducted fish-feeding assays comparing the repellent effects of the precursor and the product. When generalist reef fish in the field were offered a choice between food treated with psammaplin A sulfate and controls, they significantly preferred the controls (Fig. 4a). When offered a choice between food treated with psammaplin A sulfate and food treated with psammaplin A (Fig. 4b), the fish only occasionally took bites. By contrast, untreated food—offered to attract the fishes to our experiment—was readily taken. We found that both psammaplin A sulfate and psammaplin A barely leak from the experimental fish food. Thus, their deterrent properties are presumably only perceived by the fish when bites are taken. During the experiment, tasting of both compounds by one individual fish or repetitive feeding were rarely observed, making the occasional fish bites in this experiment rather random. This precludes conclusions on differences in anti-feeding activity between the two compounds. However, the observation that both types of treated food were largely avoided whereas untreated food was readily taken, suggests that both psammaplin A sulfate and psammaplin A had a pronounced feeding deterrent effect against the generalist reef fish.

Despite this pronounced repellence, we observed feeding scars on *A. rhax* in the field, suggesting that there are predators that are able to overcome the strong constitutive chemical defense of the sponge. We, therefore, performed another feeding experiment—this time with a "non-specialist" omnivorous sponge predator, i.e., a predator that is not adapted to the defense compounds of a particular sponge species, but may be less susceptible to sponge chemical defense compared to common reef fish. We selected the pufferfish *C. solandri* for this assay, since it is known to feed on sessile invertebrates such as sponges (Myers 1991). Moreover, this species is highly abundant on the coral reefs around Guam. Similar to the generalist reef fish in the field experiment, *C. solandri* strongly preferred control food over food treated with the *A. rhax* compounds (Fig. 5a and b). However, when no control but only a choice between the two *A. rhax* compounds was offered, *C. solandri* was significantly more deterred by the bioconversion product psammaplin A (Fig. 5c). Thus, the wound-activated conversion reaction in *A. rhax* resulted in an enhanced chemical protection against the potential predator *C. solandri*.

Taken together, we found a rapid, most likely enzyme-mediated bioconversion in *A. rhax* that was activated by tissue wounding and enhanced protection of the sponge against a known sponge predator. Thus, all criteria for an activated chemical defense as defined by Paul and Van Alstyne (1992) are fulfilled. Apparently, *A. rhax* is sufficiently protected against generalist reef fish by its constitutive chemical defense (i.e., the storage of psammaplin A sulfate). However, if despite this defense *A. rhax* is wounded—for instance by a sponge predator that is less susceptible to defensive chemicals than the average reef fish—the sponge's protection against predators is rapidly boosted by the wound-activated formation of psammaplin A.

When simulating fish grazing by picking tissue pieces from *A. rhax*, we observed the release of mucus-like clouds from the wounded tissue. We quantified the psammaplin A sulfate and psammaplin A concentrations released with these clouds and found that they were similarly high as the concentrations in the removed tissue bits (Fig. 3). The latter had proven sufficient to efficiently repel fishes. The mucus clouds may facilitate a rapid transport of the rather lipophilic defense compounds psammaplin A sulfate and psammaplin A to the olfactory organs of the predator, thus accelerating and intensifying their repellent effect.

To our knowledge, the bioconversion in *A. rhax* is so far only the second reported example of an activated chemical defense in sponges—the third among sessile marine invertebrates. Interestingly, all compounds in the sponges *A. rhax* and *Aplysina* spp., as well as in the hydroid *Tridentata marginata*, were initially considered constitutive (Fattorusso et al. 1970; Lindquist et al. 1996; Pham et al. 2000; Shin et al. 2000). In all cases, it was only later discovered that they are, in fact, products of wound-activated conversions (Teeyapant and Proksch 1993; Lindquist 2002; this study). Recently, a cleavage of acetylated sesterterpenes in the sponge *L. variabilis* was reported to occur when fresh tissue of the sponge is frozen and subsequently thawed (Ettinger-Epstein et al. 2007). Here, again, the cleavage products had originally been considered constitutive (de Silva and Scheuer 1981; Kernan et al. 1987). Ettinger-Eppstein et al. proposed an enzyme-mediated cleavage reaction and speculated about its relevance as an activated defense. We have lately observed pronounced changes in the chemical profiles of yet unidentified deep-water sponges collected off the coast of Guam that occurred upon damage to their tissue (Thoms C. and Schupp P.J., unpublished data). More detailed investigations on these preliminary findings need to be done. Based on all these recent observations, however, we deem it reasonable to address the question whether more activated defenses in sponges—and perhaps also in other sessile marine invertebrate phyla—may have been overlooked in the past. In fact, this would be analogous to the development in research on marine algae. Here, it took almost a decade from the first discovery of activated defenses, until it was realized that this strategy is, in fact, widespread among both micro- and macroalgal species (Pohnert 2004). Even for algae, it was recently proposed that the prevalence

of this strategy may still be underestimated due to a "restricted methodological approach by marine chemical ecologists that focuses on constitutive chemical defense" (Pohnert 2004).

In this context, it is interesting to note that the reaction mechanisms of the bioconversions in *A. rhax* and in the *Aplysina* spp. (the genus that accommodates the only other wound-activated defense reported in sponges as yet) are dissimilar. In the latter, isoxazoline alkaloids are cleaved between the isoxazoline moiety and the carbonyl function common to all these molecules (Fig. 6a; Ebel et al. 1997; Thoms et al. 2006). The cleavage is catalyzed by a yet unknown enzyme and results in the formation of the antimicrobially active compounds aeroplysinin-1 and dienone. In contrast, the activated defense reaction in the hydroid *Tridentata marginata* shares many similarities with the conversion reaction we report in this study. In *T. marginata*, as well, a phenolic sulfate ester is enzymatically hydrolyzed and a fish-feeding-deterrent phenol is formed (Fig. 6b; Lindquist 2002). Interestingly, the precursors of all three reactions (in *Aplysina* spp., *A. rhax*, and *T. marginata*) are structurally similar tyrosine-derivatives (Figs. 1 and 6). This may indicate a predisposition of this class of compounds for being converted in activated defenses. An alternative explanation for the repeated discovery of activated defenses that involve tyrosine-derivatives may be found in the fact that these compounds are accumulated in extraordinarily high concentrations in the sponges and the hydroid (Lindquist et al. 1996; Pham et al. 2000; Thoms et al. 2006). Consequently, conversion reactions in these invertebrates may simply be more conspicuous than analogous processes in other species with lower compound yields and more complex chemical profiles. Moreover, if the conversion reactions involve a high number of different precursors, intermediates, or products, this may additionally conceal activated defense reactions.

We recently reviewed the various difficulties involved in studies investigating wound-activated reactions in chemical profiles of sponges in regard to both methodological constraints and limitations of conclusions that can be drawn on the ecological relevance of these reactions (Thoms and Schupp 2007). One of our conclusions is that, in order to clarify the true prevalence of activated defenses in sponges (and in other sessile marine invertebrate phyla), studies are required that systematically compare chemical profiles of these organisms before and after tissue wounding at the

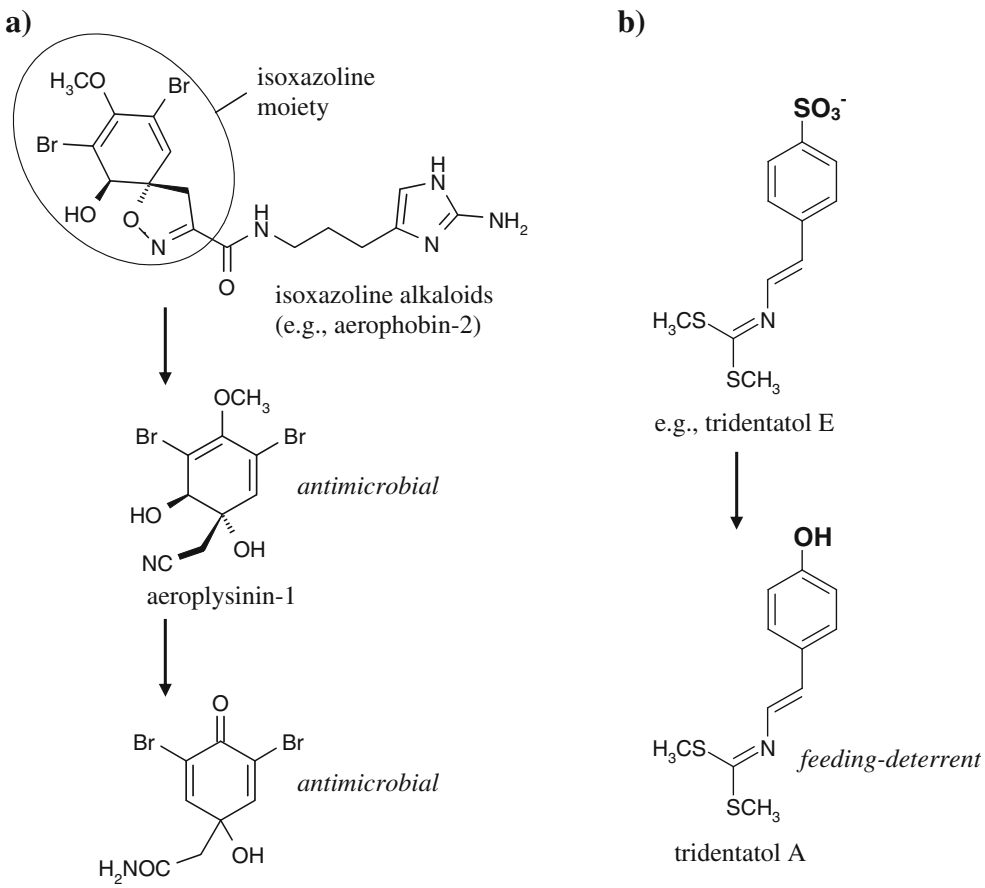

Fig. 6 Activated chemical defenses in sessile marine invertebrates: **a** wound-activated cleavage of the isoxazoline moiety of isoxazoline alkaloids in sponges of the genus *Aplysina* (Thoms et al. 2006). **b** Wound-activated hydrolysis of the sulfate ester groups of tridentatols in the hydroid *Tridentata marginata* (Lindquist 2002)

species level. An important issue that needs to be considered in this context is the methodology for preservation and extraction of invertebrate tissue that is employed in such studies. Preservation and extraction of fresh (wet) tissue in organic solvents are common practices in marine natural product chemistry and ecology. We observed that conversion reactions similar to those activated in *A. rhax* upon tissue wounding also occurred when fresh sponge tissue was stored in methanol. This finding parallels earlier observations in *Aplysina* spp. where storage of fresh sponge tissue in organic solvents elicited the cleavage of isoxazoline alkaloids to an extent similar to that of mechanical injury (Teeyapant and Proksch 1993; Thoms et al. 2006). The activation of enzymatic reactions in invertebrate tissue may be explained by the lacerating effect of organic solvents on biomembranes (Jones 1989; Weber and DeBont 1996). Similar to mechanical damage, this likely results in decompartmentalization and, thus, facilitates contact between enzymes and precursors. If the solvent concentration increases gradually (as occurs if fresh invertebrate tissue is soaked by solvents), enzymes can still sustain their activity and catalyze compound conversions (see Klibanov 2001 and references cited therein).

Another possible trigger for enzyme-mediated reactions in invertebrate tissue after sampling is freezing and thawing for preservation and subsequent extraction (see for example Ettinger-Epstein et al. 2007). Here, the formation of intracellular ice crystals may result in disruption of compartments (Gahan 1981; Hällgren and Öquist 1990). Thawing of the tissue may then re-activate the enzymes. Last but not least, mechanical damage caused during sampling and handling has to be taken into account as a potential elicitor of wound-activated defenses.

Taken together, various factors could potentially have led to the oversight of activated chemical defenses, and, in consequence, to the misinterpretation of products of these defense mechanisms as constitutive chemical profile components. If future systematic investigations on activated defenses in sessile marine invertebrates reveal additional examples of this strategy, this could add another interesting aspect to the chemical ecology of this group of animals.

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References

BYUN, D. S., KIM, D. S., GODBER, J. S., NAM, S. W., OH, M. J., SHIM, H. S., and KIM, H. R. 2004. Isolation and characterization of marine bacterium producing arylsulfatase. *J. Microbiol. Biotechnol.* 14:1134–1141.

CIMINIELLO, P., COSTANTINO, V., FATTORUSSO, E., MAGNO, S., MANGONI, A., and PANSINI, M. 1994. Chemistry of Verongida sponges.2. Constituents of the Caribbean sponge *Aplysina fistularis* forma *fulva*. *J. Nat. Prod.* 57:705–712.

CIMINIELLO, P., FATTORUSSO, E., MAGNO, S., and PANSINI, M. 1996. Chemistry of Verongida sponges. 6. Comparison of the secondary metabolic composition of *Aplysina insularis* and *Aplysina fulva*. *Biochem. Syst. Ecol.* 24:105–107.

CLAUSEN, T. P., REICHARDT, P. B., BRYANT, J. P., WERNER, R. A., POST, K., and FRISBY, K. 1989. Chemical model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against herbivores. *J. Chem. Ecol.* 15:2335–2346.

DE SILVA, E. D., and SCHEUER, P. J. 1981. Three new sesterterpenoid antibiotics from the marine sponge *Luffariella variabilis* (Polejaff). *Tetrahedron Lett.* 22:3147–3150.

EBEL, R., BRENZINGER, M., KUNZE, A., GROSS, H. J., and PROKSCH, P. 1997. Wound activation of protoxins in marine sponge *Aplysina aerophoba*. *J. Chem. Ecol.* 23:1451–1462.

ETTINGER-EPSTEIN, P., MOTTI, C. A., DE NYS, R., WRIGHT, A. D., BATTERSHILL, C. N., and TAPIOLAS, D. M. 2007. Acetylated sesterterpenes from the Great Barrier Reef sponge *Luffariella variabilis*. *J. Nat. Prod.* 70:648–651.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

FATTORUSSO, E., MINALE, L., and SODANO, G. 1970. Aeroplysinin-1, a new bromo-compound from *Aplysina aerophoba*. *J. Chem. Soc. D* 12:751–753.

GAHAN, P. B. 1981. Cell senescence and death in plants, pp. 145–169, in I. D. Bowen, and R. A. Lockshin (eds.). Cell Death in Biology and PathologyChapman & Hall, London.

HÄLLGREN, J., and ÖQUIST, G. 1990. Adaptations to low temperatures, pp. 265–293, in R. G. Alscher, and J. R. Cumming (eds.). Stress Responses in Plants: Adaptation and Acclimation MechanismsWiley, New York.

JIANG, Y. H., AHN, E. Y., RYU, S. H., KIM, D. K., PARK, J. S., YOON, H. J., YOU, S., LEE, B. J., LEE, D. S., and JUNG, J. H. 2004. Cytotoxicity of psammaplin A from a two-sponge association may correlate with the inhibition of DNA replication. *BMC Cancer* 4:70.

JONES, R. P. 1989. Biological principles of the effects of ethanol: a review. *Enzyme Microb. Technol.* 11:130–152.

JUNG, V., and POHNERT, G. 2001. Rapid wound-activated transformation of the green algal defensive metabolite caulerpenyne. *Tetrahedron* 57:7169–7172.

JUNG, V., THIBAUT, T., MEINESZ, A., and POHNERT, G. 2002. Comparison of the wound-activated transformation of caulerpenyne by invasive and noninvasive *Caulerpa* species of the Mediterranean. *J. Chem. Ecol.* 28:2091–2105.

KERNAN, M. R., FAULKNER, D. J., and JACOBS, R. S. 1987. The luffariellins, novel antiinflammatory sesterterpenes of chemotaxonomic importance from the marine sponge *Luffariella variabilis*. *J. Org. Chem.* 52:3081–3083.

KIM, D., LEE, I. S., JUNG, J. H., LEE, C. O., and CHOI, S. U. 1999. Psammaplin A, a natural phenolic compound, has inhibitory effect on human topoisomerase II and is cytotoxic to cancer cells. *Anticancer Res.* 19:4085–4090.

KLIBANOV, A. M. 2001. Improving enzymes by using them in organic solvents. *Nature* 409:241–246.

LINDQUIST, N. 2002. Tridentatols D–H, nematocyst metabolites and precursors of the activated chemical defense in the marine hydroid *Tridentata marginata* (Kirchenpauer 1864). *J. Nat. Prod.* 65:681–684.

LINDQUIST, N., LOBKOVSKY, E., and CLARDY, J. 1996. Tridentatols A–C, novel natural products of the marine hydroid *Tridentata marginata*. *Tetrahedron Lett.* 37:9131–9134.

MATILE, P. 1984. The toxic compartment of plant cells. *Naturwissenschaften* 71:18–24.

MIRALTO, A., BARONE, G., ROMANO, G., POULET, S. A., IANORA, A., RUSSO, G. L., BUTTINO, I., MAZZARELLA, G., LAABIR, M., CABRINI, M., and GIACOBBE, M. G. 1999. The insidious effect of diatoms on copepod reproduction. *Nature* 402:173–176.

MRAZ, W., and JATZKEWITZ, H. 1974. Cerebroside sulphatase activity of arylsulphatases from various invertebrates. *Hoppe-Seyler's Z. Physiol. Chem.* 355:33–44.

MYERS, R. F. 1991. Micronesian reef fishes. Coral, Barrigada, Guam.

NEWMAN, D. J., and CRAGG, G. M. 2004. Marine natural products and related compounds in clinical and advanced preclinical trials. *J. Nat. Prod.* 67:1216–1238.

PAUL, V. J., and PUGLISI, M. P. 2004. Chemical mediation of interactions among marine organisms. *Nat. Prod. Rep.* 21:189–209.

PAUL, V. J., and VAN ALSTYNE, K. L. 1992. Activation of chemical defenses in the tropical green algae *Halimeda* spp. *J. Exp. Mar. Biol. Ecol.* 160:191–203.

PHAM, N. B., BUTLER, M. S., and QUINN, R. J. 2000. Isolation of psammaplin A 11¢-sulfate and bisaprasin 11¢-sulfate from the marine sponge *Aplysinella rhax*. *J. Nat. Prod.* 63:393–395.

POHNERT, G. 2000. Wound-activated chemical defense in unicellular planktonic algae. *Angew. Chem. Int. Ed.* 39:4352–4254.

POHNERT, G. 2002. Phospholipase A(2) activity triggers the wound-activated chemical defense in the diatom *Thalassiosira rotula*. *Plant. Physiol.* 129:103–111.

POHNERT, G. 2004. Chemical defense strategies of marine organisms, pp. 179–219, in S. Schulz (ed.). Topics in Current Chemistry, Volume 239: The Chemistry of Pheromones and Other Semiochemicals ISpringer, Berlin.

PUYANA, M., FENICAL, W., and PAWLIK, J. R. 2003. Are there activated chemical defenses in sponges of the genus *Aplysina* from the Caribbean? *Mar. Ecol. Prog. Ser.* 246:127–135.

RICHELLE-MAURER, E., DE KLUIJVER, M. J., FEIO, S., GAUDENCIO, S., GASPAR, H., GOMEZ, R., TAVARES, R., VAN-DER-VYVER, G., and SOEST, R. W. M. V. 2003. Localization and ecological significance of oroidin and sceptrin in the Caribbean sponge *Agelas conifera*. *Biochem. Syst. Ecol.* 31:1073–1091.

SCHUPP, P. J., and PAUL, V. J. 1994. Calcium carbonate and secondary metabolites in tropical seaweeds—variable effects on herbivorous fishes. *Ecology* 75:1172–1185.

SCHUPP, P., EDER, C., PAUL, V., and PROKSCH, P. 1999. Distribution of secondary metabolites in the sponge *Oceanapia* sp. and its ecological implications. *Mar. Biol.* 135:573–580.

SHIN, J., LEE, H. S., SEO, Y., RHO, J. R., CHO, K. W., and PAUL, V. J. 2000. New bromotyrosine metabolites from the sponge *Aplysinella rhax*. *Tetrahedron* 56:9071–9077.

STERNER, O., BERGMAN, R., KIHLBERG, J., and WICKBERG, B. 1985. The sesquiterpenes of *Lactarius vellereus* and their role in a proposed chemical defense system. *J. Nat. Prod.* 48:279–288.

STOEWSAND, G. S. 1995. Bioactive organosulfur phytochemicals in *Brassica oleracea* vegetables—a review. *Food Chem. Toxicol.* 33:537–543.

TABUDRAVU, J. N., EIJSINK, V. G. H., GOODAY, G. W., JASPARS, M., KOMANDER, D., LEGG, M., SYNSTAD, B., and VAN AALTEN, D. M. F. 2002. Psammaplin A, a chitinase inhibitor isolated from the Fijian marine sponge *Aplysinella rhax*. *Bioorg. Med. Chem.* 10:1123–1128.

TEEYAPANT, R., and PROKSCH, P. 1993. Biotransformation of brominated compounds in the marine sponge *Verongia aerophoba*. Evidence for an induced chemical defense? *Naturwissenschaften* 80:369–370.

THOMPSON, J. E., BARROW, K. D., and FAULKNER, D. J. 1983. Localization of two brominated metabolites, aerothionin and homoaerothionin, in spherulous cells of the marine sponge *Aplysina fistularis*. *Acta Zool. Stockh.* 64:199–210.

THOMS, C., and SCHUPP, P. J. 2007. Chemical defense strategies in sponges: a review, pp. 627–637, in M. R. Custódio, G. Lôbo-Hajdu, E. Hajdu, and G. Muricy (eds.). Porifera Research—Biodiversity, Innovation and Sustainability. Série Livros 28Museu Nacional, Rio de Janeiro.

THOMS, C., EBEL, R., and PROKSCH, P. 2006. Activated chemical defense in *Aplysina* sponges revisited. *J. Chem. Ecol.* 32:97–123.

TURON, X., BECERRO, M. A., and URIZ, M. J. 2000. Distribution of brominated compounds within the sponge *Aplysina aerophoba*: coupling of X-ray microanalysis with cryofixation techniques. *Cell Tissue Res.* 301:311–322.

VAN ALSTYNE, K. L., and HOUSER, L. T. 2003. Dimethylsulfide release during macroinvertebrate grazing and its role as an activated chemical defense. *Mar. Ecol. Prog. Ser.* 250:175–181.

VAN ALSTYNE, K. L., WOLFE, G. V., FREIDENBURG, T. L., NEILL, A., and HICKEN, C. 2001. Activated defense systems in marine macroalgae: evidence for an ecological role for DMSP cleavage. *Mar. Ecol. Prog. Ser.* 213:53–65.

WAJANT, H., and EFFENBERGER, F. 1996. Hydroxynitrile lyases of higher plants. *Biol. Chem.* 377:611–617.

WEBER, F. J., and DEBONT, J. A. M. 1996. Adaptation mechanisms of microorganisms to the toxic effects of organic solvents on membranes. *Biochim. Biophys. Acta-Rev. Biomembr.* 1286:225–245.

WEISS, B., EBEL, R., ELBRÄCHTER, M., KIRCHNER, M., and PROKSCH, P. 1996. Defense metabolites from the marine sponge *Verongia aerophoba*. *Biochem. Syst. Ecol.* 24:1–12.

WITTSTOCK, U., and GERSHENZON, J. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol.* 5:300–307.

WOLFE, G. V., STEINKE, M., and KIRST, G. O. 1997. Grazing-activated chemical defence in a unicellular marine alga. *Nature* 387:894–897.

ZAGROBELNY, M., BAK, S., RASMUSSEN, A. V., JORGENSEN, B., NAUMANN, C. M., and MOLLER, B. L. 2004. Cyanogenic glucosides and plant–insect interactions. *Phytochemistry* 65:293–306.

ZAR, J. H. 1999. Biostatistical analysis. Prentice-Hall, Upper Saddle River, New Jersey.

(4 mm inner diameter) 15-ml glass vial (Sigma-Aldrich, Dorset, UK) by piercing the septum in the cap with the SPME needle. The vial was kept under the armpit by an individual; this sampling device is the subject of UK Patent Application WO/2006/111748. To standardize environmental effects on odor profiles, the volunteers followed an established hygiene regime (Roberts et al. 2005). After 50 min of axillary sampling at room temperature, the fiber was introduced into the gas chromatograph for sample desorption. The air in the sampling room was monitored for background volatiles using the same absorbent material.

Gas Chromatography–Mass Spectrometry Analysis Separation and analysis of the odor compounds was performed by using an Agilent 6890N gas chromatograph. The gas chromatograph injection port liner used was a SPME injection sleeve of 0.75 mm inner diameter (Supelco). SPME desorption was at 230°C, in pulsed splitless mode for 2 min at 25 psi. The gas chromatograph was coupled to an Agilent 5973 mass-selective detector (EI+, electron voltage 70 eV, full scan mode in a range of *m*/*z* 35–400 amu, interface temperature of 300°C; Agilent Technology, Stockport, Cheshire, UK). Separation of compounds was performed on a Zebron, ZB-Wax (30 m× 0.25 mm×0.25 μm film thickness) capillary column coated with 100% polyethylene glycol (Phenomenex, Cheshire, UK). The carrier gas used was helium at a flow rate of 1 ml min^{-1}. The oven temperature program was 40°C (held for 5 min), then raised to 100°C at 3°C/min (5 min), followed by 5°C/min to 150°C (5 min), and reaching 230°C at 5°C/min (8 min).

Culturing of Axillary Microflora A sterile swab, pretreated in 0.8% NaCl, was applied for 10 sec to the left axilla to harvest its microbial flora. The swab was transferred into 20 ml of the glucose-enriched medium (glucose 1%, ammonium chloride 0.5%, sodium dihydrogen orthophosphate 0.2% with final pH adjusted to 7.2) (Sigma-Aldrich) prepared in a screw-cap bottle. The bottle was kept in an orbital shaker at 37°C, 160 rpm, for 12 hr. One milliliter of the culture was then transferred into a sterile 1.8-ml Eppendorf centrifuge tube that was spun for 10 min at 11,500×*g*. The supernatant was removed, and the pellet was washed eight times with sterile distilled water. The pellet was resuspended in the sterile distilled water, and a total microbial count was equated to McFarland turbidity standard 10 (6×10^7cfu/ml).

Preparation of Substrate Solution The LRGYYNQSED and GSHSMRYFST HLA decapeptides (97% purity) were synthesized by Sigma Genosys (Pampisford, Cambridge, UK). To prepare a substrate solution, 1.5 mg of each HLA peptide were dissolved in 1 ml filtered (0.25 mm) sterile distilled water. Each substrate solution also contained 4.4 mg KH_2PO_4, 4.8 mg Na_2HPO_4, 1.0 mg NH_4Cl, and 0.5 mg $MgSO_47H_2O$.

Headspace Analysis of Medium An aliquot (20 μl) of the resuspended pellet and 115 μl of the final substrate solution were added to a 2-ml gas chromatography–mass spectrometry (GC-MS) glass vial containing 2.5 μl of vitamin solution, made of 0.05% thiamine (aneurine) hydrochloride, 0.05% riboflavin, 0.05% niacin, 0.05% pyridine hydrochloride, 0.05% inositol, 0.05%, calcium pantothenate, 0.05% *p*-aminobenzoic acid, and 0.025% biotin (Sigma-Aldrich). The vial was capped, and the mixture was incubated in an orbital shaker (160 rpm) at 37°C for 48 hr. The cap septum was then pierced with the protective needle covering the SPME fiber, and the fiber was extruded to sample the headspace over the medium. The sampling was carried out for 30 min over a heating block set at 50°C. After sampling, the fiber was immediately introduced into the gas chromatograph for sample desorption. In all cases, a negative control of the headspace of the microbial culture without added peptide was analyzed. Each sample was analyzed in duplicate.

Data Analysis Ion 41 (*m*/*z* 41) was selected as one of the main diagnostic ions of 3-methylbutanal (3-MB) from the GC-MS profile of an individual, and its abundance was recorded. Six other compounds that also contained *m*/*z* 41 were selected from the same profile of the individual, their *m*/*z* 41 abundances were also measured, and the mean abundance across all six compounds was calculated. The abundance of *m*/*z* 41 for 3-MB was divided by this mean to obtain a standardized value of *m*/*z* 41 for 3-MB. The standardized value was calculated for every individual profile

Table 1 Standardized abundance of *m*/*z* 41 of 3-methylbutanal from axillary samples from 18 human subjects sampled over 3 wk[a]

Subject	Week 1	Week 2	Week 3
ORE_1	2.21	0.79	1.65
ORE_2	1.6	2.13	1.05
ORE_3	0.62	0.78	1.44
ORE_4	0	0.64	1.74
ORE_5	0	0	0
ORE_6	0.1	0.07	0.29
ORE_7	0	0	0
ORE_8	0.33	0.05	0.04
ORE_9	1.78	0.89	1.19
ORE_10	0.4	0.34	1.21
ORE_11	0.61	0.51	1.32
ORE_12	0.14	0.28	0.28
ORE_13	0.14	0.17	0.21
ORE_14	0.62	1.69	2.3
ORE_15	0	0	0
ORE_16	0	0.57	0.31
ORE_17	0.78	0.21	1.09
ORE_18	0.15	0.13	0.21

[a] ANOVA ($F_{1,\ 17}$=40.125, P<0.001) showed significant variation in abundance of 3-methylbutanal among 18 individuals.

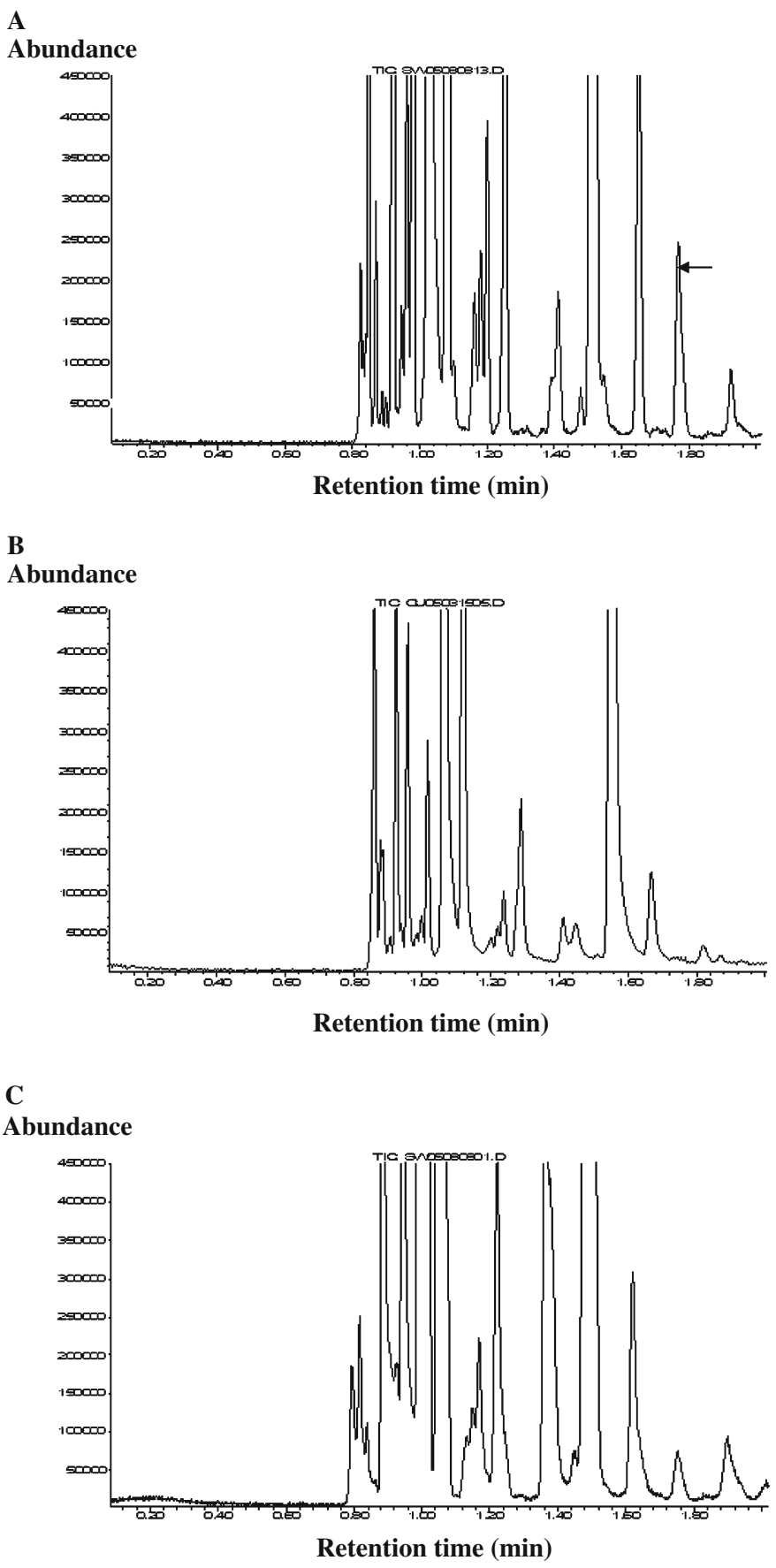

Fig. 1 GC-MS profiles of volatiles trapped above three incubation media including **a** medium containing LRGYYNQSED and microflora cultured from an individual (ORE_2), who consistently emitted 3-MB *in vivo* (*arrow* indicates 3-MB, which eluted at 1.75 min and was verified with the authentic standard from Sigma-Aldrich), **b** medium as in **a** except that the decapeptide GSHSMRYFST was used as the substrate (3-MB was not detected *in vitro*), and **c** medium containing LRGYYNQSED and microflora cultured from an individual (ORE_5), who did not emit detectable 3-MB *in vivo* (3-MB was not detected *in vitro*)

(Table 1). Comparisons of the standardized abundances of 3-MB among individuals were performed by using repeated-measures analysis of variance (ANOVA) with α=0.05 as the threshold for significance (SPSS statistical software).

Results and Discussion

The GC-MS analysis of the axillary odor of 18 volunteers revealed a number of compounds among which 3-MB was noted as a potential link to HLA (Montag et al. 2001). The signal was not reported by Curran et al. (2005), who also used SPME for the analysis of the axillary odor. This discrepancy may be attributed to differences in sampling methodologies between the two studies. The abundance of 3-MB among individuals varied during 3 wk of sampling (Table 1) without a discernable general pattern. Five of 18 subjects yielded at least one zero measurement for 3-MB, and since zero values do not represent a confirmed absence of the chemosignal, abundance measurements from these individuals were excluded initially from the statistical analysis of the significance of the variation in emission of 3-MB among individuals. ANOVA revealed significant variation in the signal among the 13 subjects ($F_{1,\ 12}$=22.9, P<0.001), whereas variation among weeks was not significant ($F_{2,\ 24}$=1.9, P>0.05). There were also significant disparities in the signal among all 18 subjects when zero values were included in the analysis ($F_{1,\ 17}$=40.125, P< 0.001; data were square root-transformed prior to analysis due to significant departures from normality). In this instance, there was also no significant variation in the signal across weeks ($F_{2,\ 34}$=3.07, P>0.05). These statistical analyses may indicate a possible genetic origin of 3-MB, especially after taking into account the observation of Montag et al. (2001) that strains of environmentally controlled congenic mice that differ only in the major histocompatibility complex (MHC or HLA in humans) expression can be distinguished on the basis of differences in the ratio of their urine volatile signals, including 3-MB.

An *in vitro* experiment was carried out to determine whether 3-MB may be formed from the interaction between HLA peptides and dermal microflora. Two decapeptides, whose sequences are expressed in the α1 domain of the HLA class 1 region (Mason and Parham 1998), namely LRGYYNQSED and GSHSMRYFST, were synthesized. The first sequence was selected because it was shown previously that leucine and arginine substitutions in congenic mutant mice, differing only in three amino acids in the MHC region (Schulze et al. 1983), allowed olfactory distinction between the strains (Yamazaki et al. 1983) and that these two amino acids, L and R, were neighbors in the MHC sequence. The other peptide was chosen for the lack of L and L–R linkage in its sequence.

In the first instance, microflora cultured from individual ORE_2, whose axillary odor contained a relatively large abundance of 3-MB during all three weekly samplings (Table 1), was incubated in two separate media. One medium contained LRGYYNQSED, whereas the other contained GSHSMRYFST. The headspace above the surface of each medium was sampled and analyzed for 3-MB. The target compound was detected in the headspace above the LRGYYNQSED incubate (Fig. 1a) but not in the headspace above the GSHSMRYFST incubate (Fig. 1b). If the cutaneous microbial population is affected by an individual's genotype and possibly phenotype, we would predict that the sampled microflora from individual ORE_5, who was not associated with a positive 3-MB signal (Table 1), would not yield the signal when incubated with LRGYYNQSED. Subsequent analysis did not detect 3-MB in the headspace of the medium containing LRGYYNQSED or microflora from this individual (Fig. 1c). The results of the *in vitro* experiment were reproduced by using the microflora cultured from ORE_8 and ORE_7; that is, abundances of 3-MB were 123,560 and 0 (undetected), respectively. The negative controls provided evidence that different HLA peptides can alter production of the signal and that individuals possess differences in microbial populations that influence production of the signal. Based on Leyden et al. (1981), we speculate that aerobic diptheroids, *Corynebacterium*, were involved in the interaction with the HLA peptides. These findings are in agreement with the hypothesis that genes and microflora may influence formation of the odor (Penn and Potts 1998), although it remains unclear whether the production of the chemosignal: (1) is HLA sequence specific, (2) depends on the L–R linkage, or (3) is entirely due to the encoded presence of leucine in the peptide. A further study on the substitutions of the amino acids in the LRGYYNQSED may reveal the extent of HLA contribution to the production of the chemosignal.

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References

CURRAN, M. A., RABIN, I. S., PRADA, A. P., and FURTON, G. K. 2005. Comparison of the volatile organic compounds present in human odor using SPME-GC/MS. *J. Chem. Ecol.* 31:1607–1619.

IMAYAMA, S., YASHIMA, Y., and HORKI, Y. 1992. Differing cell surface distribution of human leukocyte antigen-DR molecules on

epidermal Langerhans cells and eccrine duct cells. *J. Histochem. Cytochem.* 40:1191–1196.

LEYDEN, J. J., MCGINLEY, K. J., HOLZLE, E., LABOWS, J. N., and KLIGMAN, A. M. 1981. The microbiology of the human axilla and its relationship to axillary odor. *J. Invest. Dermatol.* 77:413–416.

MASON, P. M., and PARHAM, P. 1998. HLA class I region sequences. *Tissue Antigens* 51:417–466.

MONTAG, S., FRANK, M., ULMER, H., WERNET, D. W., and RAMMENSEE, H. G. 2001. Electronic nose detects major histocompatability complex-dependent prerenal and postrenal odor components. *Proc. Natl. Acad. Sci. USA* 98:9249–9254.

PENN, D., and POTTS, W. 1998. How do major histocompatibility complex genes influence odor and mating preferences? *Adv. Immunol.* 69:411–436.

ROBERTS, S. C., GOSLING, L. M., SPECTOR, T. D., MILLER, P., PENN, D. J., and PETRIE, M. 2005. Body odor similarity in non-cohabiting twins. *Chem. Senses* 30:651–656.

SCHULZE, D. H., PEASE, L. R., GEIER, S. S., REYES, A. A., SARMIENTO, L. A., WALLACE, R. B., and NATHENSON, S. G. 1983. Comparison of the cloned H-2Kbm1 variant gene with the H-2Kb gene shows a cluster of seven nucleotide differences. *Proc. Natl. Acad. Sci. USA* 80:2007–2011.

YAMAZAKI, K., BEAUCHAMP, G. K., EGOROV, I. K., BARD, J., THOMAS, L., and BOYSE, E. A. 1983. Sensory distinction between H-2^b and H-2^{bm1} mutant mice. *Proc. Natl. Acad. Sci. USA* 80:5685–5688.

ZAVAZAVA, N., WESTPHAL, E., and MULLER-RUCHHOLTZ, W. 1990. Characterization of soluble HLA molecules in sweat and quantitative HLA differences in serum of healthy individuals. *J. Immunogenet.* 17:387–394.

by larval sea lampreys (Haslewood and Tokes 1969; Polkinghorne et al. 2001). A series of experiments demonstrated that PS is released by larvae, detected by adults at picomolar concentrations, and is behaviorally attractive to adult lampreys (Li et al. 1995; Polkinghorne et al. 2001; Vrieze and Sorensen 2001; Fine and Sorensen 2005). However, the behavioral activity of PS was considerably less than whole larval odor, suggesting that PS was part of a multi-component pheromone (Vrieze and Sorensen 2001). By using bioassay-guided fractionation, two novel sulfated steroids, petromyzonamine disulfate (PADS) and petromyzosterol disulfate (PSDS), were identified in larval water (Sorensen et al. 2005).

Methodological details of this isolation and identification, as well as biological tests with highly purified natural and synthetic compounds, have not previously been reported. In this paper, the isolation of PADS, PSDS, and PS is described for the first time, along with the results of new, confirmatory tests of the biological activity of both purified natural and synthetic compounds. This is the first complete account of the isolation and biological activity of a multi-component migratory pheromone in a fish. This manuscript has four goals: (1) to provide a path that others may follow to identify multi-component aquatic pheromones; (2) to provide key details not included in our initial report; (3) to provide new data on the specificity with which these components are discerned; and (4) to prove that this important pheromone has been correctly identified by providing descriptions of tests of highly purified and synthetic compounds.

Methods and Materials

Experimental Design and Rationale Pheromone isolation and purification proceeded in three phases: (I) extraction of the pheromone from larval sea lamprey holding water; (II) isolation of compounds from larval holding water extract; and (III) establishment of the olfactory and behavioral activity of the isolated compounds. The overarching goal was to isolate and identify the active compounds (see Hoye et al. 2007) and then to test these compounds to ascertain biological activity. Protocols were approved by the Animal Care and Use Committee of the University of Minnesota.

Phase I: Extraction of Pheromone from Larval Lamprey Holding Water A method to extract the complete pheromone from larval holding water was developed. Initial tests employed reversed-phase C18 resin (RP-C18) to extract compounds of interest from larval water (Fine et al. 2006). Larval water was collected following established protocols in which groups of 500 larval sea lampreys were maintained in 200-l flow-through tanks with the water turned off for 12 h after feeding (see Polkinghorne et al. 2001). Ten liter aliquots of this water (a quantity known to activate approximately 100,000 l of water and to represent a biologically relevant concentration; Vrieze and Sorensen 2001) were filtered through paper and passed through methanol-activated RP-C18 sep-paks (1 l/sep-pak, Waters Corp., MA, USA; Polkinghorne et al. 2001). Columns were washed with 5 ml 20% methanol (to remove unwanted highly polar compounds) followed by 5 ml hexane (to remove non-polar compounds) and then eluted with 5 ml of methanol.

Column eluates and other odors were tested for ability to attract migratory sea lampreys in 9-m-long preference mazes that had two side channels through which a mixture of Lake Huron water and river water lacking lampreys (1:100) was pumped (see Fine et al. 2004 for details). For tests, groups of four recently caught lampreys were placed into upstream cages and then released one cage at a time after sunset (when lampreys typically migrate) while experimental and control odors were added to the side channels. Time spent by the lampreys in each side was monitored for 15 min using overhead cameras and infrared lighting and later compared to an expected 50% (i.e., no preference) value using two-tailed t tests after a Zar transformation. Experiments employed 12–14 groups of lampreys. Calculations of pheromone concentration were based on concentration of larval holding water extract (number of larvae held in static holding water multiplied by the time they were held divided by final dilution in the maze).

Initial tests compared the activity of 10 l aliquots of larval water extract (0.018 larval hours per liter) to methanol (carrier) control passed through the columns. Next, extract potency was tested against 10 l of whole (unmodified) larval odor to ascertain whether all activity had been extracted. Behavioral experiments occurred during the lamprey migratory season (May–June) over 5 years (2001–2005).

Phase II: Isolation of Pheromonal Compounds from Larval Water using Bioassay-Guided Fractionation RP-C18 resin appeared to extract all pheromone compounds so a bioassay-guided fractionation scheme based on this extraction was designed. This scheme had seven steps and concluded with a test designed to determine if the isolated component(s) was as active as whole larval odor. The scheme was designed so that steps could be repeated to isolate additional components if/as needed.

II-1. Fractionation Larval holding water was extracted (as described above), dried under nitrogen, reconstituted in 60% methanol (110 μl), injected onto a RP-C18 column (Nova-pak, 4 μm column, Waters Chromatography, Milford, MA, USA) and eluted with a gradient of methanol and water (1 ml/min; 15% methanol for the first 4 min., followed by a

linear increase from 15% to 100% methanol from 4 to 60 min.) Twenty fractions (3 min/fraction) were collected during the course of each high-performance liquid chromatography (HPLC; Gilson HPLC system equipped with 305 and 306 pumps and a FC203 fraction collector) run. This step was repeated twice. The first used extract collected from 280 l of larval water, while the second used extract from 360 l of larval water to which ^{14}C-chenodeoxycholic acid (^{14}C-CDCA) was added as a tracer so as to monitor retention times by using an in-line radiation detector (INUS Systems, FL, USA).

II-2. Identification of Promising Fractions by Olfactory Activity Fractions were tested using electro-olfactogram (EOG) recording to identify those with the greatest promise. Briefly, migratory sea lampreys were anesthetized, immobilized, and positioned on a stand, while odors were pulsed across their olfactory epithelia and DC voltage transients, believed to reflect receptor binding activity, measured (see: Li et al. 1995). One-thousand-fold dilutions of each fraction (0.03 larval hour per liter), similar to that used in the behavioral assay, were tested.

II-3. Identification of Fractions with Strong Behavioral Activity Fractions were tested for behavioral activity using two-choice mazes (described above). First, mixtures of the EOG-active (with adjacent fractions) and the EOG-inactive fractions were tested to confirm that the EOG assay was correctly isolating all compounds with potential behavioral activity. Next, the activity of each EOG-active fraction was tested on its own to identify those with greatest promise. Aliquots of these fractions (representing 10 l of larval water, which produced a final maze concentration of 0.018 larval hours per liter) were tested against methanol control or whole extract.

II-4. LC/MS Sub-Fractionation of Active Fractions As a first step in isolating compounds with pheromonal activity, behaviorally active fractions (representing 20 l of larval holding water) were fractionated and analyzed by mass spectrometry (MS). These fractions were injected onto an RP-C18 column and eluted with a gradient of methanol and water (40% methanol for 4 min and increased to 65% methanol for 52 min and then to 100% methanol for 8 min; 1 ml/min). The eluate was routed to a splitter with 90% collected, and the remainder was passed to an ion trap mass spectrometer equipped with an ESI source (LCQ Classic, Thermo Electron Corporation, Waltham, MA, USA) using a spray voltage of 5 kV and sheath gas of 99% pure nitrogen at 60 psi.

II-5. Isolation of Individual Compounds with Olfactory Activity The fractions were fractionated into 20 sub-fractions (3 min/fraction), each of which was tested with EOG at a 1,000-fold dilution (0.6 larval hour per liter) on three fish. After EOG-active sub-fractions were identified, another three 20 l aliquots of that fraction were re-fractionated in the same manner using liquid chromatography (LC)/MS, except this time, compounds of interest were collected and tested for EOG activity (1,000-fold dilution).

II-6. Verification that Isolated Compounds Had Behavioral Activity Compound(s) isolated in step II-5 were tested in mazes against methanol control (0.018 larval hours per liter).

II-7. Determination of Whether Isolated Compounds Had a Majority of Pheromonal Activity The mixture of all known active compound(s) was tested in the maze against whole extract at equivalent concentrations (0.018 larval hours per liter). If the behavioral activity of the isolated compounds was less than the whole extract, step II-1 was repeated to obtain additional fractions/compounds and the basic procedure repeated with appropriate modifications using new combinations of isolated compounds before testing once again against the whole extract.

Phase III: Olfactory and Behavioral Activity of Compounds Isolated from Larval Water As a final step, the olfactory and behavioral activities of the compounds isolated in phase II were extensively tested for behavioral and olfactory (EOG) potency across a range of concentrations. In this experiment, three candidate pheromonal compounds (PS, PSDS, and PADS) were isolated and identified, one of which (PS) was commercially available and purchased (Toronto Research Chemicals, Canada). The other two compounds (PSDS and PADS) were novel and had to be collected and isolated from larval holding waters in mg quantities. To collect these quantities, 8,000 l of larval holding water was passed though XAD7HP resin, which was found to be more efficient than RP-C18 for extracting large volumes of water (Fine et al. 2006), and the extract fractionated following procedures described above. In addition, approximately 1 mg of PADS was synthesized from chenodeoxycholic acid (Hoye et al. 2007). While Sorensen et al. (2005) reported EOG data collected from PSDS and PADS that were ~65% pure, in this study, we tested new samples that were approximately 85% pure.

The sensitivity of the lamprey olfactory system to each compound was determined with EOG recording. Log molar dilutions ranging from 10^{-14} to 10^{-8} M of PS, PSDS, and PADS were tested on four lampreys, and responses were compared using *t* tests with 10^{-5} M L-arginine (a non-pheromonal odorant) as a standard. Following established protocols, EOG responses were calculated relative to the standard after subtracting responses (typically, zero) to blank water control. To determine whether PADS, PSDS, and PS were discerned by independent olfactory receptor

sites/mechanisms, cross-adaptation tests were conducted. This procedure exposes the olfactory epithelium to a compound (saturating olfactory receptors) before adding other stimuli into the background; if a response is measured, it is assumed that a different receptor mechanism is activated (see, Li and Sorensen 1997). In addition to PADS, PSDS, and PS, the sea lamprey sex pheromone component, 3-keto petromyzonol sulfate (3k-PZS; Li et al. 2002; Toronto Research Chemicals), was tested as a positive control. Each compound was tested at a concentration that elicited responses of about 25–30% of the standard (at 10^{-10} M). Results are expressed as percent unadapted response (PUR). A high PUR with a value close to 100% implies independent receptor sites, while a low PUR denotes shared receptor sites.

Finally, the behavioral activities of purified PADS, PSDS, and PS were tested at a range of concentrations in the maze as described above. Behavioral activity of synthesized PADS was tested alone and then against authentic PADS at concentrations of 10^{-12} M to confirm that the synthesized compound was correct (had full activity).

Results

Phase I: Extraction of Pheromone from Larval Holding Water When tested against methanol control, adults were strongly attracted to RP-C18-extracted larval water (average relative time in extract=67%±8% [95% CI] vs. expected value of 50%; $P<0.05$). Further, adults did not distinguish between larval water and RP-C18 extract (average time in extract vs. control=52%±7%; $P>0.05$).

Phase II: Isolation of Pheromonal Compounds Two HPLC fractionations (Fig. 1) were required for the isolation of all the active compounds. In the first fractionation, the strongest olfactory responses were, on average, elicited by fractions 1, 7, 9, 10, and 11 (Fig. 2a) with some variation among injected samples. Pooled fractions 1–20 from the first fractionation were attractive ($P<0.05$; Fig. 3a) to lampreys in the behavioral bioassay, while groups of EOG-inactive fractions were not ($P>0.05$). The group of EOG-active fractions was highly attractive ($P<0.05$) in the behavioral bioassay, and when tested against all 20 fractions, no difference was noted ($P>0.05$; Fig. 3a). This suggested that all key components were in these fractions. When fractions were tested individually in the behavioral bioassay (step II-3; Fig. 1), only fraction 10 was attractive ($P<0.05$; Fig. 3b). This fraction was fractionated by LC/MS (step II-4), with only sub-fraction 3 possessing EOG activity (step II-5a, Fig. 1; data not shown). Seven compounds were subsequently isolated from sub-fraction 3 using LC/MS, two of which elicited significant EOG responses. One elicited an average relative response of 49±

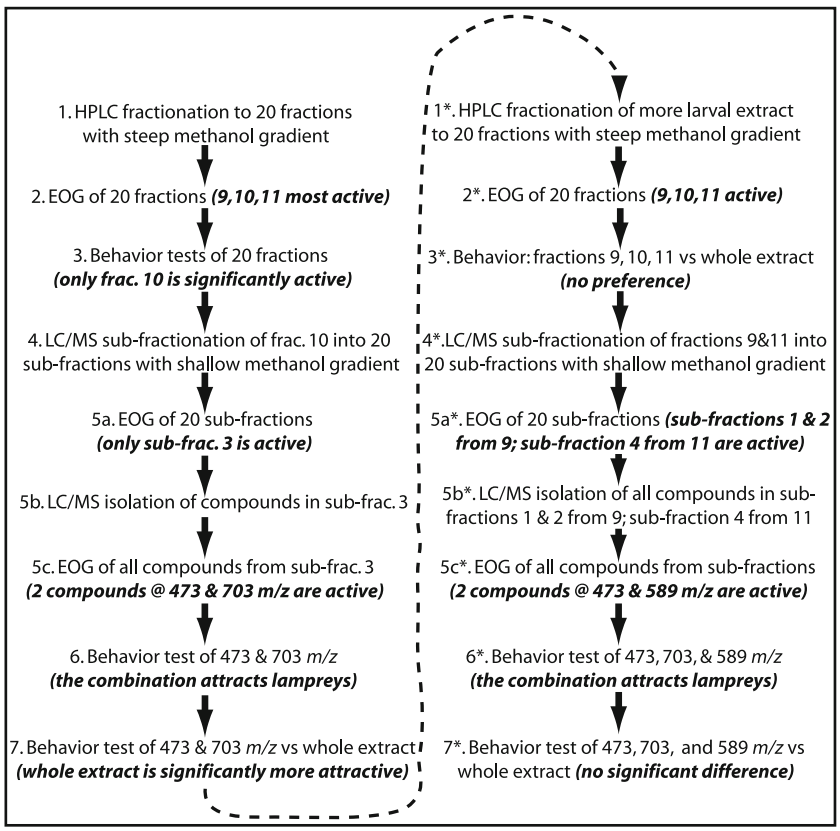

Fig. 1 Stepwise scheme of the specific methods and results employed in this study. Results are in *parentheses*, *bold* and *italics*. Note that two fractionations were employed; steps associated with the second have an *asterisk* and differ slightly from those associated with the first because of our findings

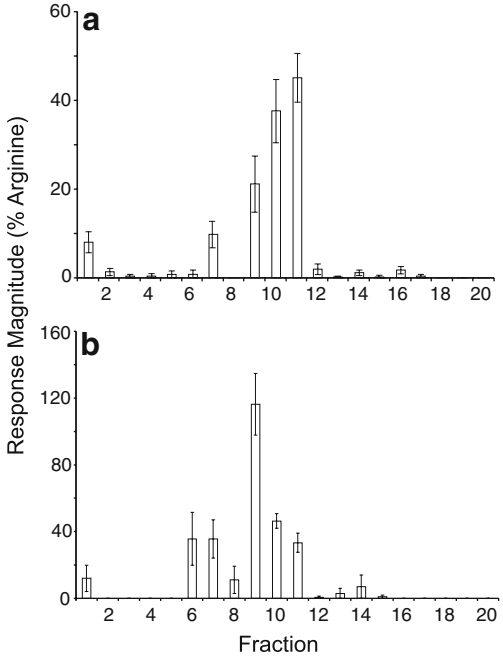

Fig. 2 Average electro-olfactogram (*EOG*) responses (±SE) recorded from migratory adult sea lamprey to fractionated larval extract (N=3). Results are expressed relative to the standard, 10^{-5} M L-arginine. Odors are RP-C18 extracts of 1 l of larval sea lamprey holding water that had been fractionated and diluted 1,000-fold to a concentration of 0.03 larval hour per liter. Two fractionations were conducted: **a** EOG responses to the first fractionation (shown in Sorensen et al. 2005); **b** EOG responses to fractions from the second fractionation which was used to confirm petromyzonamine disulfate and petromyzonol sulfate and isolate petromyzosterol disulfate

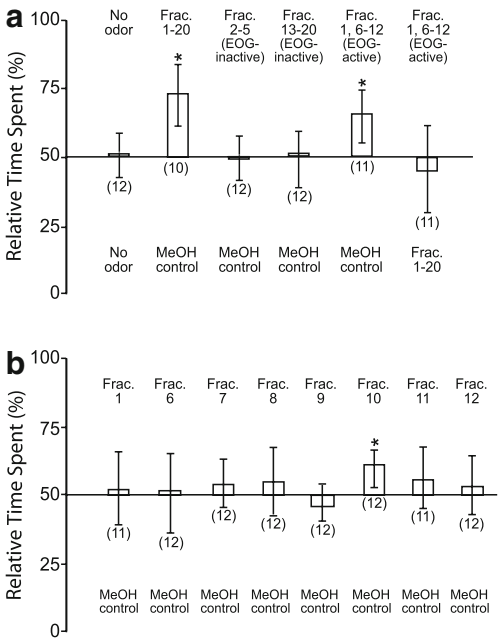

Fig. 3 Mean percent time (±95% CI) spent by adult sea lampreys in fractions of larval sea lamprey holding water in the maze (fractionation 1). Tests employed groups of fractions (**a**) and individual fractions (**b**). Test odor concentration was 0.018 larval hour per liter. *Numbers in parentheses* represent the number of trials (groups of four lampreys). Means were compared to a no-preference value of 50% by t test; *P<0.05. The first, second, fifth, and sixth tests (from *left*) in **a** are also shown in Sorensen et al. (2005)

7% (N=3) and had a negative ion at m/z 473.4, while the other elicited a relative average response of 81±18% and had negative ions at m/z 703.3 and 351.4. Closer inspection of m/z 351.4 showed an isotopic distribution pattern of 0.5, demonstrating that it was the dianion of m/z 703.3 and, therefore, the same compound. These compounds were combined and tested in the behavioral bioassay against whole extract (step II-7; Fig. 1); the mixture had significantly less activity than the extract (Fig. 4a).

Because the mixture of the two compounds did not fully explain the activity of larval extract, a second aliquot of larval extract was collected, extracted, fractionated (step II-1*, Fig. 1), and tested by EOG (step II-2*, Fig. 1). Fractions 6, 7, 8, 9, 10, and 11 had the greatest olfactory activity (Fig. 2b). The retention time of ^{14}C-CDCA in this set of fractionations suggested that on average, individual compounds ran about 1 min more quickly. Nevertheless, in both fractionations, fractions 9–11 explained the bulk of the activity. MS of a subsample of fraction 10 confirmed that it contained both compounds with respective m/z 473.4 and 703.3/351.4 m/z.

To check whether fractions 9 and 11 contained other components, these fractions were added to fraction 10 and tested against whole extract in the behavior maze (step 3*, Fig. 1). Lamprey did not discriminate between these two odors (Fig. 4b). Fractions 9 and 11 were further fractionated and the resulting sub-fractions tested by EOG (steps 4* and

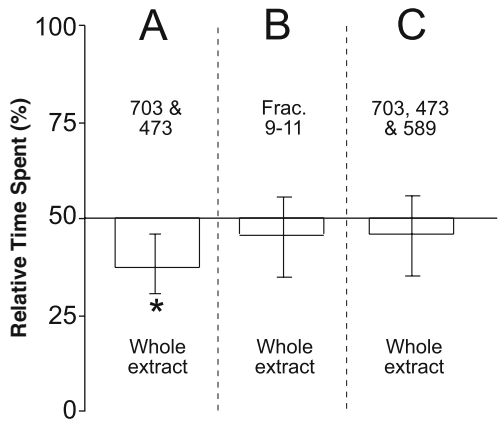

Fig. 4 Mean percent time (±95% CI) spent by adult sea lampreys in various fractionated odors tested against each other in the maze to confirm the complete presence of the pheromone. Three experiments were conducted: **a** The electro-olfactogram-active compounds from fraction 10 vs. whole extract (first fractionation); **b** fractions 9–11 vs. whole extract (second fractionation); **c** the three isolated compounds vs. whole extract (second fractionation). Concentration was 0.018 larval hours per liter. N=14 groups of four lampreys. Means were compared to a no-preference value of 50% using a t test; *P<0.05

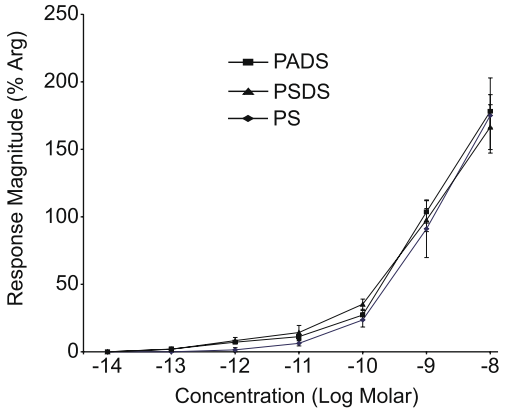

Fig. 5 Average electro-olfactogram responses (±SE) of adult sea lampreys (N=6) to natural, highly purified petromyzonamine disulfate (*PADS*), petromyzosterol disulfate (*PSDS*), and petromyzonol sulfate (*PS*) at a range of concentrations presented as a percentage of that elicited by the standard odorant, 10^{-5} M L-arginine

5a*, Fig. 1). Sub-fractions 1 and 2 from fraction 9 had EOG activity. Seven compounds were isolated from these sub-fractions, with one compound having EOG activity (data not shown). This compound had *m/z* 589.1/294.2 and elicited an EOG response of 99±9%. Closer inspection of *m/z* 294.2 *m/z* showed an isotopic distribution pattern of 0.5, demonstrating that it was the dianion of *m/z* 589.1. Sub-fractions 3 and 4 from fraction 11 both had EOG activity, but this was attributable to a single compound (*m/z* 473.3) which appeared to be the same compound as that found in fraction 10 previously; it had moderate olfactory activity (52±10%). When a sample of the compound with *m/z* 589.1 was added to a mixture (at natural ratios) of compounds with *m/z* 473.3 and 703.3 and tested against control water in the maze, the resulting three-component mixture was highly attractive (data not shown). Finally, there was no difference (P>0.05; Fig. 4c) in lamprey responses in the behavioral bioassay to this mixture and whole extract (step 6*, Fig. 1). Therefore, the respective compounds with *m/z* 473.3, 589.1, and 703.3 appear to elicit the majority of pheromonal activity.

Phase III: Biological Activity of the Pheromonal Compounds The three compounds isolated in phase II were identified (see, Sorensen et al. 2005; Hoye et al. 2007): *m/z* 473.4 was the negative molecular ion of PS, *m/z* 589.1 was the negative molecular ion of PSDS, and *m/z* 703.3 was the negative molecular ion of PADS. Preliminary tests using semi-purified samples of these compounds also found them to be attractive (Sorensen et al. 2005), but the biologically activity of purified samples has not been reported. Here we report the results of these studies using new samples. Approximately 85% pure PADS elicited strong EOG responses at 10^{-8} M and significant (P<0.05) responses at concentrations down to 10^{-13} M, but not at 10^{-14} M (N=6; P>0.05; Fig. 5). As measured by EOG, the detection thresholds of PSDS and PS were approximately 10^{-13} and 10^{-12} M, respectively (P<0.05; Fig. 5). When used as adapting stimuli, each compound suppressed EOG responsiveness to itself (Fig. 6a–d). Further, when the lamprey olfactory epithelium was adapted to PADS, the other three compounds (3k-PZS,

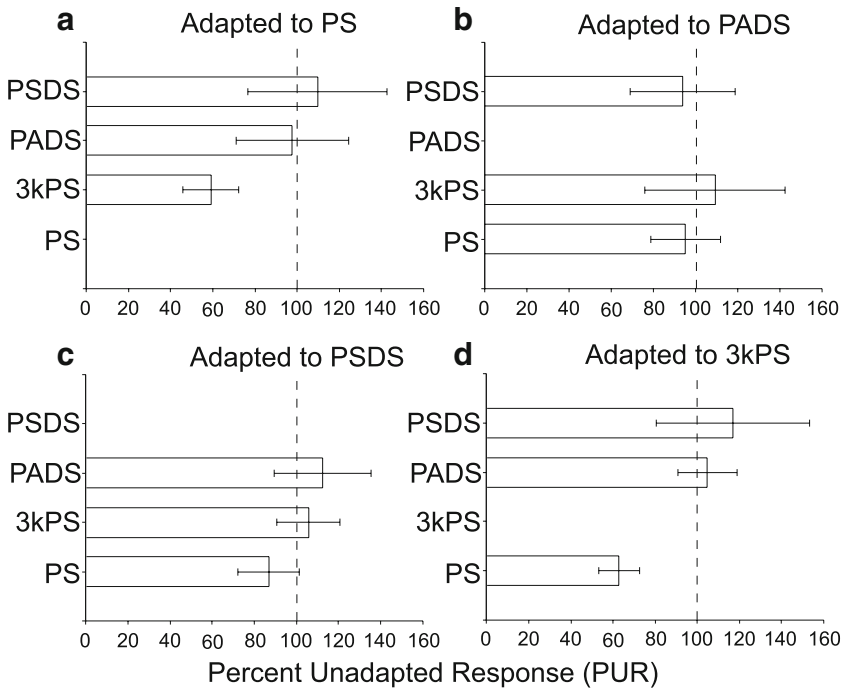

Fig. 6 Average electro-olfactogram responses (±SE) of adult sea lampreys (N=4) to petromyzonamine disulfate (*PADS*), petromyzosterol disulfate (*PSDS*), 3-keto petromyzonol sulfate (*3k-PZS*), and petromyzonol sulfate (*PS*) when adapted to one of these four compounds: **a** PS, **b** PADS, **c** PSDS, and **d** 3k-PZS. *Bars* (±SD) are the average percent unadapted response (*PUR*)

PSDS, and PS) elicited PURs of ~100% (Fig. 6b). The result was the same when the lamprey nose was adapted to PSDS (Fig. 6c). When adapted to PS and 3k-PZS, PURs for PADS and PSDS were ~100%. However, when adapted to PS, the PUR for 3k-PZS was only ~60% ($P<0.05$; Fig. 6a). Conversely, when the lamprey olfactory epithelium was adapted to 3k-PZS, the PUR for PS was 63% (Fig. 6d).

Pure natural PADS elicited behavioral responses at 10^{-13} M ($P<0.05$), but not at 10^{-14} M (Fig. 7a). Synthetic PADS elicited identical behavioral activity as natural PADS (Fig. 7b). Both PSDS and PS were attractive at 10^{-11} M ($P<0.05$), but not at 10^{-12} M (Fig. 7c,d). When natural and synthetic PADS were tested against each other in the maze at 10^{-12} M, the lampreys did not distinguish between them (avg=50.5%; $P=0.88$).

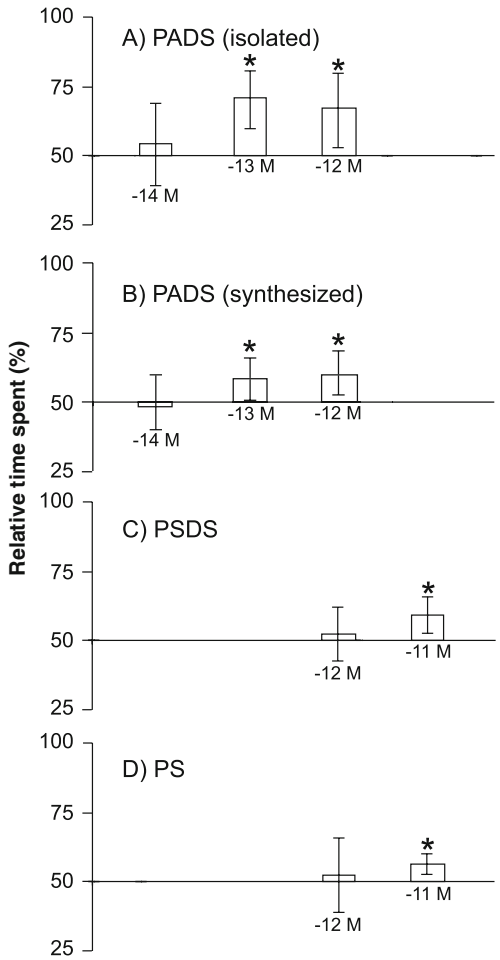

Fig. 7 Mean percent time (±95% CI) spent by adult sea lampreys in various concentrations of: **a** authentic petromyzonamine disulfate (*PADS*); **b** synthesized PADS; **c** petromyzosterol disulfate (*PSDS*); and **d** petromyzonol sulfate (*PS*) tested vs. methanol in the maze. $N=14$ trials for each experiment. Means were compared to a no-preference value of 50% using a *t* test; *$P<0.05$. The data used in **a**, **c**, and **d** is also described by Sorensen et al. (2005) who tested components with lower purity

Discussion

This study describes the isolation of pheromone components from larval lamprey holding water and the testing of their olfactory and behavioral activity and complements details already reported in Sorensen et al. (2005). First, and most importantly, the present dataset demonstrates for the first time that synthesized PADS, the most active component of the pheromone, is as biologically active as PADS isolated from larval holding water. Second, this study demonstrates for the first time that a mixture of the three compounds (not just the fractions that contain them) is as behaviorally attractive as whole larval odor when tested in our maze, demonstrating that these are the principal components of the pheromone. Third, cross-adaptation tests showed that each of the isolated compounds is detected with independent olfactory receptors. Fourth, we describe how to isolate multiple components from a pheromonal mixture—a tricky task in the case of the lamprey pheromone whose components synergize each other's activity (Fine 2006). Especially valuable is the ability of our assay to isolate key individual components one/several at a time and test their effects in a pheromonal mixture in an iterative fashion. Finally, this study confirms (through new behavior and EOG dose–response data) the potency of the pheromone, which is the first migratory pheromone identified in a vertebrate. Similar protocols could be used for structural elucidation of other complex aquatic pheromones.

Because the mixture of PADS, PSDS, and PS was not significantly different from the whole extract in the behavior maze, it seems reasonable to conclude that these compounds do indeed constitute the key components of the pheromone (Sorensen et al. 2005). Notably, the present data confirm our earlier preliminary study that used HPLC fractions (Sorensen et al. 2005) and olfactory studies of semi-purified components. However, the fact that the extract was consistently, albeit not significantly, more potent than the mixture of the three components as well as the mixture of fractions 9, 10, and 11 suggests that there may be other minor components which a more powerful/sensitive assay or larger sample size may discern. Such minor components could nevertheless be important in natural settings where background odors exist (Sorensen et al. 2003). The high potency of the RP-C18 extracts of larval holding water and the ability of XAD extracts of larval water to attract lampreys in the field (Wagner et al. 2006) suggest that if there are missing components, they are present in the extract and could be isolated by using the scheme presented here. The consistent olfactory activity of fractions 6 and 7 suggests a place to look. Allocholic acid, another minor bile acid released by sea lampreys (Li and Sorensen 1997), is probably not an important part of the

pheromone since it was found in fraction 16. Similarly, it is unlikely that 3k-PZS is a component of this pheromone, although it was occasionally found in low concentrations in fraction 9 (two of seven samples we examined over the course of several years; data not shown).

The behavioral detection thresholds for the pheromone components are very low: 10^{-13} M for PADS, and 10^{-11} M for both PSDS and PS. Recent EOG studies of synthesized PADS have also confirmed a 10^{-13} M threshold (Jeffrey and Sorensen, unpublished results). Our EOG cross-adaptation study showed that these compounds have independent olfactory receptor sites from each other and from the receptor for the lamprey sex pheromone component, 3k-PZS (Li et al. 2002). Similar scenarios have been described for insects (Roelefs 1995). Interestingly, and without obvious explanation, the cross-adaptation tests showed some overlap between receptor sites for PS and 3k-PZS, as seen in the study by Siefkes and Li (2004).

Why and how the sea lamprey evolved a multi-component pheromone is an interesting question, especially because it does not appear to be related to species specificity (Fine et al. 2004). Perhaps lampreys evolved a multi-component pheromone because it (or portions of it) might be easier to discern at greater distances/lower concentrations in the complex and variable natural environments that this creature has experienced over the past several hundred million years.

The results of this study are important for practical reasons. Complete identification of the pheromone now allows it to be developed for sea lamprey control in the Great Lakes (Sorensen and Hoye 2007). Similarly, this pheromone could be used to help restore threatened or endangered lamprey species around the world (Close et al. 2002; Yun et al. 2003) because unlike most pheromones identified in teleost fish (Stacey and Sorensen 2005; Sisler and Sorensen 2008), it seems to lack species specificity (Fine et al. 2004; Fine 2006). Research on the evolution and use of lamprey pheromones may now proceed with confidence that the three unique sulfated steroids we have isolated and identified constitute the key components of the migratory cue.

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References

CHRISTIE, G. C., and GODDARD, C. I. 2003. Sea lamprey international symposium (SLIS II): Advances in the integrated management of sea lamprey in the Great Lakes. *J. Great Lakes Res.* 26:1–14.

CLOSE, D. A., FITZPATRICK, M. S., and LI, H. W. 2002. The ecological and cultural importance of a species at risk of extinction, Pacific lamprey. *Fisheries* 27:19–25.

DANIELS, R. A. 2001. Untested assumptions: the role of canals in the dispersal of sea lamprey, alewife, and other fishes in the Eastern United States. *Environ. Biol. Fishes.* 60:309–329.

FINE, J. M. 2006. Isolation, identification, and biological characterization of the sea lamprey (*Petromyzon marinus*) migratory pheromone. PhD dissertation. University of Minnesota, Twin Cities.

FINE, J. M., and SORENSEN, P. W. 2005. Biologically relevant concentrations of petromyzonol sulfate, a component of the sea lamprey migratory pheromone, measured in stream water. *J. Chem. Ecol.* 31:2205–2210.

FINE, J. M., VRIEZE, L. A., and SORENSEN, P. W. 2004. Evidence that Petromyzontid lampreys employ a common migratory pheromone which is partially comprised of bile acids. *J. Chem. Ecol.* 30:2091–2110.

FINE, J. M., SISLER, S. P., VRIEZE, L. A., SWINK, W., and SORENSEN, P. W. 2006. A practical method for obtaining useful quantities of pheromones from sea lamprey and other fishes for identification and control. *J. Great Lakes Res.* 23:832–838.

HARDISTY, M. W., and POTTER, I. C. 1971. The general biology of adult lampreys, pp. 127–206, in M. W. Hardisty, and I. C. Potter (eds.). The Biology of Lampreys, vol. 1 Academic, London.

HASLEWOOD, G. A. D., and TOKES, L. 1969. Comparative studies of bile salts: Bile salts of the sea lamprey, *Petromyzon marinus*. *J. Biochem.* 114:179–184.

HOYE, T. R., DVORNIKOVS, V., FINE, J. M., ANDERSON, K. R., JEFFREY, C. S., MUDDIMAN, D. C., SHAO, F., SORENSEN, P. W., and WANG, J. 2007. Details of the structure determination of the sulfated steroids PSDS and PADS—new components of the sea lamprey (*Petromyzon marinus*) migratory pheromone. *J. Org. Chem.* 72:7544–7550.

LI, W., and SORENSEN, P. W. 1997. Highly independent olfactory receptor sites for naturally occurring bile acids in the sea lamprey, *Petromyzon marinus*. *J. Comp. Physiol. A* 180:429–438.

LI, W., SORENSEN, P. W., and GALLAHER, D. D. 1995. The olfactory system of migratory adult sea lamprey is acutely and specifically sensitive to unique bile acids released by conspecific larvae. *J. Gen. Physiol.* 105:569–587.

LI, W., SCOTT, A. P., SIEFKES, M. J., YAN, H., LIU, Q., and YUN, S.-S. 2002. Bile acid secreted by male sea lamprey that acts as a sex pheromone. *Science* 296:138–141.

MOORE, H. H., and SCHLEEN, L. P. 1980. Changes in spawning runs of sea lamprey (*Petromyzon marinus*) in selected streams in Lake Superior after chemical control. *Can. J. Fish. Aquat. Sci.* 37:1851–1860.

POLKINGHORNE, C. N., OLSON, J. M., GALLAHER, D. D., and SORENSEN, P. W. 2001. Larval sea lamprey release two unique bile acids to the water at a sufficient rate to produce a detectable pheromonal plume. *Fish Physiol. Biochem.* 24:15–30.

ROELEFS, W. L. 1995. Chemistry of sex attraction. *Proc. Natl. Acad. Sci. U. S. A.* 92:44–49.

SIEFKES, M. J., and LI, W. 2004. Electrophysiological evidence for detection and discrimination of pheromonal bile acids by the olfactory epithelium of female sea lampreys (*Petromyzon marinus*). *J. Comp. Physiol. A.* 190:193–199.

SISLER, S. P. and SORENSEN, P. W. 2008. Common carp and goldfish discern conspecific identity using chemical cues. *Behaviour* 145:1409–1429.

SMITH, B. R., and TIBBLES, J. J. 1980. Sea lamprey (*Petromyzon marinus*) in Lake Huron, Michigan, and Superior: history of invasion and control, 1936–1978. *Can. J. Fish. Aquat. Sci.* 37:1780–1801.

SORENSEN, P. W., and HOYE, T. R. 2007. A critical review of the discovery and application of a migratory pheromone in an invasive fish, the sea lamprey, *Petromyzon marinus* L. *J. Fish Biol.* 71(Supplement D):100–114.

SORENSEN, and VRIEZE, L. A. 2003. Recent progress understanding the chemical ecology and potential application of the sea lamprey migratory pheromone. *J. Great Lakes Res.* 29:66–84.

SORENSEN, P. W., VRIEZE, L. A., and FINE, J. M. 2003. A multi-component migratory pheromone in the sea lamprey. *Fish Physiol. Biochem*. 28:253–257.

SORENSEN, P. W., FINE, J. M., DVORNIKOVS, V., JEFFREY, C. S., SHOA, F., WANG, J., VRIEZE, L. A., ANDERSON, K. R., and HOYE, T. R. 2005. Mixture of new sulfated steroids functions as a migratory pheromone in the sea lamprey. *Nat. Chem. Biol.* 1:324–328.

STACEY, A. P. and SORENSEN, P. W. 2005. Hormones, pheromones, and reproductive behaviors. Chapter 9. in K. A. Sloman, S. Balshine; and R. W. Wilson (eds.). Behaviour: Interactions with Fish Physiology [Volume 24 in Fish Physiology (series editors W.S. Hoar, D.J. Randall, and A.P. Farrell]. Academic, New York.

TEETER, J. 1980. Pheromone communication in sea lampreys (*Petromyzon marinus*): implications for population management. *Can. J. Fish. Aquat. Sci.* 37:2123–2132.

VRIEZE, L. A., and SORENSEN, P. W. 2001. Laboratory assessment of the role of a larval pheromone and natural river odor in spawning river localization by migratory sea lamprey (*Petromyzon marinus*). *Can. J. Fish. Aquat. Sci.* 58:2374–2385.

WAGNER, C. M., JONES, M. L., TWOHEY, M. B., and SORENSEN, P. W. 2006. A field test verifies pheromones can be useful for sea lamprey (*Petromyzon marinus*) control in the Great Lakes. *Can. J. Fish. Aquatic Sci.* 63:475–479.

YUN, S.-S., SCOTT, A. P., BAYER, J. M., SEELYE, J. G., CLOSE, D. A., and LI, W. 2003. HPLC and ELISA analyses of larval bile acids from Pacific and western brook lampreys. *Steroids* 68:515–523.

sis, *B. griseocollis*, *B. morrisoni*, and *B. rufocinctus* in North America. In many of these species, scent marking of the perching site has been observed (Haas 1949; Alcock and Alcock 1983; O'Neill et al. 1991; Williams 1991; Kindl et al. 1999). Male labial gland secretions of only a few species with perching behavior have been investigated chemically (Hovorka et al. 1998; Bertsch et al. 2004; Rasmont et al. 2005). In this paper, we investigated the cephalic labial glands of males of the North American bumblebees *B. morrisoni* and *B. rufocinctus*.

Methods and Materials

Materials Males of *B. morrisoni* Cresson and *B. rufocinctus* Cresson were collected at 40°22′41″ N, 120°19′42″ W near Litchfield (about 30 km east of Susanville, CA, USA) where these species are abundant. Males of both species are low in numbers, shy, and fast in flight. Ten individuals of each species were caught when they were feeding at *Chrysothamnus* in late afternoon. Individual gland contents may be variable and change quantitatively and qualitatively (a) during a lifetime (see Sobotnik et al. 2008 for *B. terrestris*) and (b) during daytime (when secretions are used for scent marking in the morning, glands may be depleted later in the day; see Bergman 1997 for *B. lapidarius*). In our field collections, old males were recognized by their frayed wings and were discarded. The borders of the wings of all males used for gland preparation were smooth, indicating active males. Males were transported alive to the laboratory and fed with a honey solution for 2 days in flight cages so that they could refill their glands. Then, they were frozen after a short flight activity very early in the morning. The cephalic part of the labial glands was dissected from the head of frozen males and placed in vials (glands from five males per vial) containing 0.2-ml pentane. As the aim of this investigation was not to study the variability of gland content but to elucidate the complexity of a composition of bumblebee male labial glands, we pooled the glands of five males into a single vial. This method allowed us to detect even minor compounds present in the glands.

Gas Chromatography/Mass Spectrometry A Finnigan MAT TSQ700 gas chromatograph (GC)/tandem mass spectrometer (MS) was employed. GC was carried out on a Hewlett-Packard Ultra 1 column (50 m, 0.2 mm i.d., 0.11 μm film thickness) in a splitless mode with helium as carrier gas at an inlet pressure of 300 kPa. The split valve was opened for 1 min. Initial temperature of 120°C was held for 1 min, then increased at 8°C/min to 280°C, at 3°C/min to 310°C, and at 1°C/min to 320°C. This temperature was held for 10 min. Mass spectrometer conditions were interface temperature 300°C, source temperature 130°C, electron energy 70 eV, emission current 0.2 mA, and electron multiplier 1,400 V. When using the positive ion chemical ionization (CI) mode, ammonia CI gas pressure was 70 Pa. Compounds were identified by comparing their mass spectra with those of the NIST'02 Library (National Institute of Standards and Technology, USA) and by retention times and molecular ions from CI spectra. To determine the position of double bonds, derivatization with dimethyl disulfide was used as described by Buser et al. (1983).

Results

The labial glands of both *B. morrisoni* and *B. rufocinctus* males contained a mixture of acyclic diterpenes (alcohols, acetates, and hydrocarbons), two cyclic terpenes, and various straight-chain fatty acid derivates (alcohols, esters, and both saturated and unsaturated hydrocarbons with C21–C31). Typical chromatograms of the male cephalic labial gland secretions of *B. rufocinctus* and *B. morrisoni* are shown in Fig. 1. The compounds are summarized in Table 1.

In labial glands of male *B. morrisoni*, the major compound was 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (Fig. 1, 82% of total peak area, peak 20). In addition, considerable amounts of (6*E*, 10*E*)-3,7,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (peak 6), 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 9), 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol (peak 18), and hexadecyl dodecanoate (peak 30) were detected. Traces of 3,7,11-trimethyl-2,6,10-dodecatrien-1-ol (peak 3), two more isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 7 and 11), two unidentified cyclic diterpenes (peak 13a and 14), 9-hexadecenyl 9-tetradecenoate (peak 31), 9-octadecenyl 9-tetradecenoate (peak 33), and eicosenyl 9-tetradecenoate (peak 34) complete the pattern of substances found. Small amounts of 9-hexadecenol (peak 4) and hexadecanol (peak 5) were identified. The peak area of hexadecyl dodecanoate (30) with about 4.5% of the total peak area shows that considerable amounts of hexadecanol may be used by this species to form esters.

In labial glands of male *B. rufocinctus*, the major compound was 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (Fig. 1, 79% of total peak area, peak 20). Furthermore, considerable amounts of 9-hexadecenol (peak 4), (6*E*, 10*E*)-3,7,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene (peak 6), and 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 9) were detected. Minor amounts of two more isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene (peak 7 and 11) and 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol (peak 18)

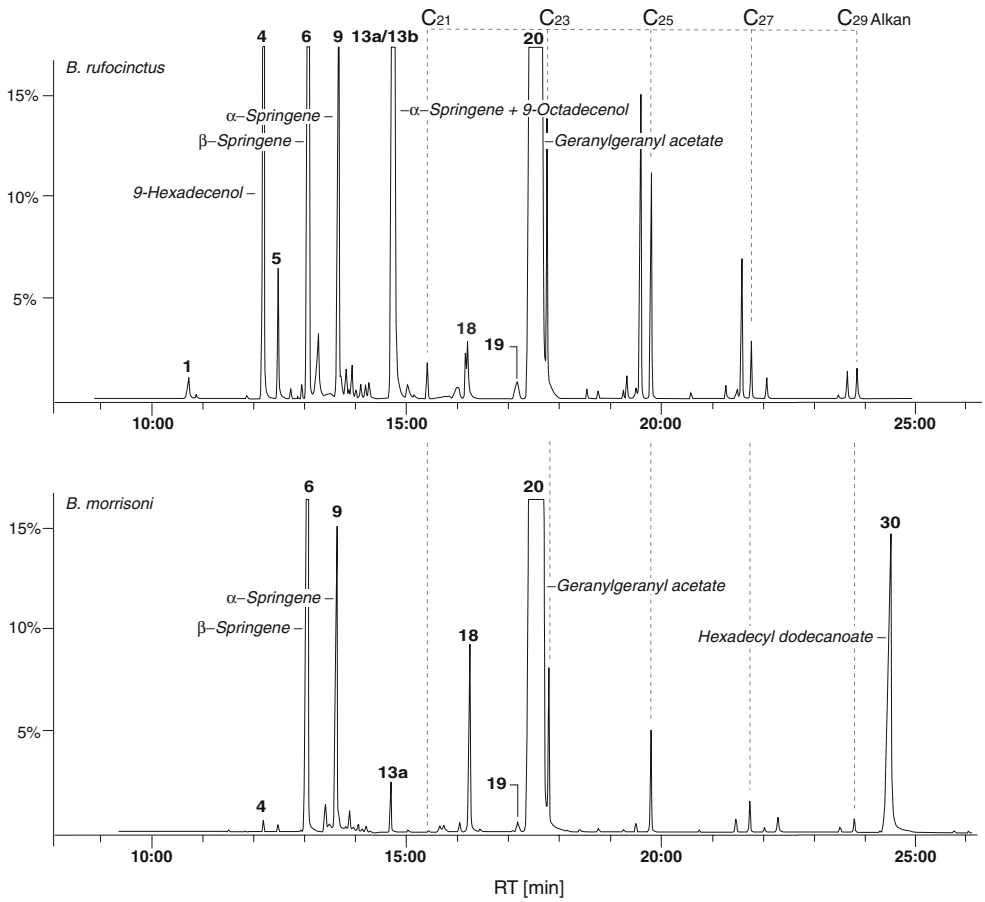

Fig. 1 Total ion chromatograms of the pentane extract of the cephalic labial gland from *B. morrisoni* and *B. rufocinctus*. *Numbers* correspond to the numbers in the peak list (Table 1). Some compounds listed in Table 1 were present in quantities too low to be visible in these chromatograms

were found. 9-Octadecenol (peak 13b) was also present in considerable amounts co-eluting with the unidentified cyclic diterpene detected in the GC of *B. morrisoni* (peak 13a). Small amounts of an isomer of this unidentified cyclic diterpene (peak 14), 9-tetradecenoic acid (peak 1), tetradecanoic acid (peak 2), hexadecanoic acid (peak 8), 9-octadecenoic acid (peak 17), 9-hexadecenyl acetate (peak 10), hexadecyl acetate (peak 12), and 9-octadecenyl acetate (peak 17) were detected.

Compared to the chemistry of labial glands of other male bumblebee species, the proportion of hydrocarbons and esters was low in the species studied here. Only few wax-type esters were found (Table 1). Typical GCs of the species studied here also contained four diterpenes, i.e., isomers of springene. Their molecular ion was *m*/*z*=272. Their mass spectra were identical with those of (6*E*, 10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [β-springene] (peak 6) and α-springene (peaks 7, 9, 10). The relative retention times of (6*E*, 10*E*)-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [β-springene], (3*Z*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene, and (3*E*,6*E*,10*E*)-3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene [the α-springenes] were as reported by Burger et al. (1981). As expected, the mass spectra of the three isomers of 3,7,11,15-tetramethyl-hexadeca-1,3,6,10,14-pentaene [α-springene] were nearly identical.

Peaks 13a and 14 in the secretions could not be identified. The mass spectra of both compounds exhibited molecular ions at *m*/*z*=272, indicative of a hydrocarbon of the molecular formula $C_{20}H_{32}$. The molecular mass was confirmed by CI (NH_3) and the pseudo molecular ion *m*/*z* 273 ($[M+H]^{+\cdot}$), whereas the pseudo molecular ion *m*/*z* 290 ($[M+NH_4]^+$) could not be detected. The mass spectrum of peak 13a was characterized by the following fragment ion *m*/*z* (%) values: 272 (19, M^+), 135 (100), 120 (11), 119 (10), 107 (90), 105 (36), 93 (43), 91 (33), 79 (11), 77 (13), 69 (46), 53 (13), 41 (33). The mass spectrum of peak 14 was nearly identical. The most intense fragment ions *m*/*z* 135 ($[C_{10}H_{15}]^+$) and *m*/*z* 107 ($[C_8H_{11}]^+$) were observed in cyclic and polycyclic hydrocarbons indicating peak 13a and peak 14 to be isomers of a cyclic terpene.

Discussion

The labial glands of most bumblebee males with the *patrolling* premating behavior contain a pattern of straight chain primary alcohols [C12–C26] (Bergström et al. 1981,

Table 1 Compounds of the cephalic labial glands of males of *B. rufocinctus* and *B. morrisoni* and structural evidence

No.	Compound	RT	RI	*B. rufocinctus* [% of total peak area]	*B. morrisoni* [% of total peak area]	$M^{+\bullet}$	Diagnostic mass spectral fragments (*m*/*z*)
1	9-Tetradecenoic acid	10:48		0.11	–	226	**55**, 69, 81, 166, 208, {117, 203, $M^{+\bullet}$ 320}
2	Tetradecanoic acid	10:50	1,720	0.67	–	228	60, **73**, 129
3	3,7,11-Trimethyldodecatrien-1-ol	12:00	1,744	Trace	Trace	222	**69**, 93, 107, 133, 161, 204
4	9-Hexadecenol	12:18	1,836	0.54	Trace	240	M-18=222, {145, 189, $M^{+\bullet}$ 348}
5	Hexadecanol	12:35	1,859	0.36	Trace	242	M-18=224
6	7,11,15-Trimethyl-3-methylene-1, 6,10,14-hexadecatetraene	13:13	1,906	3.79	6.67	272	41, 55, **69**, 133, 161, 187, 257
7	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	13:33	1,935	0.13	Trace	272	41, 55, **69**, 107, 119, 135,
8	Hexadecanoic acid	13:38	1,950	0.10	–	256	60, **73**, 129, 256
9	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	13:46	1,955	1.45	2.40	272	41, 55, **69**, 107, 119, 134, 191, 257
10	9-Hexadecenyl acetate	13:55	1,967	0.06	–	282	**43**, 55, 61, M-60=222
11	3,7,11,15-Tetramethyl-hexadeca-1, 3,6,10,14-pentaene	14:02	1,974	0.08	Trace	272	41, 55, **69**, 107, 119, 133, 187
12	Hexadecyl acetate	14:17	2,009	0.03	–	284	**43**, 55, 61, M-60=224
13 a	Cyclic terpene ? ($C_{20}H_{32}$)	14:48	2,039	~0.7	0.25	272	55, 69, 107, **135**
13 b	9-Octadecenol	14:52	2,045	~6.5	–	268	41, **55**, 67, 82 M-18=250, {145, 299, $M^{+\bullet}$ 444}
14	Cyclic terpene ? ($C_{20}H_{32}$)	15:07	2,068	0.04	Trace	272	55, 69, **107**, 135
15	Heneicosane	15:31	2,100	0.08	–	296	41, 43, **57**, 71, 85
16	9-Octadecenyl acetate	16:15	2,152	0.10	–	310	**43**, 55, 61, M-60=250
17	11-Octadecenoic acid	16:17	2,162	0.20	–	282	**55**, 69, 83, 125, 222, {145, 231, $M^{+\bullet}$ 376}
18	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraen-1-ol	16:20	2,169	0.10	1.17	290	41, 55, **69**, 81, 93, 95, 265, 271
19	3,7,11,15-Tetramethyl-6,10, 14-hexadecatrienyl acetate	17:17	2,250	0.15	0.02	334	41, 43, **69**, 81, 93, 265, 291, M-60=274
20	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl acetate	17:45	2,278	78.77	82.18	332	41, 43, **69**, 81, 93, 263, 289, M-60=272
21	Tricosane	17:51	2,300	0.81	0.67	324	41, 43, **57**, 71, 85
22	3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl butyrate	19:21	2,445	0.02	–	360	**69**, 81, 93, M-88=272
23	?,?-Pentacosadiene	19:25	2,452	0.06	–	348	41, 43, **55**, 69, 83
24	7-Pentacosene	19:46	2,485	0.84	0.02	350	41, 43, **55**, 69, 83, {145, 299, $M^{+\bullet}$ 444}
25	Pentacosane	19:55	2,500	0.58	0.66	352	41, 43, **57**, 71, 85
26	7-Heptacosene	21:35	2,683	0.03	0.03	378	41, 43, **55**, 69, 83, {145, 327, $M^{+\bullet}$ 472}
27	Heptacosane	21:44	2,700	0.15	0.18	380	41, 43, **57**, 71, 85
28	3,7,11,15,19-Pentamethyl-2,6,10, 14,18-eicosapentaenyl acetate	22:02	2,729	0.05	–	400	**69**, 81, 93, M-60=340
29	Nonacosane	23:48	2,900	0.09	0.10	408	41, 43, **57**, 71, 85
30	Hexadecyl dodecanoate	24:39		–	4.51	424	[183, 201; 224][a]
31	9-Hexadecenyl 9-tetradecenoate	26:25		0.17	–	448	43, **55**, 69, [a][209, 227; 222]
32	Ergosta-5,24-dien-3β-ol	27:13		0.04	–	398	55, 69, **314**
33	9-Octadecenyl 9-tetradecenoate	28:53		1.15	–	476	[a][208, 227; 250]
34	Eicosenyl 9-tetradecenoate	31:30		0.65	–	504	[a][208, 227; 278]

RT retention time; *RI* retention index; $M^{+\bullet}$ molecular ion; ester: [a] [acylium ion of acid, protonated acid; alcohol M-18], DMDS adducts {xxx, xxx, $M^{+\bullet}$ xxx}

Valterová and Urbanová 1997). The corresponding acetates are often found only in minor amounts or as traces. Indeed, sometimes they are completely absent and occur only because of an aging process of the prepared glands. Our study and other previous studies show that the secretions of male bumblebees with the *perching* premating behavior differ from bumblebees with *patrolling* premating behavior in the occurrence of acetates as the leading components (Table 2).

In *B.* (*Cullumanobombus*) *rufocinctus*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 9-octadecenol contributed 84% to the total peak area. In *B.* (*Separatobombus*) *morrisoni*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol [geranylgeraniol] contributed 84% to the total peak area. In *B.* (*Separatobombus*) *griseocollis*, tetradecyl acetate and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] contributed 97% to the total peak area (Bertsch et al. 2004). In *B.* (*Confusibombus) confusus, (Z)*-9-octadecenyl acetate and 3,7,11,15-tetramethyl-6,10,14-hexadecatrien-1-ol [geranylcitronellol] contributed 77% to the total peak area (Hovorka et al. 1998), and in *B.* (*Sibiricobombus*) *vorticosus*, 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate] and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraen-1-ol [geranylgeraniol] contributed 87% to the total peak area (Rasmont et al. 2005). In all labial gland secretions of males with perching behavior, two compounds dominate the marking secretions, with an acetate being the dominant compound.

A mixture of substances with high and lower volatility is characteristic of the scent glands in male bumblebees. Bergman and Bergström (1997) detected the main component, farnesol, of the labial glands of *B.* (*Pyrobombus*) *pratorum* in headspace samples from marked leaves, but they could not detect 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate [geranylgeranyl acetate], also produced by the glands, in these samples. Kindl et al. (1999) showed that 3,7,11,15-tetramethyl-6,10,14-hexadecatrienyl acetate [geranylcitronellyl acetate], though present only in minor amounts in the labial glands of *B.* (*Confusibombus*) *confusus*, could be detected in headspace samples of the male-marked perch (dry flower head of *Centaurea stoebe*). O'Neill et al. (1991) studied marking behavior and labial glands of male *B. rufocinctus*. Their results suggest that males of this species mark leaves of *Symphoricarpus* and *Ribes* with at least three components of labial gland secretion. Even though these compounds were not identified, the retention times given for them indicate that they might be hexadecenol (O'Neill et al., Fig. 4, peak 12.31), octadecenol (peak 14.29), and 3,7,11,15-tetramethyl-2,6,10,14-hexadecatetraenyl acetate (peak 16.37). Less volatile compounds left on leaves may remain detectable until the next day, helping the bumblebees to find and reconstruct the activity, of the previous day. Evaporation of alcohols and acetates is a first-order process (release rate is proportional to the amount of pheromone present) with long half-lives (Butler and McDonough 1981; McDonough et al. 1989) strongly depending on size, weight, and polarity of the compound molecule.

In labial gland secretions of *B.* (*Pyrobombus*) *pratorum*, two isomers of 3,7,11-trimethyl-2,6,10-dodecatriene [farnesene] were identified, the only isoprenoid hydrocarbons detected in bumblebee labial glands so far (Valterová et al. 1997). (3*E*, 6*E*, 10*E*)-3,7,11,15-Tetramethyl-hexadeca-1,3,6,10,14-pentaene and 7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene [α- and β-springene, respectively] detected in the labial glands of *B.* (*Separatobombus*) *griseocollis* were newly identified isoprenoid hydrocarbons in bumble bee secretions (Bertsch et al. 2004). Meanwhile, these substances also have been detected in *B.* (*Sibiricobombus) vorticusus* (Rasmont et al. 2005), a male bumblebee with perching habit. (6*E*, 10*E*)-β-Springene has been isolated from a diversity of organisms, including the paracloacal gland of reptiles [the American alligator (Ibrahim

Table 2 Main compounds (% of peak area) of the cephalic labial glands of *Bombus* males with perching behavior

Compound	*B. rufocinctus*	B. morrisoni	*B. griseocollis*[a]	*B. confusus*[b]	B. vorticosus[c]	$M^{+\bullet}$
Tetradecyl acetate	–	–	82%	–	–	256
9-Octadecenol	5%	–	–	–	–	268
3,7,11,15-Tetramethyl-6,10, 14-hexadecatrien-1-ol	–	–	–	30%	–	292
3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraen-1-ol	–	2%	–	–	13%	290
9-Octadecenyl acetate	–	–	–	47%	–	310
3,7,11,15-Tetramethyl-2,6,10, 14-hexadecatetraenyl acetate	79%	82%	15%	–	74%	332
Total	84%	84%	97%	77%	87%	

[a] (Bertsch et al. 2004), [b] (Hovorka et al. 1998), [c] (Rasmont et al. 2005)

et al. 1998) and the smooth-fronted caiman (Avery et al. 1993)], the dorsal secretions of mammals [collared peccary (Waterhouse et al. 1996), white-lipped peccary (Waterhouse et al. 2001), and springbok (Burger et al. 1978, 1981)], the Dufour glands of insects [Australian ant *Nothomyrmecia macrops* (Billen et al. 1988), the Old World army ant *Aenictus rotundatus* (Oldham et al. 1994), the ectoparasitoid *Habrocon hebetor* (Fukushima et al. 1990; Howard et al. 2003), and as a trace component in the stingless bee *Nannotrigona testaceicornis* (Cruz-Lopez et al. 2001)]. Additional isomers of α-springene described so far are 3,7,11,15-tetramethyl-hexadeca-1,4,6,10,14-pentaene (Waterhouse et al. 1996; *Tayassu tajacu*, Mammalia) and 2,6,11,15-tetramethyl hexadeca-2,6,8,10,14-pentaene (Burger et al. 1981; see Table 1; *Antidorcas marsupialis*, Mammalia, and Zhou et al. 2006; *Citrus grandis*, Spermatophyta).

Studies of the perching behavior of bumblebees have revealed that no activity of virgin females (gynes) is detected near perches that have been marked by males with labial glandular secretion. This effect of male labial glandular secretion in male–female interactions has been shown in *B.* (*Separatobombus*) *griseocollis* (Alcock and Alcock 1983), in *B.* (*Cullumanobombus*) *rufocinctus* (O'Neill et al. 1991), and in *B.* (*Confusibombus*) *confusus* (Kindl et al. 1999). The question whether male labial glandular secretions mark small territories or individual perches and thus, mediate male–male interactions, need to be addressed in future studies.

References

ALCOCK, J., and ALCOCK, J. P. 1983. Behaviour in two bumblebees, *Bombus nevadensis auricomus* and *B. griseocollis* (Hymenoptera: Apidae). *J. Zool.* 200:561–570.

AVERY, J. W., SHATAGATI, A., TURNER, A., WHEELER, J. W., and WELDON, P. J. 1993. β-Springene in the paracloacal gland secretions of the smooth-fronted caiman (*Paleosuchus trigonatus*). *Biochem. Syst. Ecol.* 21:533–534.

BERGMAN, P. 1997. Chemical Communication in Bumblebee Premating Behaviour. PhD Thesis, Göteborg University, Sweden.

BERGMAN, P., and BERGSTRÖM, G. 1997. Scent marking. Scent origin, and species specificity in male premating behaviour of two Scandinavian bumblebees. *J. Chem. Ecol.* 23:1235–1251.

BERGSTRÖM, G., SVENSSON, B. G., APPELGREN, M., and GROTH, I. 1981. Complexity of bumble bee marking pheromones: Biochemical, ecological and systematical interpretations, pp. 175–183, in P. E., Howse, J. L., and Clément (eds.). Biosystematic of Social Insects. Systematic Association Special Volume No. 19, Academic Press, London, New York.

BERTSCH, A., SCHWEER, H., and TITZE, A. 2004. Analysis of the labial gland secretions of the male bumblebee *Bombus griseocollis* (Hymenoptera: Apidae). *Z. Naturforsch.* 59c:701–707.

BILLEN, J. P. J., JACKSON, B. D., and MORGAN, E. D. 1988. Secretion of the Dufour gland of the ant *Nothomyrmecia macrops* (Hymenoptera: Formicidae). *Experientia* 44:715–719.

BURGER, B. V., LEROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R.C. 1978. Mammalian pheromone studies III. *(E, E)*-7,11,15-trimethyl-3-methylene-hexadeca-1,6,10,14-tetraene, a new diterpene analogue of beta-farnesene from the dorsal gland of the springbok, *Antidorcas marsupialis*. *Tetrahedon Lett.* 5221–5224.

BURGER, B. V., LEROUX, M., SPIES, H. S. C., TRUTER, V., and BIGALKE, R. C. 1981. Mammalian pheromone studies. 4. Terpenoid compounds and hydroxy esters from the dorsal glands of the springbok *Antidorca marsupialis*. *Z. Naturforsch.* 36c: 340–343.

BUSER, H. R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond positions in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

BUTLER, L. I., and MCDONOUGH, L. M. 1981. Insect pheromones: Evaporation rates of alcohols and acetates from natural rubber septa. *J. Chem. Ecol.* 7:627–633.

CRUZ-LOPEZ, I., FLAVIA, E., PATRICIO, L. R. A., and MORGAN, E. D. 2001. Secretion of stingless bees: The Dufour gland of *Nannotrigona testaceicornis*. *J. Chem. Ecol.* 27:69–80.

FUKUSHIMA, J., KUWAMARA, Y., YAMADA, A., and SUZUKI, T. 1990. New non-cyclic homo-diterpene from the sting glands of *Bracon hebetor* Say (Hymenoptera, Braconidae). *Agric. Biol. Chem.* 54:809–810.

HAAS, A. 1949. Arttypische Flugbahnen von Hummelmännchen. *Z. Vergl. Physiol.* 31:281–307.

HOVORKA, O., URBANOVÁ, K., and VALTEROVÁ, I. 1998. Premating behavior of *Bombus confusus* males and analysis of their labial gland secretion. *J. Chem. Ecol.* 24:183–193.

HOWARD, R. W., BAKER, J. E., and MORGAN, E. D. 2003. Novel diterpenoids and hydrocarbons in the Dufour gland of the ectoparasitoid *Habrobracon hebetor* (Say) (Hymenoptera: Braconidae). *Arch. Insect Biochem. Physiol.* 54:95–109.

IBRAHIM, S. A., AVERY, J. W., WELDON, P. J., and WHEELER, J. W. 1998. Age-class differences in lipids from the paracloacal glands of the American alligator (*Alligator missippiensis*). *Z. Naturforsch.* 53c:201–209.

KINDL, J., HOVORKA, O., URBANOVÁ, K., and VALTEROVÁ, I. 1999. Scent marking in male premating behavior of *Bombus confusus*. *J. Chem. Ecol.* 25:1489–1500.

LLOYD, J. E. 1981. Sexual selection: Individuality, identification, and recognition in a bumblebee and other insects. *Florida Entomol.* 64:89–107.

MCDONOUGH, L. M., BROWN, D. F., and ALLER, W. C. 1989. Insect sex pheromones: Effect of temperature on evaporation rates of acetates from rubber septa. *J. Chem Ecol.* 15:779–790.

OLDHAM, N. J., MORGAN, E. D., GOBIN, B., SCHOETERS, E., and BILLEN, J. 1994. Volatile secretions of an Old World army ant, *Aenictus rotundatus*, and chemotaxonomic implications of army ant Dufour gland chemistry. *J. Chem. Ecol.* 20:3297–3305.

O'NEILL, K. M., EVANS, H. E., and BJOSTAD, L. B. 1991. Territorial behavior in males of three North American species of bumblebees (Hymenoptera, Apidae, *Bombus*). *Can. J. Zool.* 69: 604–613.

RASMONT, P., TERZO, M., AYTEKIN, A. M., HINES, H., URBANOVA, K., CAHLIKOVA, K., and VALTEROVA, I. 2005. Cephalic secretions of the bumblebee subgenus *Sibiricobombus* Vogt suggest *Bombus niveatus* Kriechbaumer and *Bombus vorticusus* Gerstaecker are conspecific (Hymenoptera, Apidae, *Bombus*). *Apidologie* 36:571–584.

SCHREMMER, F. 1972. Beobachtungen zum Paarungsverhalten der Männchen von *Bombus confusus* Schenk. *Z Tierpsychol.* 31: 503–512.

SOBOTNIK, J., KALINKOVÁ, B., CAHLIKOVÁ, L., WEYDA, F., PTACEK, V., and VALTEROVÁ, I. 2008. Age-dependent changes in structure and function of the male labial glands in *Bombus terrestris*. *J. Insect Physiol.* 54:204–214.

VALTEROVÁ, I., and URBANOVÁ, K. 1997. Chemiké signály cmeláku [Chemical signals of bumble bees]. *Chem. Listy* 91:846–857 (in Czech).

WATERHOUSE, J. S., KE, I., PICKETT, J. A., and WELDON, P. J. 1996. Volatile components in dorsal gland secretions of the collared peccary, *Tayassu tajacu* (Tayassuidae, Mammalia). *J. Chem. Ecol.* 22:1307–1314.

WATERHOUSE, J. S., HUDSON, M., PICKETT, J. A., and WELDON, P. J. 2001. Volatile compounds in dorsal secretions of the white-lipped peccary, *Tayassu peccari*, from Bolivia. *J. Chem. Ecol.* 27:2459–2469.

WILLIAMS, P. 1991. The bumble bees of the Kashmir Himalaya (Hymenoptera: Apidae, Bombini). *Bull. Br. Mus. Nat. Hist. (Ent.)* 60:1–204.

ZHOU, J., ZHOU, Ch., JIANG, X., and XIE, L. 2006. Extraction of essential oil from shaddock peel and analysis of its components by gas chromatography-mass spectrometry. *J. Cent. South Univ. Technol.* 13:44–48.

than males produced by their mother (r=0.25). Although worker reproduction may be favored due to relatedness, other factors such as maintaining colony efficiency and conflict over sex allocation favor reproductively restrained workers (Cole 1986; Ratnieks 1988). Under queenright conditions, workers usually either behaviorally inhibit (police) the reproductive efforts of other workers or are selected to exhibit reproductive self-restraint (Ratnieks 1988; Wenseleers et al. 2004).

The two most common policing behaviors in ant societies are physical policing of potential egg layers and egg policing (Monnin and Ratnieks 2001; Ratnieks et al. 2006). The reproductive efforts of individuals that are physically policed are inhibited by nestmates attacking the policed individual (Hölldobler and Carlin 1989; Dietemann et al. 2003; Hartmann et al. 2003). Egg policing occurs when worker-laid eggs are destroyed by nestmates (Kikuta and Tsuji 1999; D'Ettorre et al. 2004; Endler et al. 2004). In order for either one of these policing behaviors to be carried out effectively the policing individuals must have reliable information that indicates which workers should be policed or which eggs should be destroyed. Hydrocarbon blends present on the cuticle of workers and queens, and on the surface of eggs, are thought to contain these signals.

Cuticular hydrocarbons provide resistance to desiccation (Lockey 1988) and have the potential for holding information through recognizable quantitative and qualitative blend differences (Monnin 2006; Le Conte and Hefetz 2008). Changes in cuticular hydrocarbons in numerous ant species reliably correlate with changes in reproductive ability (e.g., Liebig et al. 2000; Dietemann et al. 2003, 2005; Cuvillier-Hot et al. 2004; Lommelen et al. 2006). This potentially allows the effective policing of individuals that change their hydrocarbon blends while becoming reproductively active. However, experimental data to prove a causal relationship has not yet been presented. Similar correlative evidence exists for egg policing. Policing occurs where eggs can be identified according to the reproductive caste of the egg-laying worker (Monnin and Peeters 1997; D'Ettorre et al. 2004; Endler et al. 2004). A causal relationship between surface hydrocarbons and egg policing has been shown in the ant *Camponotus floridanus* (Endler et al. 2004). In this species, workers destroy significantly fewer worker-produced eggs when the eggs are given a queen-derived profile. This indicates that egg surface hydrocarbons are the signal that provides the information necessary for effective egg policing.

In the current study, we examined behavioral regulation of reproduction and fertility signaling within the worker caste of the desert-ant *Aphaenogaster cockerelli*. The derived status of this species is indicated by a distinct worker–queen dimorphism and large-size colonies with a single queen. Workers of this species have highly active ovaries that produce trophic eggs in the presence of a queen and viable male eggs in as few as 2 weeks after the removal of the queen (Hölldobler and Carlin 1989). In the presence of a queen, workers have been shown to police reproductive nestmates physically (Hölldobler and Carlin 1989), thus limiting workers to trophic egg production in queenright colonies. However, egg policing has not yet been examined in this species. Furthermore, the hydrocarbon signals that potentially underlie both of these behaviors have yet to be identified. Herein, we study egg policing and identify the hydrocarbon signals available for both egg and physical policing behaviors.

Studies of fertility signaling and policing in ants have mostly focused on more primitive species with low to non-existent worker–queen reproductive dimorphism. Our findings on the signaling and policing behavior of *A. cockerelli* provide new insights into the evolution of fertility signals. We conclude that the policing behaviors performed in this species are a direct result of the signals available to the policing workers. In comparison to other ant species, our results provide the first insights into the physiological adaptations that may influence patterns of reproductive signaling.

Methods and Materials

Study Species Mature colonies of *A. cockerelli* were collected from the Chihuahuan desert between Portal, Arizona, and Rodeo, New Mexico, from the end of August through the beginning of October 2006 and in August 2007. Colonies were collected by triggering nest evacuation using army ants (*Neivamyrmex nigrescens*; Smith and Haight 2008). Collected colonies averaged 1,500 workers; all had a single queen. Mature colonies consist of a nest with a single queen and one to four distinct satellite nests containing workers and brood (Hölldobler and Carlin 1989). The colonies used for this experiment consisted primarily of workers collected from the nest containing the queen. Previous work based on 31 colonies and 487 workers genotyped for two microsatellites has shown that queens are singly mated (Mösl and Gadau, unpublished data).

Laboratory Conditions In the laboratory, ants were housed in a dental-plaster nest with molded chambers, darkened inside by red acetate over glass. The nests were attached to a foraging arena in which they received a constant supply of water, sugar–water, and pieces of cricket (*Acheta*

domestica) and beetle larvae (*Zophobas morio*). For this study, temperature was maintained at 25°C, and the foraging arenas were kept in constant light.

Egg Policing To determine whether or not workers selectively destroy viable eggs produced by sister workers, we measured egg acceptance by using discriminator worker groups (a method used to show egg policing; Endler et al. 2004, 2006). These worker groups consisted of 50 randomly chosen nest workers. Each group was placed into a nest connected to a foraging arena similar in construction to the queenright colonies from which they originated. These workers were given food (as mentioned above), water, sugar–water, and 24 h to acclimate to their new nests. There were three discriminator worker groups from each colony (N=9) in this experiment. Each group simultaneously received one of three egg treatments, introduced into the nest area. One group received 20 mother-queen-produced eggs, another received 20 sister-worker produced eggs, and the final group received a mixture of ten mother-queen eggs and ten sister-worker eggs. Sister-worker-produced eggs were taken from isolated worker groups that had become reproductive, exhibiting an egg-carrying behavior indicative of tending viable eggs rather than trophic eggs (Hölldobler and Carlin 1989). This setup allowed us to test if workers would police worker-produced eggs in the presence and absence of queen-produced eggs. *A. cockerelli* workers are often separated from the queen due to polydomous colony nest structure (mentioned above). Therefore, the short-term absence of a queen present in these discriminator groups should not have affected the outcome of our egg-policing tests.

The number of eggs remaining after 24 h was recorded in all treatments. This period of time has proven to be suitable for egg policing to occur (Endler et al. 2004; Dietemann et al. 2005; Helanterä and Sundström 2007). Egg survival in the experimental groups was analyzed by using a non-parametric Friedman's ANOVA.

Hydrocarbon Profiles Queen (N=16), non-reproductive worker (N=14), and reproductive worker (N=10) cuticular hydrocarbon profiles were collected from our laboratory colonies. Note that the sample size (above) represents ants from independent colonies, except the reproductive worker group in which two workers from the same colony were sampled, thus making colony sample size actually N=9. Queen profiles were taken from live queens that were reproductively active, as indicated by the presence of eggs. Non-reproductive workers consisted of non-callow workers that were randomly picked off of the brood pile of a queenright colony; therefore, these ants were likely to be trophic-egg producing, brood-tending workers. Reproductive workers were taken from reproductive groups of workers (100–200 individuals) that had been isolated from their queen and brood for an extended time span (3 weeks to 6 months). Workers were classified only as reproductive (1) when they had been observed laying an egg that was then deposited onto the pre-existing egg pile (to ensure that the worker was producing viable eggs, not trophic eggs, as the latter are immediately fed to brood or eaten by other workers; Hölldobler and Carlin 1989), and (2) when subsequent dissection of these workers confirmed ovarian activity and the condition or type of yolky oocytes indicated the development of viable eggs.

Eggs used for gathering egg profiles were taken directly from the egg piles of both queenright colonies and isolated worker groups (mentioned above) that had started to reproduce.

Chemical Analysis Cuticular hydrocarbon profiles were gathered from live queens and workers by using solid-phase microextraction (Arthur and Pawliszyn 1990). A fiber (Supelco Inc., coated with a 30-μm polydimethylsiloxane film) was rubbed on the gaster surface for 5 min (Monnin et al. 1998). The fiber was inserted into the injection port of an Agilent 6980N series gas chromatograph (GC) equipped with a DB-1MS (J&W Scientific) non-polar capillary column (30 m×0.25 mm×0.25 μm), connected to an Agilent 5975 series mass selective detector. The GC injection port was set to 260°C and the transfer line to 300°C. The column temperature was held at 60°C for 2 min before increasing to 200°C at 40°C min^{-1}, and then to 320°C at 5°C min^{-1}. Helium was used as carrier gas at 1 ml min^{-1}, and samples were injected in the splitless mode. Electron impact mass spectra were measured at 70 eV with a source temperature of 230°C. Identification of the compounds was tentatively based on a comparison of their mass spectra with published spectra, synthetic alkane standards, and retention indices.

Egg-surface hydrocarbon profiles were gathered by extracting groups of 20 eggs in 20 μl of hexane for 5 min. The extract was evaporated and suspended in 2 μl of hexane from which 1 μl was injected into the GC.

Compounds were included in the statistical analysis if they occurred in ≥70% of the sampled individuals within at least one of the classes (queen, reproductive worker, nest worker, worker-produced egg, and queen-produced egg). We performed non-parametric multi-dimensional scaling to analyze the similarity of cuticular and egg profiles by using Primer 6. Chord distances were used to calculate the distance matrices.

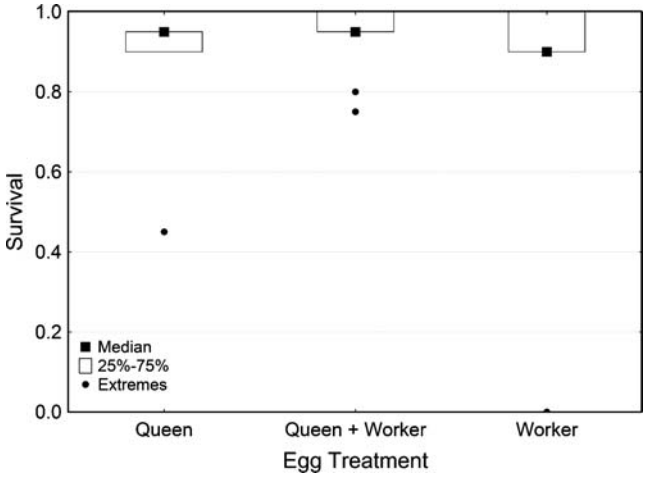

Fig. 1 Bioassay of egg survival over 24 h. Survival of 20 queen eggs is compared to ten queen eggs mixed with ten worker eggs and to 20 worker eggs. There was no statistical difference among treatments, Friedman's ANOVA, n_{groups}=9, P=0.875

Results

Egg Policing There was no significant difference in the survival of eggs among the different treatments (Fig. 1). Median survival of queen eggs and a mix of worker and queen eggs was 0.95, while median survival of worker eggs was 0.9. If workers effectively police worker-produced eggs, survival of the queen and worker mixed egg group would have been 0.5, while worker egg survival would have been zero.

Egg Surface Hydrocarbon Profiles The two classes of eggs (worker-produced and queen-produced) were not qualitatively different in their hydrocarbon profiles. Only small relative abundance differences were distinguishable (Fig. 2, Table 1), which was confirmed by the lack of a clear separation of the two classes of eggs in the non-parametric multi-dimensional scaling (Fig. 3). The stress value of 0.09 indicates a good graphical representation of the data structure.

Cuticular Hydrocarbon Profiles Quantitative and qualitative differences separated the classes of individuals (queen, nest worker, and reproductive worker; Fig. 2, Table 1). For instance, compounds 1–9 (Fig. 2) were unique to reproductive individuals. There were only three of these compounds that occurred in all reproductive workers sampled (peaks 3, 7, and 8; Table 1). In two of these three compounds, quantitative differences separated reproductive workers and queens; e.g., median relative abundance of pentacosane (peak 3; Table 1), queen = 25.2 and reproductive worker = 6.8.

Non-parametric multi-dimensional scaling separated queens and non-reproductive workers well, with a large gap between the two groups (Fig. 4). The reproductive workers were between the two groups as expected, including profiles that were relatively similar to those of queens and those of non-reproductive workers. The stress value of 0.07 indicates a good graphical representation of the data structure.

Discussion

The crucial requirement for effective reproductive policing is the ability to identify which workers or which eggs should be policed. Our findings suggest that the policing behaviors of *A. cockerelli* can be explained by discriminatory information present in hydrocarbon blends on the worker cuticle and the surface of eggs. Workers of *A. cockerelli* do not police worker-produced eggs; viable worker egg survival was not different from that of queen eggs in the egg policing experiment. Correspondingly, workers produce eggs with a surface hydrocarbon blend nearly identical to that of queen-produced eggs (Table 1, Fig. 2). In contrast, the cuticular profiles of non-reproductive workers showed strong qualitative differences from queens. Reproductive egg-laying workers fall between the two groups. This difference provides potential to identify reproductive workers, while eggs cannot be distinguished.

A. cockerelli is a species with workers that have active ovaries used for trophic egg production within a queenright nest. There are striking parallels between *A. cockerelli* and another trophic egg-producing ant, *Myrmecia gulosa*, in which policing and hydrocarbon signaling has been studied. Like *A. cockerelli*, *M. gulosa* do not police worker-produced viable eggs. Instead, reproductive workers are singled out and policed. Correspondingly, reproductive workers develop a distinct cuticular hydrocarbon profile, and their eggs are covered with a suite of hydrocarbons similar to queen eggs (Dietemann et al. 2003, 2005). In both phylogenetically distant systems (*Aphaenogaster*, Myrmicinae; *Myrmecia*, Myrmeciinae), workers can distinguish only reproductive castes and not their eggs. Thus, policing is limited to physical attacks and immobilization of reproductive workers.

In contrast to the mode of policing seen in *M. gulosa* and *A. cockerelli* is that of the ant *Camponotus floridanus* (subfamily Formicinae). When workers become reproductive, their cuticular hydrocarbon profile does not change.

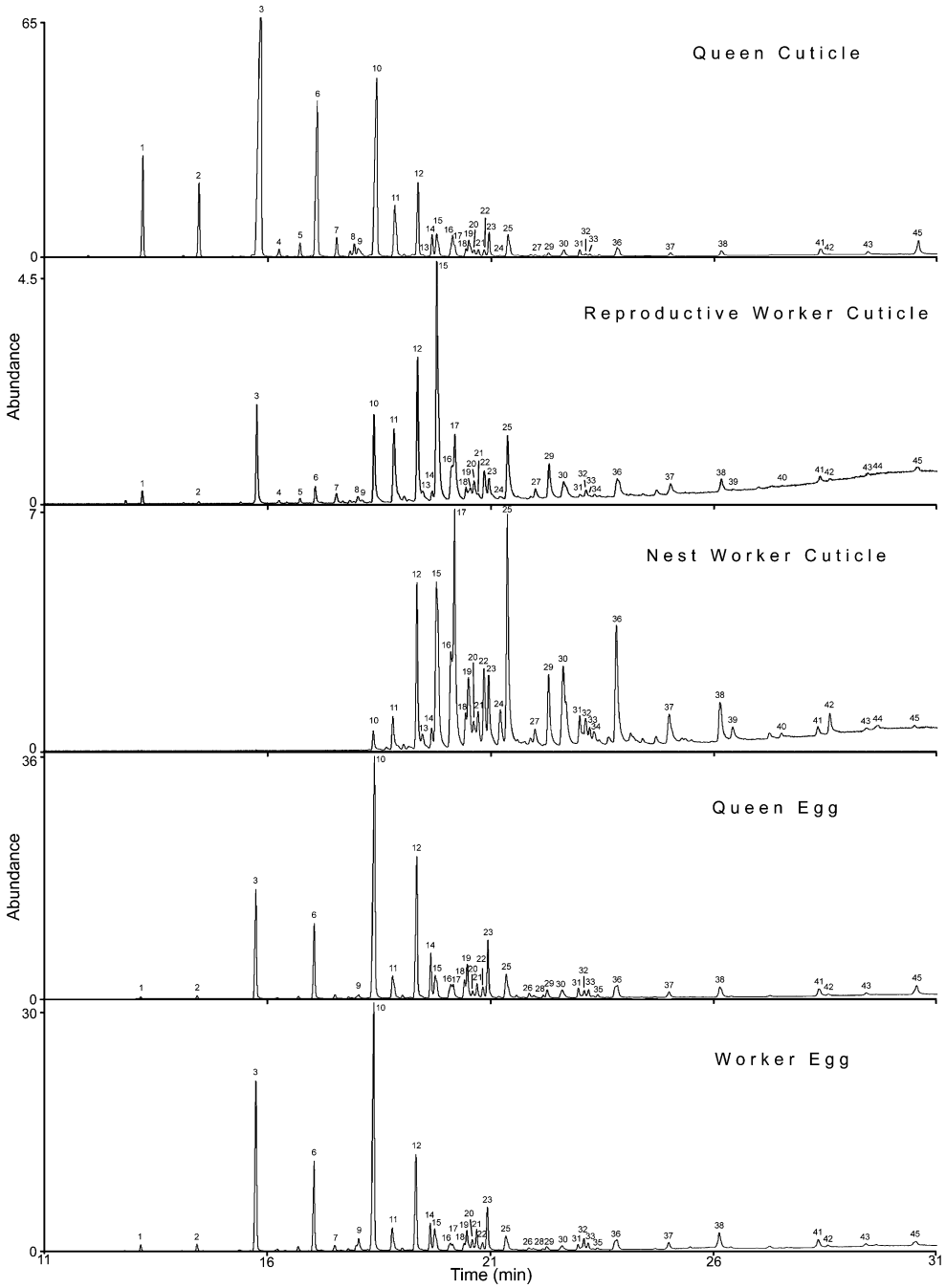

Fig. 2 Representative chromatograms showing hydrocarbon profiles among sampled classes of individuals and eggs. *Numbered peaks* correspond to compounds in Table 1 and are the compounds included in the statistical analyses

Their eggs receive hydrocarbon blends that are similar to those occurring on the cuticle of workers. This makes worker eggs distinguishable from queen eggs, which contain hydrocarbons that vary with fertility and indicators of fertility such as colony size (Endler et al. 2006). Correspondingly, workers in this system are not physically policed, but their eggs are (Endler et al. 2004, 2006, 2007). In contrast to *M. gulosa* and *A. cockerelli*, *C. floridanus* workers are not trophic egg producers and do not have active ovaries under normal queenright conditions.

The ability to produce queen-like hydrocarbon blends may be associated with workers retaining active ovaries. It has been suggested recently (Endler et al. 2007) that workers of more derived ant genera, with low reproductive potential (inactive ovaries, e.g., *C. floridanus*), may have lost the ability to produce a fertility signal due to their

Table 1 Tentative identification of chemical compounds found on *Aphaenogaster cockerelli,* along with retention indices and relative concentration

Peak number	Compound	Retention index	Group				
			QC	RWC	NWC	QE	WE
1	Tricosane	2,300	3.3 (1.5, 14.9)*	0.8 (0, 5.5)*	0 (0, 0)	0 (0, 0)	0 (0, 0)
2	Tetracosane	2,400	1.9 (0.7, 6.8)**	0.1 (0, 2.3)**	0 (0, 0)	0.2 (0, 0.4)	0.4 (0, 0.6)
3	Pentacosane	2,500	25.2 (11.8, 36.8)**	6.8 (0.6, 24.5)**	0 (0, 0)	8.1 (5.8, 13.4)	10.9 (5.8, 18.1)
4	9-Methylpentacosane	2,530	0.4 (0, 1.3)***	0 (0, 0.7)***	0 (0, 0)	0 (0, 0)	0 (0, 0)
5	3-Methylpentacosane	2,567	0.4 (0, 1.0)	0.3 (0, 1.7)	0 (0, 0)	0 (0, 0)	0 (0, 0)
6	Hexacosane	2,600	12.5 (10.0, 4.3)**	1.2 (0.4, 6.0)**	0 (0, 0)	6.0 (3.4, 7.0)	4.7 (3.1, 7.9)
7	10-;14-Methylhexacosane	2,630	0.9 (0, 2.7)	0.7 (0.2, 1.6)	0 (0, 0)	0.4 (0, 0.6)	0 (0, 0.6)
8	x,y-Heptacosadiene	2,658	0.9 (0.2, 3.7)*	0 (0, 1.3)*	0 (0, 0)	0 (0, 0)	0 (0, 0)
9	x-Heptacosene	2,665	1.0 (0, 3.5)**	0 (0, 0.5)**	0 (0, 0)	1.5 (0, 2.9)	0.8 (0, 2.1)
10	Heptacosane	2,700	20.4 (11.6, 32.1)	8.0 (3.0, 19.3)	1.0 (0.6, 3.5)	24.6 (18.0, 36.8)	24.2 (15.5, 30.3)
11	9-;11-;13-Methylheptacosane	2,730	3.5 (0.5, 13.8)	3.5 (0, 9.6)	1.5 (0, 3.8)	3.4 (1.2, 4.5)	1.7 (0.9, 3.0)
12	3-Methylheptacosane	2,775	6.1 (3.7, 8.7)	7.1 (0.6, 16.1)	6.2 (3.4, 12.1)	12.1 (10.1, 15.5)	12.7 (9.5, 16.3)
13	unknown	2,782	0 (0, 0.5)	0 (0, 1.6)	0.7 (0, 1.2)	0 (0, 0)	0 (0, 0)
14	Octacosane	2,800	2.2 (1.2, 4.0)	2.4 (0, 16.2)	1.0 (0.4, 2.0)	3.9 (3.0, 4.4)	3.5 (2.5, 5.3)
15	3,7-;3,11-Dimethylheptacosane	2,808	2.3 (0.6, 6.0)	5.6 (0, 31.0)	11.1 (4.0, 14.8)	2.5 (1.4, 4.1)	3.2 (2.0, 5.0)
16	10-;12-;14-Methyoctacosane	2,835	1.1 (0, 4.5)	5.8 (0.7, 11.4)	12.6 (1.1, 20.2)	1.5 (1.1, 2.2)	1 (0.9, 1.5)
17	8,12-Dimethyloctacosane	2,839	0.6 (0, 2.4)	0 (0, 7.7)	0 (0, 4.8)	1.6 (0.5, 2.4)	1.3 (0.6, 1.9)
18	unknown	2,859	0.5 (0, 1.1)	1.3 (0, 5.0)	1.1 (0, 1.9)	1.4 (0, 2.4)	1.2 (0.6, 1.8)
19	2- or 4-Methyloctacosane	2,864	1.6 (1.0, 2.2)	2.5 (0, 4.3)	4.2 (3.3, 8.7)	3.1 (2.1, 4.3)	4.2 (1.2, 6.5)
20	x-Nonacosene	2,872	0 (0, 1.5)	1.5 (0, 2.8)	1.4 (0, 1.9)	0.8 (0, 1.0)	1.2 (0, 2.3)
21	x-Nonacosene	2,880	0 (0, 0.9)	0.9 (0, 2.9)	1.8 (0, 3.9)	1.4 (0.8, 2.0)	2.1 (0.6, 2.6)
22	4,12-Dimethyloctacosane	2,890	0.2 (0, 1.2)	2.6 (0.4, 30.2)	3.5 (1.1, 4.5)	1.1 (0, 1.4)	1.1 (0.6, 1.6)
23	Nonacosane	2,900	3.0 (1.5, 5.9)	0.3 (0, 5.4)	3.7 (2.3, 9.7)	5.4 (4.4, 8.6)	6.2 (4.4, 9.9)
24	unknown	2,918	0 (0, 0.3)	0 (0, 0.9)	1.3 (0, 2.6)	0 (0, 0)	0 (0, 0)
25	9-;11-;13-;15-Methylnonacosane	2,933	1.3 (0, 5.9)	8.0 (3.4, 12.5)	11.1 (8.9, 18.7)	3.4 (2.7, 5.2)	3.3 (2.2, 6.1)
26	3-Methylnonacosane	2,975	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.4 (0, 1.0)	0.5 (0, 0.8)
27	5,11-;5,13-;5,15-Dimethylnonacosane	2,983	0 (0, 0.3)	0.8 (0, 1.5)	1.1 (0, 1.6)	0 (0, 0)	0 (0, 0)
28	Triacontane	3,000	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.3 (0, 0.9)	0 (0, 0.8)
29	3,9-;3,11-;3,13-Dimethylnonacosane	3,007	0 (0, 1.0)	3.2 (0.7, 4.6)	4.4 (1.5, 8.0)	0.9 (0, 1.4)	1.2 (0, 1.5)
30	10-;12-;14-Methyltriacontane	3,035	0 (0, 0.3)	0.8 (0, 1.5)	1.1 (0, 1.6)	1.5 (0, 2.4)	1.5 (0.6, 2.5)
31	2- or 4-Methyltriacontane	3,064	0.4 (0, 0.6)	1.2 (0, 3.2)	1.5 (0.9, 3.1)	0.8 (0, 2.7)	1.5 (0.5, 2.7)
32	x-Hentriacontene	3,075	0 (0, 0.5)	0.7 (0, 2.4)	1.4 (1.0, 3.2)	0.8 (0, 1.4)	1.4 (0.7, 3.7)
33	x-Hentriacontene	3,083	0 (0, 0.2)	0 (0, 1.1)	0.9 (0, 2.4)	0.9 (0, 1.3)	1.0 (0, 1.4)
34	unknown	3,092	0 (0, 0)	0.2 (0, 3.4)	0.6 (0, 1.7)	0 (0, 0)	0 (0, 0)
35	Hentriacontane	3,100	0 (0, 0)	0 (0, 0)	0 (0, 0)	0.4 (0, 1.7)	0 (0, 1.9)
36	9-;11-;13-;15-Methylhentriacontane	3,136	0.8 (0, 2.6)	3.5 (1.1, 8.7)	6.5 (5.1, 12.5)	2.2 (0.9, 4.8)	2.7 (1.1, 5.4)
37	10-;12-;14-Methyldotriacontane	3,235	0.1 (0, 0.9)	1.7 (0.8, 3.2)	2.2 (1.7, 3.7)	1.1 (0, 1.6)	1.1 (0.8, 1.7)
38	9-;11-;13-;15-Methyltritriacontane	3,335	0.4 (0, 1.3)	1.9 (1.1, 4.4)	3.4 (1.8, 4.9)	2.4 (1.1, 3.7)	2.3 (1.6, 3.0)
39	13,17-;15,17-Dimethyltritriacontane	3,357	0 (0, 0)	0 (0, 1.2)***	1.0 (0, 2.1)***	0 (0, 0)	0 (0, 0)
40	unknown	3,358	0 (0, 0)	0 (0, 0.5)***	0.3 (0, 0.4)***	0 (0, 0)	0 (0, 0)

Table 1 (continued)

Peak number	Compound	Retention index	Group				
			QC	RWC	NWC	QE	WE
41	9-;11-;13-Methylpentatriacontane	3,535	1.0 (0.3, 2.4)	0.6 (0, 1.9)	2.0 (0.8, 3.1)	1.7 (0.7, 2.6)	1.3 (0.7, 1.6)
42	13,19-;15,19-Dimethylpentatriacontane	3,555	0 (0, 0.3)	0.5 (0, 1.7)	2.0 (0.8, 3.1)	0.3 (0, 0.5)	0 (0, 0.8)
43	10-;12-Methylhexatriacontane	3,636	0.4 (0, 1.2)	0.1 (0, 1.2)	0.4 (0, 1.0)	0.6 (0, 0.9)	0 (0, 0.8)
44	unknown	3,680	0 (0, 0)	0 (0, 2.6)***	0.5 (0, 2.2)***	0 (0, 0)	0 (0, 0)
45	9-;11-;13-Methylheptatriacontane	3,736	2.5 (0.5, 6.2)	0.5 (0, 2.4)	0 (0, 1.0)	2.4 (0.8, 3.5)	1.4 (0.8, 2.0)

Compounds were tentatively identified on the basis of mass spectrum and retention index; assignment of *n*-alkanes confirmed by comparison with authentic compounds. X, Y: position of double bond unknown. 10-;14-Methylhexacosane=a mixture of 10- and 14-methyl isomers. Mann–Whitney *U* test, $n_{QC}=16$, $n_{RWC}=10$, and $n_{NWC}=14$) in relative compound concentrations. Concentrations are provided as mean (min, max).
Group: *QC* queen cuticle, *RWC* reproductive worker cuticle, *NWC* non-reproductive worker cuticle, *QE* queen egg, *WE* worker egg
*$P<0.01$, two-sided (significant difference)
**$P<0.001$, two-sided (significant difference)
***$P<0.05$, two-sided (significant difference)

inability to replace the primary reproductive. However, the social organization of *A. cockerelli* is highly derived with distinct worker–queen dimorphism, large colony size, and a single queen, similar to that of *C. floridanus*. The most obvious difference between the two species is the ovarian activity level of workers in the presence of the queen. *A. cockerelli* is, to our knowledge, one of the most derived species of ant in which workers can produce the reproductive signals utilized by the primary reproductive or queen. This is commonly found in more primitive ant societies, in which worker–queen dimorphism is low or non-existent (Liebig et al. 2000; Cuvillier-Hot et al. 2004; Lommelen et al. 2006). In these systems, nestmates have a reproductive potential that rivals that of the queen. The high level of ovarian activity for trophic egg production of *A. cockerelli* workers may be the underlying physiological condition that has led to workers and queen sharing the same reproductive signaling capabilities. Further fertility signaling studies in other trophic egg-producing derived species of ants are needed to verify this hypothesis.

Fig. 3 Two-dimensional configuration of non-metric, multi-dimensional scaling of surface hydrocarbon blend differences between queen-produced eggs and worker-produced eggs

Fig. 4 Two-dimensional configuration of non-metric, multi-dimensional scaling of cuticular hydrocarbon blend differences among queens, reproductive workers, and non-reproductive workers

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References

ARTHUR, C. L., and PAWLISZYN, J. 1990. Solid-phase microextraction with thermal-desorption using fused-silica optical fibers. *Anal. Chem.* 62:2145–2148.

BOURKE, A. F. G. 1988. Worker reproduction in the higher eusocial Hymenoptera. *Q. Rev. Biol.* 63:291–311.

BRIAN, M. V., and RIGBY, C. 1978. Trophic eggs of *Myrmica rubra* L. *Insectes Soc.* 25:89–110.

CHOE, J. C. 1988. Worker reproduction and social evolution in ants (Hymenoptera: Formicidae), pp. 163–187, in J. C. Trager (ed.). Advances in MyrmecologyLeiden, New York.

COLE, B. J. 1986. The social-behavior of *Leptothorax allardycei* (Hymenoptera, Formicidae)—time budgets and the evolution of worker reproduction. *Behav. Ecol. Sociobiol.* 18:165–173.

Cuvillier-Hot, V., LENOIR, A., CREWE, R., MALOSSE, C., and PEETERS, C. 2004. Fertility signaling and reproductive skew in queenless ants. *Anim. Behav.* 68:1209–1219.

D'ETTORRE, P., HEINZE, J., and RATNIEKS, F. L. W. 2004. Worker policing by egg eating in the ponerine ant *Pachycondyla inversa*. *Proc. R. Soc. Lond., Ser. B: Biol. Sci.* 271:1427–1434.

DIETEMANN, V., LIEBIG, J., HÖLLDOBLER, B., and PEETERS, C. 2005. Changes in the cuticular hydrocarbons of incipient reproductives correlate with triggering of worker policing in the bulldog ant *Myrmecia gulosa*. *Behav. Ecol. Sociobiol.* 58:486–496.

DIETEMANN, V., PEETERS, C., LIEBIG, J., THIVET, V., and HÖLLDOBLER, B. 2003. Cuticular hydrocarbons mediate discrimination of reproductives and nonreproductives in the ant *Myrmecia gulosa*. *Proc. Natl. Acad. Sci. U.S.A.* 100:10341–10346.

ENDLER, A., HOLLDOBLER, B., and LIEBIG, J. 2007. Lack of physical policing and fertility cues in egg-laying workers of the ant *Camponotus floridanus*. *Anim. Behav.* 74:1171–1180.

ENDLER, A., LIEBIG, J., and HÖLLDOBLER, B. 2006. Queen fertility, egg marking and colony size in the ant *Camponotus floridanus*. *Behav. Ecol. Sociobiol.* 59:490–499.

ENDLER, A., LIEBIG, J., SCHMITT, T., PARKER, J. E., JONES, G. R., SCHREIER, P., and HÖLLDOBLER, B. 2004. Surface hydrocarbons of queen eggs regulate worker reproduction in a social insect. *Proc. Natl. Acad. Sci. U.S.A.* 101:2945–2950.

HAMMOND, R. L., and KELLER, L. 2004. Conflict over male parentage in social insects. *PLoS Biol.* 2:1472–1482.

HARTMANN, A., WANTIA, J., TORRES, J. A., and HEINZE, J. 2003. Worker policing without genetic conflicts in a clonal ant. *Proc. Natl. Acad. Sci. U.S.A.* 100:12836–12840.

HELANTERÄ, H., and SUNDSTRÖM, L. 2007. Worker policing and nest mate recognition in the ant *Formica fusca*. *Behav. Ecol. Sociobiol.* 61:1143–1149.

HÖLLDOBLER, B., and CARLIN, N. 1989. Colony founding, queen control and worker reproduction in the ant *Aphaenogaster* (=*Novomessor*) *cockerelli* (Hymenoptera: Formicidae). *Psyche* 96:131–151.

HÖLLDOBLER, B., and WILSON, E. O. 1990. The Ants. Springer, Heidelberg, Germany.

KIKUTA, N., and TSUJI, K. 1999. Queen and worker policing in the monogynous and monandrous ant, *Diacamma* sp. *Behav. Ecol. Sociobiol.* 46:180–189.

LE CONTE, Y., and HEFETZ, A. 2008. Primer pheromones in social Hymenoptera. *Annu. Rev. Entomol.* 53:523–542.

LIEBIG, J., PEETERS, C., OLDHAM, N. J., MARKSTADTER, C., and HÖLLDOBLER, B. 2000. Are variations in cuticular hydrocarbons of queens and workers a reliable signal of fertility in the ant *Harpegnathos saltator*? *Proc. Natl. Acad. Sci. U.S.A.* 97:4124–4131.

LOCKEY, K. H. 1988. Lipids of the insect cuticle—origin, composition and function. *Comp. Biochem. Physiol. B: Biochem. Mol. Biol.* 89:595–645.

LOMMELEN, E., JOHNSON, C. A., DRIJFHOUT, F. P., BILLEN, J., WENSELEERS, T., and GOBIN, B. 2006. Cuticular hydrocarbons provide reliable cues of fertility in the ant *Gnamptogenys striatula*. *J. Chem. Ecol.* 32:2023–2034.

MONNIN, T. 2006. Chemical recognition of reproductive status in social insects. *Ann. Zool. Fenn.* 43:515–530.

MONNIN, T., MALOSSE, C., and PEETERS, C. 1998. Solid-phase microextraction and cuticular hydrocarbon differences related to reproductive activity in queenless ant *Dinoponera quadriceps*. *J. Chem. Ecol.* 24:1423–1423.

MONNIN, T., and PEETERS, C. 1997. Cannibalism of subordinates' eggs in the monogynous queenless ant *Dinoponera quadriceps*. *Naturwissenschaften* 84:499–502.

MONNIN, T., and RATNIEKS, F. L. W. 2001. Policing in queenless ponerine ants. *Behav. Ecol. Sociobiol.* 50:97–108.

RATNIEKS, F. L. W. 1988. Reproductive harmony via mutual policing by workers in eusocial Hymenoptera. *Am. Nat.* 132:217–236.

RATNIEKS, F. L. W., FOSTER, K. R., and WENSELEERS, T. 2006. Conflict resolution in insect societies. *Annu. Rev. Entomol.* 51:581–608.

SMITH, A. A., and HAIGHT, K. L. 2008. Army ants as research and collection tools. *J. Insect Sci.* (in press).

WENSELEERS, T., HELANTERÄ, H., HART, A., and RATNIEKS, F. L. W. 2004. Worker reproduction and policing in insect societies: an ESS analysis. *J. Evol. Biol.* 17:1035–1047.

var. *melanocarpa* collected in British Colombia, prunasin constituted approximately 3–6% of leaf dry weight. Santamour (1998) reported an average of 3,776±526 ppm cyanide in *P. virginiana* leaves measured from June to September in plants growing in the National Arboretum in Washington, D.C. Thus, previous studies indicated that the leaves fed by colonies of *A. cerasivoranus* are highly cyanogenic. The caterpillars of *A. cerasivoranus* are bright yellow. Their aposematic coloration and their habit of moving outside the nest during the day to pull leaves into the shelter suggest that the larvae are "advertising" their toxicity. In response to disturbance, the caterpillars regurgitate (Grant 2006) exuding a viscous liquid from their mouthparts. We tested the hypothesis that larval aposematism in *A. cerasivoranus* is associated with sequestration of host-derived HCN. Specifically, we conducted chemical assays to determine the concentration of HCN in the caterpillar's body, regurgitant, and frass. We also assessed the influence of the rate of ingestion on foregut pH and cyanogenesis.

Methods and Materials

Study Site The study was conducted during spring/early summer of 2006–2008. Colonies of *A. cerasivoranus* and leaves of *P. virginiana* used were collected from field locations in Cortland County, New York, during June.

Chemical Analysis of Cyanide To prepare leaf and fecal pellet samples for analysis, material was weighed to 0.1 mg, then ground in 0.5 ml of 0.2 M phosphate buffer ($NaH_2PO_4 \cdot H_2O$, pH 6.2 (Brinker and Seigler 1989, 1992) with a mortar and pestle (5 cm barrel length×0.3 cm diameter). Both the buffer, mortar, and pestle were chilled to approximately 4°C in an ice bath immediately prior to use to prevent the loss of cyanide as HCN (Fitzgerald et al. 2002). The buffer was transferred to an 18-ml capacity Warburg flask, the center well of which was preloaded with 0.2–0.3 ml of 1 M NaOH. The entire process of grinding and transfer took less than 10 s. The flask was then sealed and held at 35°C for a minimum of 18 h. For some analyses, as noted below, we added 7 units of β-glycosidase (Sigma-Aldrich product number G4511) to the Warburg flask (Swain et al. 1992). HCN volatilizing from the sample during the distillation process was trapped in the center well (preloaded with NaOH) as CN^-. Samples were analyzed with a Dionex Ion Chromatograph with a GP 50 gradient pump and ED 40 electrochemical detector. The machine was fitted with a 4 mm AG9-HC guard column and a 250×4 mm AS7 analytical column. The eluent consisted of 41 g $NaC_2H_3O_2$, 5 ml ethylenediamine, and 16.5 ml of 6.0 M NaOH per liter, delivered at a flow rate of 1.0 ml/min. Twenty-five microliter of the NaOH center well solution were injected into the apparatus. CN^- eluted at approximately 6 min. A standard curve was prepared prior to each run by injecting known concentrations of CN^- into the apparatus.

Cyanide Content of Leaf, Frass, and Regurgitant Samples The HCN-p of a total of 31 leaves of *P. virginiana* collected from five trees was determined. Sixteen of these leaves were partially expanded leaves growing at the tips of shoots (young leaves) and 15 were fully expanded leaves found at the base of the current years growth (aged leaves). A 2–4 mg section (3.0±0.1 mg SE) was cut from the leaves and prepared for analysis as described above.

Frass was collected from 11 caterpillars allowed to feed ad libitum in plastic containers on leaves of *P. virginiana* of mixed age in the laboratory and prepared for analysis as described above. In addition, we attempted to relate cyanide content of frass to leaf age using the following procedure. Five caterpillars of *A. cerasivoranus* (fourth instar) were housed in each of 20 vented plastic containers (10.5 cm diameter×4 cm deep). Caterpillars in ten of these containers were allowed to feed ad libitum for 4 d on young leaves and caterpillars in the other ten containers on aged leaves. Leaves were kept fresh by inserting their stems in water piks. Consumed leaves were replaced daily. Samples of dried frass (4.3±0.5 mg) were collected from five containers having old leaves and five having young at the end of the 4-day period and prepared for analysis as described above. In addition, similarly sized samples of frass from four more containers having young leaves were distilled in buffer to which 7 units of β-glycosidase were added.

Caterpillars aggregate their pupal cells in a central core of the leaf shelter. Each is encased in frass. We determined the cyanide content of frass taken from the core for four groups of caterpillars fed on young leaves and 4 groups fed on aged leaves under laboratory conditions. Stems bearing young or aged leaves of *P. virginiana* were placed in vials to provide foraging arenas. Twenty last instar caterpillars were placed into each arena. The caterpillars eventually bound the stems into tight nests. When they had finished feeding, they constructed a central core of pupa cells. Samples of the frass encasing the pupal cells (6.1±1.1 mg) were collected from each nest and processed as described above.

We collected samples of regurgitant from caterpillars housed in the 20 containers as described above at the end of the 4-day feeding period. Samples of 1–6 μl of regurgitant were collected from caterpillars in each container depending upon the amounts expressed by the caterpillars. Regurgitant was collected by holding a caterpillar between the thumb and forefingers and gently brushing the tip of a micropipette against its mouthparts causing it to express a

droplet that was drawn by capillary action into the pipette. The regurgitant samples were injected directly into microvials filled with an exact known quantity (0.1–0.2 ml) of 1 M NaOH. Some caterpillars produced little or no regurgitant, and it was usually necessary to collect droplets from several caterpillars from each container. The caterpillars in two containers maintained on young leaves failed to produce regurgitant. Thus, we analyzed eight samples of regurgitant from caterpillars maintained on young leaves and ten from caterpillars maintained on aged leaves. In addition, we analyzed 25 samples (each 5 μl) of regurgitant collected in the same manner from 12 different colonies in the field. As was the case for our experimental caterpillars, caterpillars in field colonies expressed varying amounts of regurgitant, and it was necessary to combine the regurgitant of several caterpillars from each colony to obtain 5-μl samples.

Cyanide Content of Whole Caterpillars We determined the cyanide content of 18 whole caterpillars that had been allowed to feed on either young or aged leaves. Caterpillars used for this study were those housed in the 20 containers as described where they were able to feed ad libitum. At the end of their 4-day feeding period, the caterpillars were killed by freezing and held at −21°C until processed 7 days later. To prepare specimens for analysis, a frozen caterpillar was weighed, then immediately sandwiched between the leaves of a folded sheet of aluminum (0.15 mm thick× 25 mm long×12 mm wide) and immersed in liquid nitrogen. When fully frozen, the aluminum case was withdrawn, and the caterpillar within shattered by crushing the case with a small mallet. The case was then opened, and the frozen, pulverized caterpillar was washed into a Warburg flask with chilled buffer and incubated as described above. Five of the caterpillars that were pulverized were each incubated separately with 7 units of β-glycosidase.

Cyanide Loss to Atmosphere A study was conducted to obtain an estimate of the fraction of the cyanide potential of a leaf lost to the atmosphere as HCN while the leaf was consumed by the caterpillars.

Five to 15 caterpillars were housed in a sealed feeding chamber (8 cm long×2 cm diameter) with a half leaf of *P. virginiana* of known mass (Fig. 1). A tube leading into the chamber carried a humidified air stream to the bottom of the chamber, and another tube at the top carried the air to a second, capture chamber. Air entering the capture chamber was directed to the bottom of the chamber and bubbled through 10 ml of 1 M NaOH. Glass beads were used to cause the rising bubbles to take a circuitous route through the NaOH, thus increasing the capture success. Air exited the chamber though an opening at the top. The air flow rate was adjusted to 2–3 ml/min. Caterpillars were allowed to consume the leaf for 24 h, after which the NaOH was retrieved and analyzed as described above. We determined the mass of leaf tissue that was left at the end of the feeding period to obtain a measure of the amount consumed by the caterpillars over the course of the study.

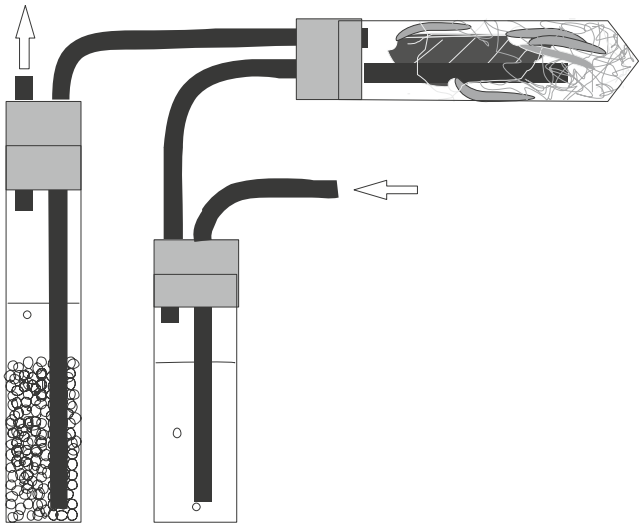

Fig. 1 Apparatus used to collect cyanide escaping into the atmosphere as *Archips* caterpillars feed

Effluent from the other half of the leaf was collected simultaneously with an identical apparatus except that the feeding chamber was used as an extraction chamber immersed in a water bath at 35°C. The purpose of this was to determine the total HCN-p of the leaf. In preparation, the leaf section was placed onto the bottom of the extraction chamber, and the chamber was immersed in liquid nitrogen. The frozen leaf was then ground to a powder. Approximately 1 ml phosphate buffer was added, and the chamber was sealed immediately. An air stream adjusted to the same rate as that used in the chamber housing the caterpillars was then passed through the chamber housing the leaf and then into the capture chamber for 24 h.

pH of Leaf and Foregut Regurgitant The pH of approximately 1 g of leaves of *P. virginiana* macerated in 100 ml of DI water was measured. The pH of leaves from four different trees was determined. We measured the pH of the foregut regurgitant of 13 *Archips* caterpillars that were allowed to feed ad libitum. For comparative purposes, we also measured the pH of the regurgitant of fourth–sixth instar eastern tent caterpillars (N=10), *Malacosoma americanum*, deprived of food for 8 h. Measurements were started immediately after they had fed on the leaves of *P. serotina*. The pH was measured with a 100-μm-diameter Beetrode® NMPH1 pH electrode and a 450-μm-diameter Dri-Ref ® 450 reference electrode. To produce standard pH

readings on an Acumet AR10 pH meter, a Bee-Cal® compensator was used. All electrode components were manufactured by World Precision Instruments, Sarasota, FL, USA. The meter was calibrated prior to each measurement with Hydrion® buffer with a pH of 12.0± 0.02 at 25°C. Meter accuracy was monitored with Hydrion pH 7 and 10 buffers. To obtain regurgitant, the opening of a 5-µl micropipette was lightly pushed against a caterpillar's mouthparts causing it to regurgitate. The liquid was drawn into the pipette by capillary action. Approximately 2 µl of the collected regurgitant were then injected into a glass sample tube (2 mm long×1.4 mm I.D.) fixed horizontally to the top of a 30-mm-long glass tube of the same diameter forming an upright 'T'. To determine the pH of the regurgitant, the pH electrode was inserted into one end of the sample tube and the reference electrode into the other. Measurements were made under the low power of a dissecting microscope to facilitate the placement of the electrodes.

Rate of Ingestion To obtain a comparative index of the rate of food intake, we weighed 4th instars (N=10, each of *Archips* and *M. americanum*) that had been deprived of food for 24 h, then were exposed to a host leaf and allowed to feed ad libitum (initial mass of *M. americanum*=44.4± 2.0 mg, of *Archips*=68.3±4.4 mg). The duration of the feeding bout was determined by direct observation. The mass gained by each caterpillar was determined immediately after it finished feeding. In addition, four *Archips* caterpillars were individually housed with a host leaf and video-recorded continuously for a total of 56 h to obtain a record of the frequency and duration of feeding bouts of individuals over extended periods. Caterpillars were maintained under a 14:10-L:D photoperiod regime. Red light was used during the scotophase to illuminate the foraging arena.

Effect of pH on Cyanogenesis We determined the effect of the pH of the buffer used to incubate leaf samples of *P. virginiana* on cyanogenesis. The same protocol for preparing leaf samples for cyanide analysis with Warburg flasks as detailed above was used except that the pH of the buffer was varied. Five 3–4 mg samples were cut from adjacent sites on the same leaf, then each sample was ground and incubated in a buffer with a different pH value. The pH ranged from 7–11. Two different leaves were treated in this same manner.

Susceptibility of Caterpillars to Cyanide The tolerance of the caterpillars to cyanide gas was determined by grinding 0.54±0.01 g of young leaves of *P. virginiana* in 2 ml of pH 6.2 buffer and placing the mixture in the bottom section of a gas chamber (Fitzgerald et al. 2002). HCN liberated from the macerated leaf passed into an upper chamber housing the caterpillar. To test the effect of liberated HCN on the caterpillars, an *Archips* caterpillar and, for control, a milkweed bug (*Oncopeltus faciatus*) were placed together in the chamber and observed for 1 hr; the point of immobility was noted (N=5 replicates of this study). The milkweed bug was used for control, since a previous study showed that this species is highly sensitive to HCN (Fitzgerald et al. 2002). In a second study, five caterpillars were housed together in the gas chamber and observed for 24 hr.

Statistical Analysis T-tests and Mann–Whitney rank sum tests, as detailed below, were carried out with ProStat and SigmaStat statistical software. All error values are standard errors.

Results

Cyanide Content of Leaf, Frass, and Regurgitant Samples Young leaves of *P. virginiana* had 2,473±130 (1,411–3,446) ppm cyanide (N=16). Aged leaves had 1,058±98 (360–1513) ppm cyanide (N=15). The difference between the HCN-p of young and aged leaves was highly significant (T-test, t=8.20, $P\leq0.001$). The frass collected from 11 caterpillars allowed to feed ad libitum on variously aged leaves contained no detectable cyanide. The frass collected from five caterpillars fed young leaves and five fed aged contained no detectable cyanide. The frass of four caterpillars fed young leaves and incubated in exogenous β-glycosidase contained no cyanide. Frass collected from the surface of pupal cells in the shelter's pupation core from colonies fed either young leaves or aged leaves contained no detectable cyanide.

Regurgitant collected from caterpillars housed with young leaves in six of the eight containers had 74.5± 28.2 ppm cyanide (2–153). Caterpillars from the other two containers produced no regurgitant. Regurgitant samples were obtained from all groups of caterpillars housed with aged leaves. Of these, six contained 2.7±1.2 ppm (1–4), and the remainder had no detectable cyanide. The difference between the cyanide content of the regurgitant of caterpillars fed young and aged leaves was significant (Mann–Whitney rank sum test, P=0.015). Six of 25 regurgitant samples collected from 12 field colonies contained cyanide (17.6±6.5), the remaining samples had no detectable cyanide.

Cyanide Content of Whole Caterpillars Seven of the 13 whole caterpillars incubated without exogenous β-glycosidase had trace quantities of cyanide (3.9±0.9 ppm). The

remaining caterpillars including the five incubated with exogenous β-glycosidase had no detectable cyanide or less that 1 ppm.

Cyanide Loss to Atmosphere Only small quantities of cyanide were recovered from air passed over caterpillars maintained in feeding chambers. The amount of cyanide collected averaged 2.6±0.33% (N=7) of the total amount in the leaf as measured by this same technique.

pH of Leaf and Foregut Regurgitant The pH of the four leaf samples of *P. virginiana* was 6.1±0.0. The mean pH of the regurgitant of 13 *Archips* caterpillars feeding ad libitum was 11.6±0.2. The pH of the ten starved caterpillars of *M. americanum* was 10.0±0.2, while the pH of the regurgitant immediately after the caterpillars had fed was 7.0±0.1. The difference between these values for *M. americanum* was significant (T-test, t=−15.9, P<.001). The difference between the regurgitant pH of *Archips* and unfed *M. americanum* was also significant (Mann–Whitney Rank Sum Test, P<0.001).

Rate of Ingestion Archips caterpillars had brief feeding bouts and took in little food compared to *M. americanum*. The ratio of mass gain to initial mass following a bout of feeding in previously starved *Archips* caterpillars was 0.02± 0.00 while that of the tent caterpillars was 0.37±0.04. The difference between mass gain in the two species was highly significant (Mann–Whitney rank sum test, P<0.001). The duration of the feeding bout was 5.7±0.63 min in *Archips* and 27.4±2.2 min in *M. americanum*. The difference between time spent feeding for the two species was highly significant (Mann–Whitney rank sum test, P<0.001). Four *Archips* caterpillars observed for a total of 56 h fed for intervals of 4.1±0.4 min (n=58 feeding bouts) at the rate of 1.8±0.7 bouts/h. Intervals of feeding punctuated near continuous bouts of silk spinning. Inspection of the foreguts of the two species opened by dissection after a meal, showed that the foregut of the tent caterpillars was fully packed with food and emitted a strong odor of benzaldehyde. The foregut content of *Archips* caterpillar was watery and loosely packed with food particles; no odor of benzaldehyde was evident. Previous studies of *M. americanum* showed that the caterpillars feed at intervals of 6–8 h.

Effect of pH on Cyanogenesis Cyanide production decreased precipitously when leaves were ground and incubated in buffers with pH>9. Leaf samples incubated in buffer with pH 11 yielded no cyanide (Fig. 2).

Susceptibility of Caterpillars to Cyanide All of the milkweed bugs (N=5) tested for control succumbed with complete immobility occurring after 136±6 s. All of the

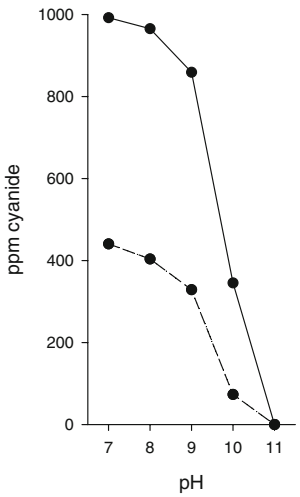

Fig. 2 The relationship between buffer pH and the release of cyanide from samples of two leaves of *Prunus virginiana*

Archips caterpillars (N=5) survived the 1-h exposure time without apparent effects of HCN. All of five caterpillars housed in the chamber were alive after 24 h. That the chamber contained HCN during the 24-h experiment period was verified by placing a milkweed bug in the chamber with the caterpillars after the 24 h interval and noting that it rapidly succumbed to the fumes. All five of the caterpillars were then placed on food. They continued to feed and grew normally when observed over a 3-day period.

Discussion

Our study shows that the caterpillars of *A. cerasivoranus* feed on leaves with moderate to high HCN-p. A small quantity of cyanide escapes into the atmosphere as the caterpillars feed, but most of a leaf's potential cyanide is ingested by the caterpillar. Nonetheless, our investigation indicates that aposematic coloration is probably not attributable to sequestration of cyanide. The first line of defense of *Archips* caterpillars is regurgitation, but the regurgitant of the caterpillars sampled in this study typically contained little or no cyanide. Only when caterpillars were fed exclusively in the lab on a tree's youngest leaves with the highest HCN-p, their regurgitant contained more than a trace amount of cyanide. The regurgitant of laboratory-reared caterpillars fed with aged leaves that make up the bulk of the insect's diet under field conditions contained only trace amounts or no detectable cyanide. The regurgitant of caterpillars feeding ad libitum in the field also contained only trace amounts of cyanide.

Assays of whole caterpillars showed that the toxin is not stored in appreciable quantities in any other of body compartments than the foregut (regurgitant). Although the pupae might gain protection by encasing themselves in

cyanide-containing frass, no cyanide occurs in the fecal pellets that surround their cocoons nor was any cyanide detected in the frass the caterpillars expel as they feed within the leaf shelter.

The results of this study contrast with those of a study of the tent caterpillar *M. americanum*. Peterson et al. (1987) determined that the regurgitant of *M. americanum* caterpillars fed with leaves of *P. serotina* having cyanide levels comparable to those ingested by caterpillars in our study contained approximately 400 ppm cyanide. The regurgitated droplets were shown to repel predacious ants but the deterrent effect was due to benzaldehyde rather then HCN. While we did not measure the concentration of benzaldehyde in the regurgitant of *Archips*, the release of benzaldehyde is stoichiometric with that of HCN. Since few of the caterpillars in our samples had HCN in their regurgitant, neither of these two products of cyanogenesis can be considered essential for any noxious effects the regurgitant may have in this species.

Our study shows that cyanogenesis in *P. virginiana* is largely inhibited at the upper pH range that characterizes the foregut fluids of *Archips* feeding ad libitum. The fluids of the empty foregut of *M. americanum* are also alkaline but the caterpillars take large meals (Snodgrass 1961), packing their guts with sufficient leaf material to lower gut pH to a range that enables cyanogenesis. In contrast to *Malacosoma*, *Archips* caterpillars feed for comparatively brief periods and ingest little food during a bout of feeding so that the pH of the meal has little influence on the pH of the foregut fluids. The foregut of *Archips* is relatively long, constituting 31% of the total gut length compared to 21% for *M. americanum*. It is the fourth longest foregut among 33 species of phytophagous caterpillars (Grant 2006). The more capacious foregut of *Archips* coupled with the small amounts of food the caterpillars process during bouts of feeding may facilitate the maintenance of high gut pH in the presence of food. Inspection of the foreguts of the two species opened by dissection after a meal, showed that the foregut of the tent caterpillar is fully distended with food and emits a strong odor of benzaldehyde. Analysis of the foregut boluses of recently fed tent caterpillars shows they contain a mean of over 600 ppm cyanide (Fitzgerald et al. 2002) consistent with the neutral pH of the regurgitant of recently fed tent caterpillars we measured in the present study. The foregut of recently fed *Archips* caterpillars is watery, loosely packed with food particles. It does not emit odor of benzaldehyde and contains little or no cyanide. The occurrence of some cyanide in the regurgitant of *Archips* caterpillars fed the most cyanogenic leaves of the host plant shows that the process of cyanogenesis in not entirely prevented by the maintenance of high gut pH. Moreover, the lack of susceptibility of the caterpillars to cyanide poisoning shows that they are adapted to deal with cyanide. This is not surprising since the insect is a *Prunus* specialist. The extent to which prunasin molecules that enter the foregut are not cleaved by β-glycosidase in the alkaline environment is unknown. However, the lack of any cyanogenic potential in the frass, even when incubated with exogenous β-glycosidase, indicates that an intact cyanogenic compound does not survive gut transit.

Fall webworms (*Hyphantria cunea*) feeding upon black cherry, *P. serotina*, employ a mechanism similar to that of *Archips* to inhibit cyanogenesis (Fitzgerald 2008). Webworms have a low rate of ingestion and capacious foregut enabling them to maintain a foregut pH of approximately 12 in the presence of the bolus. The relationship between buffer pH and cyanogenesis in *P. serotina* is the same as that for *P. virginiana*. However, webworms exposed to the fumes of macerated *P. serotina* leaves in gas chambers collapsed after 10 min exposure, and none recovered. The webworm also differs from *Archips* in that a significant percentage of the cyanogen survives gut transit. Pellets of webworms processed in the same manner as those in the present study contained a mean of approximately 2,900 ppm cyanide.

In contrast to both *Archips* and *Hyphantria*, the caterpillars of *M. americanum* do not inhibit cyanogenesis (Fitzgerald et al. 2002). It is not known how tent caterpillars detoxify cyanide, but the activity of L-3-cyanoalanine synthase at the mitochondrial level, as described for other species, may be involved (Witthohn and Naumann 1987; Meyers and Ahmad 1991). The enzyme catalyzes the reaction of HCN and cysteine to produce 3-cyanoalanine and H_2S, thus, rendering the cyanide ion innocuous. Tent caterpillars feed preferentially on a tree's most cyanogenic leaves, and while Peterson et al. (1987) suggested that this may enhance the deterrency value of the regurgitant, preference for these leaves may maximize the harvest of nitrogen from the plant's cyanogens. Compared to this detoxification mechanism and others previously described (Conn 1979; Engler et al. 2000) for other caterpillars, the inhibition of cyanogenesis by the maintenance of high foregut pH in the presence of food and paced feeding is a relatively simple means of dealing with a cyanogen.

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References

BAIRD, A. B. 1918. Some notes on the natural control of the cherry-tree ugly-nest tortricid, *Archips cerasivorana* Fitch. *Agric. Gaz. Can.* 5:766–771.

BALDUF, W. V. 1965. Observations on *Archips cerasivoranus* (Fitch) (Tortricidae: Lepidoptera) and certain parasites (Diptera: Hymenoptera). *Ohio J. Sci.* 65:60–67.

BRINKER, A. M., and SEIGLER, D. S. 1989. Methods for the detection and quantitative determination of cyanide in plant materials. *Phytochem. Bull.* 21:24–31.

BRINKER, A. M., and SEIGLER, D. S. 1992. Determination of cyanide and cyanogenic glycosides from plants, pp. 360–381, in H. F. Linskens, and J. F. Jackson (eds.). Plant toxin analysisSpringer, Berlin.

CONN, E. R. 1979. Cyanide and cyanogenic glycosides, pp. 387–412, in G. A. Rosenthal, and D. H. Janzen (eds.). Herbivores: their Interaction with secondary plant metabolitesAcademic, New York.

ENGLER, H. S., SPENCER, K. C., and GILBERT, L. E. 2000. Preventing cyanide release from leaves. *Nature* 406:144–145.

FITZGERALD, T. D. 1993. Trail and arena marking by caterpillars of *Archips cerasivoranus* (Lepidoptera: Tortricidae). *J. Chem. Ecol.* 19:1479–1489.

FITZGERALD, T. D. 2008. Larvae of the fall webworm, *Hyphantria cunea*, inhibit cyanogenesis in *Prunus serotina*. *J. Exp. Biol.* 211:671–677.

FITZGERALD, T. D., CLARK, K., VANDERPOOL, R., and PHILLIPS, C. 1991. Leaf shelter-building caterpillars harness forces generated by axial retraction of stretched and wetted silk. *J. Insect Behav.* 4:21–32.

FITZGERALD, T. D., JEFFERS, P. M., and MANTELLA, D. 2002. Depletion of host derived cyanide in the gut of the eastern tent caterpillar, *Malacosoma americanum*. *J. Chem. Ecol.* 28:257–268.

GRANT, J. B. 2006. Diversification of gut morphology in caterpillars is associated with defensive behavior. *J. Exp. Biol.* 209:3018–3024.

MAJACK, W., MCDIARMID, R. E., and HALL, J. W. 1981. The cyanide potential of Saskatoon serviceberry (*Amelanchier alnifolia*) and chokecherry (*Prunus virginiana*). *Can. J. Sci.* 61:681–686.

MEYERS, D. M., and AHMAD, S. 1991. Link between L-3-cyanoalanine synthase activity and differential cyanide sensitivity of insects. *Biochem. Biophys. Acta* 1075:195–197.

PETERSON, S. C., JOHNSON, N. D., and LEGUYADER, J. L. 1987. Defensive regurgitation of allelochemicals derived from host cyanogenesis by eastern tent caterpillars. *Ecology* 68:1268–1272.

SANTAMOUR, F. S. Jr. 1998. Amygdalin in *Prunus* leaves. *Phytochemistry* 47:1537–1538.

SNODGRASS, R. E. 1961. The caterpillar and the butterfly. Smithsonian Misc. Collect. 143(6).

SWAIN, E., LI, C. P., and POULTON, J. E. 1992. Tissue and subcellular localization of enzymes catabolizing (*R*)-amygdalin in mature *Prunus serotina* seeds. *Plant Physiol.* 100:291–300.

WITTHOHN, K., and NAUMANN, C. M. 1987. Cyanogenesis—a general phenomenon in the Lepidoptera? *J. Chem. Ecol.* 13:1789–1809.

compounds be replaced by more environmentally friendly protection methods. One promising strategy is the application of plant-derived protective antifeedant compounds onto plants (Klepzig and Schlyter 1999; Bratt et al. 2001; Sibul et al. 2001; Thacker et al. 2003; Schlyter 2004).

The adult pine weevil accepts numerous non-host species for feeding but prefers Scots pine (*Pinus sylvestris* L.) over most woody species of the Scandinavian flora (Manlove et al. 1997; Månsson and Schlyter 2004). However, there are at least 11 woody species upon which the weevil will avoid feeding even in no-choice tests (Månsson and Schlyter 2004). These are alder (*Alnus glutinosa* (L.) Gaertner), aspen (*Populus tremula* L.), beech (*Fagus sylvatica* L.), guelder rose (*Viburnum opulus* L.), holly (*Ilex aquifolium* L.), horse chestnut (*Aesculus hippocastanum* L.), linden (*Tilia cordata* Mill.), lilac (*Syringa vulgaris* L.), spindle tree (*Evonymus europaeus* L.), walnut (*Juglans regia* L.), and yew (*Taxus baccata* L.). Our general hypothesis is that the avoidance of weevils feeding on these species is caused by antifeedants present in the bark. Thus, we investigated chemical compositions.

The bark of one species, linden (*Tilia cordata*), contains nonanoic acid, which is a potent antifeedant against the pine weevil (Månsson et al. 2005). Several analogues of nonanoic acid are also active antifeedants (Månsson et al. 2006). While reports of the occurrence of similar carboxylic acids in bark tissue are sparse, several have been found in other tissues from some of the 11 woody species (Nahrstedt et al. 1981; Buttery et al. 2000).

To determine if the bark of the ten species rejected by the weevil, except for *Tilia*, also contains antifeedants against *H. abietis*, their barks were extracted with non-polar and polar solvents, and the extracts were tested for activity. Tests were made with doses corresponding to those occurring naturally in bark. The content of known antifeedants in the extracts was determined by gas chromatography (GC)–mass spectrometry (MS). The major compounds identified in the extracts were tested for activity. Additionally, an antifeedant active methanol extract of horse chestnut was fractionated. Stimulatory and inhibitory compounds in the resulting fractions were identified and bio-assayed.

Methods and Materials

Bark Extraction Branches and shoots (5- to 30-mm diam) from the ten plants, *Viburnum*, *Evonymus*, *Alnus*, *Juglans*, *Fagus*, *Aesculus*, *Taxus*, *Ilex*, *Populus*, and *Syringa*, were collected during the summer in southern Sweden. Only young plant material (second-year shoots and branches) was used. Preparation and extraction of the bark were performed as previously described (Månsson 2005; Månsson et al. 2005). Prior to removal of bark from twigs, the total bark area was measured. Outer and inner bark were cut or scraped off the stem, immersed in liquid nitrogen, and ground in a mortar. Pulverized bark was extracted first in a Soxhlet apparatus (Furniss et al. 1989) with boiling pentane (300 ml). After 2 h, the solvent was changed to methanol (MeOH; 300 ml), and the extraction continued for another 2 h. The resulting extracts (one non-polar pentane and one polar MeOH for each species) were filtered through silica gel (~1 g), which was, in both cases, washed with MeOH (~5 ml) prior to being concentrated in a rotary evaporator.

Bioassays Adult *H. abietis* were collected and stored with a food source (pine twigs) and water in buckets kept in darkness at 8–10°C as described earlier (Klepzig and Schlyter 1999; Schlyter et al. 2004a). Before starting an assay, buckets were moved into a growth chamber in which the parameters were set to those found in the field at the time of the weevil collection [24°C, 75% RH, and a photo period of 20:4 h (L/D)]. Twigs were removed 6 days before the start of a micro-feeding assay (24 h before a twig test), and water was removed 1 day before (Schlyter et al. 2004a).

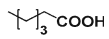
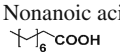
The micro-feeding assay is a choice test that uses two plates, cut from thin-layer cellulose chromatography Al plates (5×5 mm, Merck #1.0552), that were placed in Petri dishes (9-cm diam) and were allowed to run for 4 h. The method is described in detail previously (Schlyter et al. 2004a). Concentrated extracts were diluted with either pentane or methanol. The amount of solvent was determined on the basis of the total bark area extracted. Thus, if the bark area corresponded to, for example, 200 thin-layer chromatography (TLC) plates, each of 25 mm^2, solvent was added until a volume of 200×10 µl (2×5 µl) was reached. Then, 2×5 µl of the diluted extract were applied to the TLC test plate (Månsson et al. 2005). Hence, the dose (amount/area unit) of any compound on the test plate equaled that in the bark, assuming no loss during extraction and a bark thickness equal to the cellulose layer. These assumptions are not met exactly, but the deviations from the two assumptions will be of opposite directions and will partly cancel out.

Synthetic compounds tested (Tables 1, 2, and 3) were applied in 1.5-µl aliquots in a 10% MeOH solution. After solvent evaporation from both the treatment and solvent blank plates, 5 µl of 1 M sucrose in water were added as a feeding stimulant to both (Schlyter et al. 2004a).

Twig no-choice tests were performed on 12-mm long *P. sylvestris* twig sections of approximately equal diameter (6- to 10-mm diam), which were dipped in solutions of the compound to be tested. The method is described in detail (Klepzig and Schlyter 1999; Schlyter et al. 2004a).

The Anti-Feedant Index, AFI, was the variable used to quantify results from the assays (Schlyter et al. 2004a). The

Table 1 Content of known antifeedants identified in pentane and methanol extracts of ten woody non-host species and their activity against *Hylobius abietis*

Compound	AFI ±SE (*n*)	*Viburnum opulus*	*Evonymus europaeus*	*Alnus glutinosa*	*Juglans regia*	*Fagus sylvatica*	*Aesculus hippocastanum*	*Taxus baccata*	*Ilex aquifolium*	*Populus tremula*	*Syringa vulgaris*	Reference
Hexanoic acid 	0.98* ± 0.01 (26)			X					X			Månsson et al., 2006
Nonanoic acid 	0.94* ±0.05 (41)								X			Månsson et al., 2006, Månsson et al., 2005
Carvone** 	0.80* ±0.06 (49) *R*-enantiomer					X			X			Schlyter et al., 2004b
	0.92* ±0.14 (19) *S*-enantiomer											Schlyter et al., 2004b
Carvacrol 	1.00* ±0.00 (24)						X					Schlyter et al., 2004b

*) AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations (negative values indicate feeding stimulants)

**) Stereoisomeric composition in the extract not determined

area eaten on the control plate (*C*) is related to the area eaten on the test plate (*T*): AFI = (*C* − *T*)/(*C* + *T*). Negative values (<0) indicate a feeding stimulation, while a value of 1.00 corresponds to total feeding inhibition. A value of 0.50 indicates a half effect and 0 no effect. AFI values are presented as their arithmetic means ± SE (standard error of the mean), and activity is considered significant if the 95% confidence interval for the mean (95% CI) does not overlap zero.

The AFI obtained from the dose-response test with 10%, 1%, and 0.1% solutions in the micro-assays provides an ED_{50} value. The ED_{50} is the effective dose (expressed as concentration in percent of the treatment solution) that is needed for achieving a 50% feeding deterrent effect (i.e., the dose required to get an AFI=0.5).

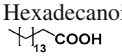
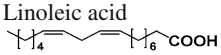
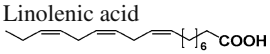
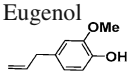
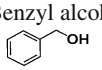
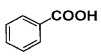
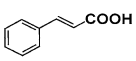
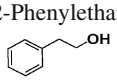
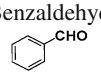
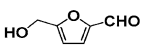
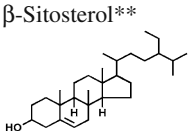
Fractionation of Aesculus Methanol Extract All solvents were distilled prior to use. The MeOH extract from *Aesculus* bark (M1) was fractionated by reverse phase LC by using octadecyl functionalized silica gel (12 g, Sigma-Aldrich Sweden AB) in a flash column (10 mm i.d.). The gradient used was one-column volume (10 ml) of MeOH followed by 10 ml of 50% CH_2Cl_2 in MeOH, 10 ml CH_2Cl_2, 10 ml 50% pentane in CH_2Cl_2, and finally, 20 ml of pentane. In total, 14 fractions, each of ≈4 ml, were collected. The fractions containing similar compounds (as judged by TLC) were pooled. Each of the resulting eight fractions (M1:1–8) were concentrated to 1 ml by flushing a gentle stream of argon over the solution, and they were subsequently tested in the micro-feeding assay.

One fraction (M1:3) had antifeedant activity and was further fractionated on silica gel 60 (6.5 g, Fluka) on a flash column (10 mm i.d.). The elution gradient was 20 ml of CH_2Cl_2 followed by 10 ml of 10% MeOH in CH_2Cl_2, 10 ml 20% MeOH in CH_2Cl_2, 10 ml 50% MeOH in CH_2Cl_2, 10 ml 75% MeOH in CH_2Cl_2, and finally, 50 ml MeOH. In total, 30 fractions, each of ≈4 ml, were collected. By merging fractions of similar chemical content as determined by TLC, the number of fractions was reduced to nine (M1:3:1–9), concentrated, and tested in the micro-feeding assay.

Chemical Analysis Bark extracts were analyzed by GC–MS on a Varian 3800 GC instrument with a CP-sil 5CB low-bleed column (Varian, 30 m×0.25 mm i.d., 0.25 μm film thickness), in series with a Saturn 2000 MS, ion trap detector, EI mode. All compounds in Tables 1 and 2 were identified by comparing their retention times and mass spectra with those of the reference compounds co-injected on the same instrument under the same analytical conditions.

Chemical Compounds (*Z*)-3-Hexenyl hexanoate was purchased from Bedoukian Research, β-sitosterol was purchased from Fluka. Tetradecyl hexanoate was synthesized

Table 2 Identified and tested compounds in pentane and methanol extracts of the ten woody non-host species and their activity against *Hylobius abietis*

Compound	AFI ±SE (*n*)	*Viburnum opulus*	*Evonymus euraopaeus*	*Alnus glutinosa*	*Juglans regia*	*Fagus sylvatica*	*Aesculus hippocastanum*	*Taxus baccata*	*Ilex aquifolium*	*Populus tremula*	*Syringa vulgaris*
Hexadecanoic acid 	0.16 ±0.09 (28)	X	X	X	X	X	X	X	X	X	X
Linoleic acid 	–0.15 ±0.10 (29)	X	X	X	X	X	X	X	X	X	X
Linolenic acid 	–0.09 ±0.08 (36)									X	
Limonene** 	0.05 ±0.13 (19)		X				X		X		
Eugenol 	1.00* ±0.00 (14)	X				X	X	X			
Benzyl alcohol 	0.59* ±0.17 (17)				X				X	X	
Vanillin 	0.01 ±0.25 (14)		X	X		X		X	X		
Benzoic acid 	–0.18 ±0.13 (19)		X		X	X	X	X	X	X	X
Cinnamic acid 	–0.02 ±0.11 (28)							X		X	
2-Phenylethanol 	1.00* ±0.01 (19)								X	X	
Benzaldehyde 	–0.16 ±0.12 (20)		X				X	X	X	X	X
2-Furaldehyde 	–0.16 ±0.13 (24)	X	X	X	X	X	X			X	X
5-(Hydroxymethyl)-2-furaldehyde 	–0.32* ±0.13 (19)				X		X			X	X
β-Sitosterol** 	–0.27* ±0.09 (38)	X	X	X	X	X	X	X		X	X

*) AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations (negative values indicate feeding stimulants)

**) Stereoisomeric composition in the extract not determined

Table 3 The antifeedant activity in *Hylobius abietis* of hexanoate esters and branched aliphatic alcohols, lead compounds tentatively identified from horse chestnut extracts

Compound	AFI ±SE (*n*)	ED_{50} (%) ±SE (*n*)	AFI twig ± SE (*n*)
(*Z*)-3-Hexenyl hexanoate	0.99[a]±0.01 (10)		
Hexyl hexanoate	0.87[a]±0.10 (19)		
2-Methylbutyl hexanoate	0.86[a]±0.07 (7)		
3-Methylbutyl hexanoate	0.88[a]±0.06 (10)		
iso-Butyl hexanoate	0.83[a]±0.10 (10)		
Tetradecyl hexanoate	0.32±0.16 (19)		
2-Butyl-1-octanol	0.86[a]±0.10 (27)	0.76±0.59 (2)	0.21[a]±0.07 (20)
2-Ethyl-1-hexanol	0.93[a]±0.08 (27)		0.11±0.16 (10)
2-Hexyl-1-decanol	0.17±0.09 (29)		0.24[a]±0.10 (10)

[a] AFI significantly different from 0 (zero) by 95% confidence interval for the mean of *n* observations

from hexanoic acid and tetradecanol; spectral data (^1H, ^{13}C NMR, and MS) were satisfactory. All other chemical compounds in Tables 1, 2, and 3 were purchased from Sigma-Aldrich Sweden AB.

Results

Twenty extracts (one pentane and one methanol of each species) of A*lnus*, *Populus*, *Fagus*, *Ilex*, *Aesculus*, *Viburnum*, *Syringa*, *Evonymus*, *Juglans*, and *Taxus* were tested twice in the micro-assay. In the first run, the highest antifeedant activity was found for the methanol extract of *Aesculus* (*N*=7). Combining the results from both test runs, the methanol extracts of *Populus*, *Ilex*, *Aesculus*, and *Taxus* were all active in inhibiting feeding (AFI 95% CI were all >0, Fig. 1). None of the pentane extracts displayed any antifeedant activity in these species. In contrast, the pentane extracts of *Fagus*, *Viburnum*, *Aesculus*, and *Syringa* significantly stimulated feeding (AFI 95% CI were all <0, Fig. 1).

The content of known antifeedants, such as straight-chained aliphatic carboxylic acids in the range C6–C9 (Månsson et al. 2006), carvone, and carvacrol (Schlyter et al. 2004b) in the 20 extracts was determined by GC–MS (Table 1). Among known active carboxylic acids, the feeding deterrents hexanoic acid (in the pentane extract of *Ilex* and *Alnus*) and nonanoic acid (in the pentane extract of *Ilex*) were detected. The known pine weevil antifeedants carvone and carvacrol were found in the pentane extracts of *Fagus* and *Ilex*, and in the methanol extract of *Aesculus*, respectively (stereoisomeric composition of carvone in the extracts was not determined; however, both enantiomers are active antifeedants, Table 1).

Additionally, major compounds in the extracts were identified, and those available were tested in the micro-assay (Table 2). The antifeedant active methanol extracts of *Aesculus*, *Taxus*, and *Populus* contained one or more of the deterrent, aromatic compounds carvacrol, eugenol, or benzyl alcohol (Tables 1 and 2). To our knowledge, the latter, together with 2-phenylethanol that was found in the pentane extracts of *Ilex* and *Populus*, have not been reported previously as feeding deterrents for the pine weevil. Two aldehydes, 2-furaldehyde (furfural) and 5-(hydroxymethyl)-2-furaldehyde (5-hydroxymethylfurfural), were found in some extracts with antifeedant activity. While no activity was found for 2-furaldehyde, 5-(hydroxymethyl)-2-furaldehyde stimulated feeding (AFI significantly <0). Another feeding stimulant, β-sitosterol, was detected in the majority of the extracts (Table 2). Hexadecanoic acid, which was identified in almost all extracts (both pentane and methanol), did not exhibit any antifeedant activity (Table 2). Two other long-chain carboxylic acids, linoleic acid that was found in several pentane and methanol extracts and linolenic acid present in the pentane extract of *Populus*, seemed to

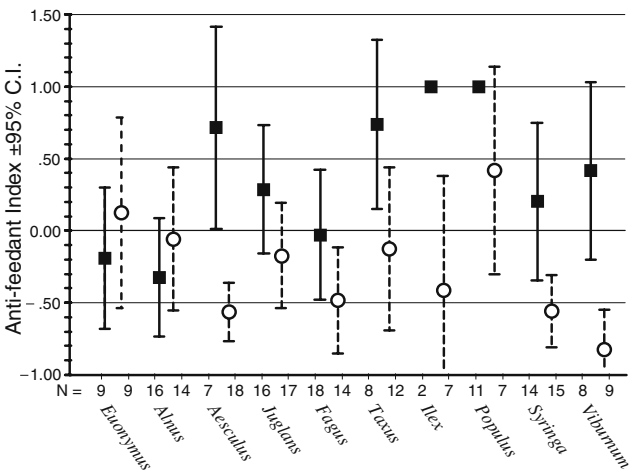


Fig. 1 Antifeedant index (*AFI*) of pentane (*lines with open circles*) and methanol (*lines with filled squares*) extracts in micro-feeding assay choice test of ten woody non-hosts. Plants extracted included spindle tree (*Evonymus europaeus*), alder (*Alnus glutinosa*), horse chestnut (*Aesculus hippocastanum*), walnut (*Juglans regia*), beech (*Fagus sylvatica*), yew (*Taxus baccata*), holly (*Ilex aquifolium*), aspen (*Populus tremula*), lilac (*Syringa vulgaris*), and guelder rose (*Viburnum opulus*). *N* on the *x*-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish. AFI relates the area eaten on the control plate (*C*) to that on the test plate (*T*): $AFI = (C - T)/(C + T)$ and ranges from −1 to +1

stimulate feeding but not significantly so (both AFI <0). Five common plant constituents, limonene, vanillin, benzoic acid, cinnamic acid, and benzaldehyde, were identified in some extracts but showed no effect on the weevil (AFI ~0, Table 2).

Since *Aesculus* was the only species that yielded both inhibitory (methanol) and stimulatory (pentane) extracts and was active in the first micro-feeding test, the active methanol extract (M1) was chosen for fractionation. Reverse-phase liquid chromatography resulted in eight fractions, of which several (M1:4 to M1:8) were phagostimulatory. Only fraction M1:3 displayed significant antifeedant activity (Fig. 2). However, when the eight fractions were combined, the activity was lost (M1 sum, Fig. 2). Fractions M1:2 (slightly antifeedant) and M1:3 (active antifeedant) contained similar patterns of compounds with one exception; fraction M1:2 contained a large amount of the feeding stimulant 5-(hydroxymethyl)-2-furaldehyde, while M1:3 did not.

A second chromathographic separation of the antifeedant active fraction M1:3 on straight phase silica gel resulted in nine fractions of which the last (M1:3:9) exhibited the highest activity (Fig. 3). In contrast, several fractions had AFI<0, and fraction M1:3:5 strongly (AFI<<0) and significantly stimulated weevil feeding on treated plates (Fig. 3). Two compounds were common in fractions M1:3:8 and M1:3:9, and their mass spectra showed they were aliphatic esters of hexanoic acid. The mass spectra and retention times of these compounds were compared with

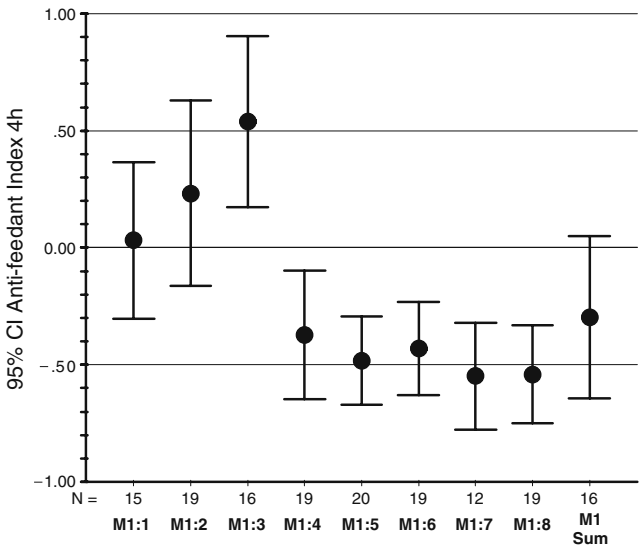


Fig. 2 Feeding activity of fractions of the first *Aesculus* methanol extract in micro-feeding assay no-choice tests. *N* on the *x*-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish. *M1:sum* joined extract from pooling all fractions

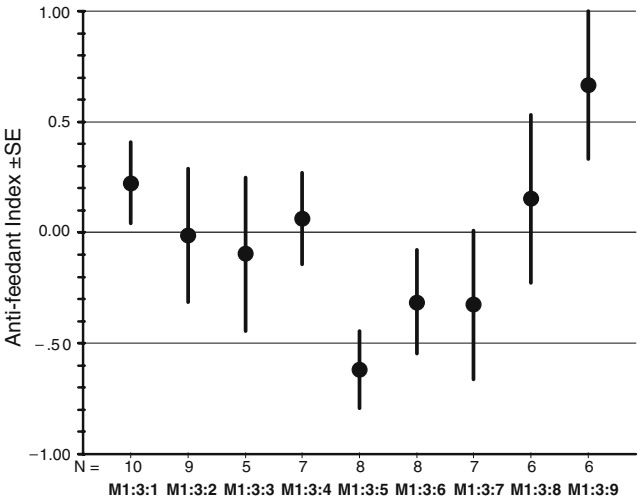


Fig. 3 Feeding activity of the sub-fractions of fraction M1:3 from the first *Aesculus* methanol extract in micro-feeding assay no-choice tests. *N* on the *x*-axis is the number of data points (individual beetles in Petri dishes) where an AFI could be calculated, i.e., when feeding occurred on at least one of the paired plates in the dish

those of reference samples of some esters of that type (tetradecyl-, tridecyl-, dodecyl-, and hexyl hexanoate), but neither the mass spectra nor the retention times were identical with any of the reference esters. Interestingly, when six available esters of this type were tested in the micro-feeding assay, five out of six were active antifeedants with AFIs>0.80 (Table 3). The most active was (*Z*)-3-hexenyl hexanoate, followed by hexyl hexanoate, 2-methylbutyl hexanoate, 3-methylbutyl hexanoate, and *iso*-butyl hexanoate. The heavier tetradecyl hexanoate was not active.

The mass spectra of other major components in the slightly antifeedant active fraction M1:3:8 indicated they were branched aliphatic alcohols. Three alcohols of that type were available, namely 2-butyl-1-octanol, 2-ethyl-1-hexanol, and 2-hexyl-1-decanol, but their retention times were not identical with those in the extracts. In the micro-feeding assay at a 10% dose, they displayed varying antifeedant activities (Table 3): high activity for 2-butyl-1-octanol and 2-ethyl-1-hexanol but low activity for 2-hexyl-1-decanol. The ED_{50} for 2-butyl-1-octanol in the choice micro-assay was as low as 0.8% (±0.6 *N*=2), indicating high activity. When tested on twigs, less activity than in the micro-feeding assay was obtained for 2-butyl-1-octanol and 2-ethyl-1-hexanol, while 2-hexyl-1-decanol showed the same low activity as in the micro-feeding assay.

Discussion

Eleven new active compounds were found during this study. Two of these, β-sitosterol and 5-(hydroxymethyl)-2-

furaldehyde (5-hydroxymethyl furfural), are the first reported feeding stimulants from non-host plants of the pine weevil. Another two, benzyl alcohol and 2-phenylethanol, were new antifeedants that were identified in several non-host plants. Aliphatic esters of hexanoic acid and branched aliphatic alcohols were tentatively identified in active extracts. Nine compounds belonging to either of these classes of compounds were tested, and seven were antifeedants.

Apart from the 11 new active compounds and the four known antifeedants identified in non-host plants (Table 1), the contrasting activity of polar and nonpolar extracts is a striking finding. Activities of extracts are related to the polarity of the solvent used for extraction (Fig. 1). Thus, only four out of ten plant barks furnished active inhibitory extracts (*Populus*, *Ilex*, *Aesculus*, and *Taxus*), and all of these were methanol extracts. In contrast, the pentane extracts of *Fagus*, *Viburnum*, *Aesculus*, and *Syringa* were strongly feeding stimulatory. Only the bark of one species, *Aesculus*, produced extracts that were both stimulatory (pentane) and inhibitory (MeOH).

We previously showed that some aliphatic carboxylic acids (in the range C6–C10) act as antifeedants against the pine weevil (Månsson et al. 2005, 2006). Of these, nonanoic acid was found in a dichloromethane/methanol bark extract of *Tilia* (Månsson 2005; Månsson et al. 2005). In addition, hexanoic, heptanoic, octanoic, and nonanoic acids have been found in *Fagus* wood (Guillén and Ibargoitia 1996). We found only hexanoic acid in the pentane bark extracts of *Ilex* and *Alnus*, and nonanoic acid in the pentane bark extract of *Ilex* (Table 1). Thus, the feeding avoidance of *H. abietis* on the ten woody species investigated in this work is not fully explained by the presence of these carboxylic acids, as it was for linden (Månsson et al. 2005, 2006).

Fractionation of the *Aesculus* methanol extracts led to the discovery of two new groups of pine weevil antifeedants, i.e., esters of hexanoic acid and branched alcohols. Additionally, the aromatic compounds benzyl alcohol and 2-phenylethanol were identified as new antifeedants. Eugenol had earlier been found in a polar extract of the feces of *H. abietis* females. Similar to our results, the compound exhibited antifeedant activity against the weevil when tested in a feeding bioassay (Borg-Karlson et al. 2006). In accordance with the decreased antifeedant activity found for carboxylic acids with chains longer than C10 (Månsson et al. 2006), no antifeedant activity was found for hexadecanoic-, linoleic-, or linolenic acids that were present in almost all extracts (Table 1).

The heteroaromatic aldehyde 5-(hydroxymethyl)-2-furaldehyde showed feeding stimulating properties. Previously, 5-(hydroxymethyl)-2-furaldehyde was reported as a constituent of the essential oil and head space extract of *Aesculus* flowers (Buchbauer et al. 1994). However, both 5-(hydroxymethyl)-2-furaldehyde and 2-furaldehyde, which was also found in some extracts, could be artifacts formed by carbohydrate dehydration (Kallury et al. 1986; Antal Jr. et al. 1990), either during the extraction procedure or in the injector of the GC.

In contrast to the high feeding-deterrent activity found for some methoxy-substituted benzaldehyde derivatives (Eriksson 2006), benzaldehyde itself showed no antifeedant activity. However, the inactivity of cinnamic acid and benzoic acid was not surprising since earlier studies have shown that methoxy-substituted benzoic- and cinnamic-acid derivatives are less active antifeedants than the corresponding aldehydes (Eriksson 2006) or methyl esters (Unelius et al. 2006).

All extracted species, except holly, contained β-sitosterol, which strongly stimulated feeding of *H. abietis*. This compound has been identified as a feeding stimulant for the obscure root weevil, *Sciopithes obscurus* Horn (Doss et al. 1982), an effect that was synergistically enhanced by adding sucrose (Shanks and Doss 1987). In our tests of β-sitosterol, sucrose was always added to the test plate.

Most extracts contained a complex mixture of antifeedant and feeding stimulatory compounds. Whether the final outcome of an extract was a feeding deterrent or a stimulatory effect was dependent on the mixture of compounds therein and on their concentrations. The feeding stimulant β-sitosterol, occurring in almost all extracts, was a minor constituent in the antifeedant active extracts, whereas in the feeding stimulatory and inactive extracts, it was a major constituent. Hence, the deterrent activity of the extracts appears to be partially dependent on the efficiency by which β-sitosterol is removed during the non-polar pentane extraction. The concentration dependence was exemplified by the fact that when the eight fractions of the active methanol extract of *Aesculus* were combined, deterrent activity was lost. Probably, this can be ascribed to the fact that the concentrations of the specific deterrents became too low relative to that of β-sitosterol.

Even after extensive fractionation of extracts, the difficulty of identifying antifeedants in the presence of feeding stimulating compounds remains. After fractionation of the first methanol extract of *Aesculus*, fractions M1:2 and M1:3 were found to have a similar composition. However, only fraction M1:3 (Fig. 2) showed feeding deterrent activity. This is possibly due to the absence of the feeding stimulant, 5-(hydroxymethyl)-2-furaldehyde, which was detected only in the less active fraction 2 (M1:2, Fig. 2).

In conclusion, eleven new active compounds were found in this study. Two of these, β-sitosterol and 5-(hydroxymethyl)-2-furaldehyde, are the first reported feeding stimulants for the pine weevil, while another two compounds, benzyl alcohol and 2-phenylethanol, were

found to be new antifeedants identified from several non-hosts of the weevil. Seven compounds belonging to either of the two classes of aliphatic esters of hexanoic acid or branched aliphatic alcohols were found to be active antifeedants. In addition, four earlier known antifeedant compounds were identified from four non-host plants. Our work suggests that further active compounds in the bark of these woody species remain to be identified.

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References

ANTAL, J. M. Jr., MOK, W. S. L., and RICHARDS, G. N. 1990. Mechanism of formation of 5-(hydroxymethyl)-2-furaldehyde from D-fructose and sucrose. *Carbohydr. Res.* 199:91–109.

BORG-KARLSON, A.-K., NORDLANDER, G., MUDALIGE, A., NORDENHEM, H., and UNELIUS, C. 2006. Antifeedants in the feces of the pine weevil *Hylobius abietis*: identification and biological activity. *J. Chem. Ecol.* 32:943–957.

BRATT, K., SUNNERHEIM, K., NORDENHEM, H., NORDLANDER, G., and LÅNGSTRÖM, B. 2001. Pine weevil (*Hylobius abietis*) antifeedants from lodgepole pine (*Pinus contorta*). *J. Chem. Ecol.* 27:2253–2262.

BUCHBAUER, G., JIROVETZ, L., WASICKY, M., and NIKIFOROV, A. 1994. Volatiles of common horsechestnut (*Aesculus hippocastanum* L.) (Hippocastanaceae) blossoms. *J. Essent. Oil Res.* 6:93–95.

BUTTERY, R. G., LIGHT, D. M., NAM, Y., MERRILL, G. B., and ROITMAN, J. N. 2000. Volatile components from green walnut husks. *J. Agric. Food Chem.* 48:2858–2861.

DOSS, R. P., LUTHI, R., EDELMAN, D. L., and HRUTFIORD, B. F. 1982. Sitosterol and quercetin 3-galactoside, obscure root weevil feeding stimulants from rhododendron. *J. Agric. Food Chem.* 30:1079–1082.

ERIKSSON, C. 2006. Isolation, synthesis and structure–activity relationships of antifeedants against the pine weevil, *Hylobius abietis*. Doctoral thesis, Chemical Science and Engineering, Royal Institute of Technology (KTH), Stockholm, 63 pp, ISBN: 91-7178-301-6.

FURNISS, B. S., HANNAFORD, A. J., SMITH, P. W. G., and TATCHELL, A. R. 1989. Vogel's Textbook of Practical Organic Chemistry. Longman Scientific & Technical, Essex.

GUILLÉN, M. D., and IBARGOITIA, M. L. 1996. Volatile components of aqueous liquid smokes from *Vitis vinifera* L. shoots and *Fagus sylvatica* L. wood. *J. Sci. Food. Agric.* 72:104–110.

KALLURY, R. K. M. R., AMBIDGE, C., TIWELL, T. T., BOOCOCK, D. G. B., ABGLEVOR, F. A., and STEWART, D. J. 1986. Rapid hydrothermolysis of cellulose and related carbohydrates. *Carbohydr. Res.* 158:253–261.

KLEPZIG, K. D., and SCHLYTER, F. 1999. Laboratory evaluation of plant derived antifeedants against European pine weevil, *Hylobius abietis*. *J. Econ. Entomol.* 92:644–650.

LÅNGSTRÖM, B., and DAY, K. R. 2004. Damage, control and management of weevil pests, pp. 415–444, in F. Lieutier, K. R. Day, A. Battisti, J.-C. Gregoire, and F. Evans (eds.). Bark and Wood Boring Insects in Living Trees in Europe, A Synthesis-Kluwer, Dordrecht, The Netherlands.

MANLOVE, J. D., STYLES, J., and LEATHER, S. R. 1997. Feeding of the adults of the large pine weevil, *Hylobius abietis* (Coleoptera: Curculionidae). *Eur. J. Entomol.* 94:153–156.

MÅNSSON, P. E. 2005. Host selection and antifeedants in *Hylobius abietis* pine weevils. Doctoral thesis, Deparment of Crop Science, Swedish University of Agricultural Sciences (SLU), Alnarp, 90 pp. ISBN: 91-576-7015-3. http://diss-epsilon.slu.se/archive/00000759/01/per200516.pdf.

MÅNSSON, P. E., and SCHLYTER, F. 2004. Hylobius pine weevils adult host selection and antifeedants: feeding behaviour on host and non-host woody Scandinavian plants. *Agric. Forest Entomol.* 6:165–171.

MÅNSSON, P. E., ERIKSSON, C., and SJÖDIN, K. 2005. Antifeedants against *Hylobius abietis* pine weevils: an active compound in extract from bark of *Tilia cordata* Linden. *J.Chem. Ecol.* 31:989–1001.

MÅNSSON, P. E., SCHLYTER, F., ERIKSSON, C., and SJÖDIN, K. 2006. Nonanoic acid, other alkanoic acids, and related compounds as antifeedants in *Hylobius abietis* pine weevils. *Entomol. Exp. Appl.* 121:191–201.

MCLEESE, D. W., METCALFE, C. D., and ZITKO, V. 1980. Lethality of permethrin, cypermethrin and fencalerate to salmon, lobster and shrimp. *Bull. Environ. Contam. Toxicol.* 25:950–955.

NAHRSTEDT, A., VETTER, U., and HAMMERSCHMIDT, F. J. 1981. Knowledge on the steam distillate from the leaves of *Juglans regia*. *Planta Med.* 42:313–332.

SCHLYTER, F. 2004. Semiochemicals in the life of bark feeding weevils, pp. 351–364, in F. Lieutier, K. R. Day, A. Battisti, J.-C. Gregoire, and F. Evans (eds.). Bark and Wood Boring Insects in Living Trees in Europe, A SynthesisKluwer, Dordrecht, The Netherlands.

SCHLYTER, F., MARLING, E., and LÖFQVIST, J. 2004a. A new microassay for antifeedants in Hylobius pine weevils (Coleoptera). *J. Pest Sci.* 77:191–195.

SCHLYTER, F., SMITT, O., SJÖDIN, K., HÖGBERG, H.-E., and LÖFQVIST, J. 2004b. Carvone and less volatile analogues as repellent and deterrent antifeedants against the pine weevil, *Hylobius abietis*. *J. Appl. Entomol.* 128:610–619.

SHANKS, C. H., and DOSS, R. P. 1987. Feeding responses by adults of five species of weevils (Coleoptera: Curculionidae) to sucrose and sterols. *Ann. Entomol. Soc. Am.* 80:41–46.

SIBUL, I., LUIK, A., and VOOLMA, K. 2001. Possibilities to influence maturation feeding of the large pine weevil, *Hylobius abietis* L., with plant extracts and neem preparations, pp. 112–119, in Practice oriented results on the use of plant extracts and pheromones in pest control: Proceedings of the International Workshop, 24–5 January, 2001, Tartu, Estonia.

THACKER, J. R. M., BRYAN, W. J., MCGINLEY, C., HERITAGE, S., and STRANG, R. H. C. 2003. Field and laboratory studies on the effects of neem (*Azadirachta indica*) oil on the feeding activity of the large pine weevil (*Hylobius abietis* L.) and implications for pest control in commercial conifer plantations. *Crop Prot.* 22:753–760.

UNELIUS, C. R., NORDLANDER, G., NORDENHEM, H., HELLQVIST, C., LEGRAND, S., and BORG-KARLSSON, A.-K. 2006. Structure–activity relationships of benzoic acid derivatives as antifeedants for the pine weevil, *Hylobius abietis*. *J. Chem. Ecol.* 32:2191–2203.

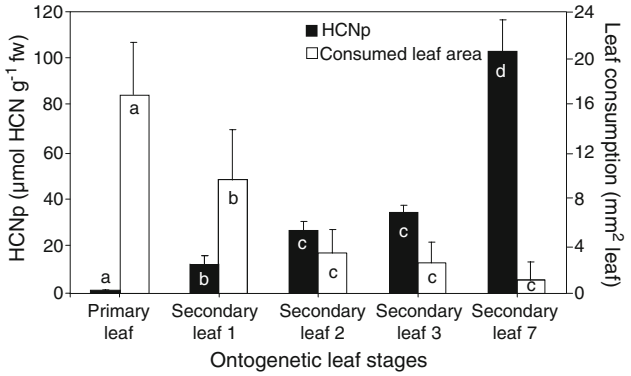

Fig. 1 Cyanogenic potential (*HCNp*) of different *P. lunatus* leaf developmental stages and preferences of herbivorous beetles. In choice experiments presenting all leaf stages simultaneously, attractiveness of leaf material to Mexican bean beetles was quantified. Values shown are means±standard deviation. Letters in/at the columns indicate significant differences in HCNp [according to LSD *post hoc* analysis ($P<0.01$) after one-way ANOVA]

(Mattson 1980). In addition, we measured the feeding rates of a natural herbivore, the Mexican bean beetle (Coccinellidae: *Epilachna varivestis* Mulsant) on these different leaf stages. This combined analysis of a defensive and a nutritive plant feature contributes to understanding the intra-individual variation of plant defensive traits in the interaction with herbivores.

Materials and Methods

Plants Lima bean plants (Fabaceae: *P. lunatus* L.) were derived from seed material collected in a natural population in Oaxaca, South Mexico (15°55′ N, 097°09′ W). Plants were cultivated in a climatic chamber adjusted to mimic conditions as recorded for the natural site in August–October 2007 (13:11 h light to dark ratio; 30:23°C; 60–70% air humidity and a photon flux density of 450–500 µmol s^{-1} m^{-1} at table height). Plants were cultivated in standard substrate (TKS®-1-Instant, Floragard, Oldenburg, Germany) mixed 1:3 with sand (grain size of 0.3–0.7 mm). Plant containers were 80 mm in diameter. We fertilized plants twice a week with an aqueous solution (0.5%) of a NPK-fertilizer (Flory-3®, EUFLOR GmbH, Munich, Germany). Plants were used for experiments when they had developed 9 to 10 leaves (6 weeks after germination; $N=8$ plants). In addition to primary leaves, we selected four secondary leaf stages for analyses. We defined the ontogenetically oldest secondary leaf as 'secondary leaf 1', while 'secondary leaf 7' was the youngest one, inserting seven positions above the primary leaves.

Cyanogenic Potential HCNp was quantified by complete enzymatic degradation of cyanogenic precursors by using specific β-glucosidase isolated from cyanogenic *Hevea brasiliensis*. Concentration of released cyanide was quantified spectrophotometrically (at 585 nm) by using the Spectroquant®Cyanide kit (Merck KGaA, Darmstadt, Germany) according to Ballhorn et al. (2007).

Protein Concentration We quantified soluble protein contents in leaves according to Bradford (1976). Polyvinylpolypyrrolidone (Sigma-Aldrich, Buchs, Switzerland) was added to leaf extracts to avoid potential interference of plant phenolics with protein.

Electron Transport Rate Photosynthetic activity of leaves was measured *in situ* in the climatic chamber. For nondestructive measurements, a chlorophyll fluorometer (Junior PAM, Walz GmbH, Effeltrich, Germany) was used. After 10 min of shading, leaf spots were illuminated with blue light at 400 µmol photons m^{-2} s^{-1} for 7 min and steady state photosynthesis was quantified as electron transport rate (ETR) values (µmol electrons m^{-2} s^{-1}).

Feeding Trials The feeding rates of adult Mexican bean beetles on leaves of different ontogenetic stages were analyzed in Petri dishes (9.5 cm) lined with moist filter paper. Beetles were starved for 4 h before leaf discs (16 mm in diameter; representing each one of the five leaf

Table 1 Photosynthetic activity and soluble protein concentration in different ontogenetic leaf stages of *P. lunatus*

Physiological characteristics of leaves used in feeding trials	Leaf developmental stages				
	Primary leaf	Secondary leaf 1	Secondary leaf 2	Secondary leaf 3	Secondary leaf 7
ETR [µmol electrons m^{-2} s^{-1}]	98.9±3.5a	101.4±2.0a	99.2±2.7a	96.6±2.6a	92.4±3.5a
Soluble protein [mg g^{-1} fw]	14.2±0.3a	14.0±0.7a	14.3±0.6a	14.5±0.4ab	15.2±0.8b

Values shown for electron transport rate and soluble protein concentration of different ontogenetic leaf stages are means±standard deviation ($N=8$ plants). Letters indicate significant differences among leaf stages according to *post hoc* analysis (LSD; $P<0.01$) after one-way ANOVA
ETR Electron transport rate

developmental stages) were offered to individual animals for 2 h. Position of leaf discs was set at random. After the experiment, leaf discs were scanned and the missing leaf area was quantified with Analysis® software (Olympus, Hamburg, Germany).

Statistical Analysis We used SPSS 16.0 (SPSS for Windows, SPSS, Chicago, IL, USA) for all statistical analyses.

Results

Cyanogenic Potential HCNp in primary leaves was significantly lower than in secondary leaves [according to least significant difference (LSD) *post hoc* analysis ($P<0.01$) after univariate analysis of variance (ANOVA); Fig. 1]. Among secondary leaves, the youngest leaves showed highest HCNp values (Fig. 1).

Protein Concentration The youngest secondary leaves contained significantly more soluble proteins than the primary leaves (one-way ANOVA with 'ontogenetic stage' as factor: $F_{4,35}=5.70$, $P<0.01$), whereas protein concentrations in older secondary leaves and primary leaves were not significantly different (Table 1).

Electron Transport Rate Chlorophyll fluorescence measurements revealed that plants showed high ETR in all leaf developmental stages analyzed. The ETR values were not significantly different among all leaf stages (Table 1).

Feeding Trials Beetles significantly preferred primary over secondary leaves (Fig. 1). Young secondary leaves were less consumed by beetles than relatively older secondary leaves. Among all leaf stages, consumed leaf area and HCNp in leaves were significantly negatively correlated (according to two-tailed Pearson correlation: $r=-0.638$, $P<0.001$).

Discussion

Primary leaves of wild-type lima bean plants were long living and showed high photosynthetic activity for a period of time that exceeded early seedling stages (>6 weeks after germination). In addition, protein concentrations in primary leaves were similar to those of the three following secondary leaves, indicating that no strong reallocation of resources from primary to the secondary leaves occurred as part of a beginning senescence of primary leaves during the considered time span.

Although presumably being valuable source organs for carbohydrates, primary leaves were poorly defended by cyanogenesis—and, consequently, Mexican bean beetles significantly preferred them over higher cyanogenic secondary leaves. Higher protein concentrations in the youngest secondary leaves ('secondary leaf 7') did not compensate for low attractiveness due to higher HCNp (Fig. 1; Table 1). In accordance with a previous study, cyanogenesis proved to be highly efficient in deterring the Mexican bean beetle (Ballhorn and Lieberei 2006).

In the present study, we focused on ontogenetic variation of cyanogenesis as a direct defense against herbivores, whereas indirect defense by VOCs was not measured. However, we demonstrated previously that the primary leaves of lima bean are not without defense, as they released high amounts of VOCs, whereas high cyanogenic secondary leaves emitted much lower amounts of VOCs (Ballhorn et al. 2008). Why do different leaf developmental stages express different types of defense? It appears to be a general phenomenon that plants do not rely on a single defense mechanism but rather express multiple defenses (Agrawal and Fishbein 2006). Nevertheless, despite beneficial effects, co-occurrence of defenses might be costly for a plant (Mauricio 1998), since investment in defensive traits is assumed to reduce the resource availability for growth and reproduction (Herms and Mattson 1992). Simultaneous resource allocation to growth and expensive nitrogen-based defenses (such as cyanogenesis) likely is constrained, particularly during the first weeks of plant development. Thus, carbon-based indirect defenses (VOCs) may be a good alternative for these early leaves, particularly when regarding their high photosynthetic capacities that were found in the present study. Although individual leaves of lima bean apparently could not express more than one type of defense at high quantities, plants may defend different leaf stages via different strategies. We suggest that multiple defense syndromes also may emerge at the 'plant level', not only at the level of individual leaves. Future studies should focus on effects of ontogenetic variation in defense mechanisms against herbivores and pathogens.

Acknowledgements We thank Monika Fillippek for excellent assistance in plant cultivation and maintenance of beetles. Financial support by the University of Duisburg Essen and the DFG (grant He 3169/4-2) is gratefully acknowledged.

References

AGRAWAL, A. A., and FISHBEIN, M. 2006. Plant defense syndromes. *Ecology* 87:132–149.

BALLHORN, D. J., and LIEBEREI, R. 2006. Oviposition choice of Mexican bean beetle (*Epilachna varivestis*) depends on host plants cyanogenic capacity. *J. Chem. Ecol.* 32:1861–1865.

BALLHORN, D. J., HEIL, M., PIETROWSKI, A., and LIEBEREI, R. 2007. Quantitative effects of cyanogenesis on an adapted herbivore. *J. Chem. Ecol.* 33:2195–2208.

BALLHORN, D. J., KAUTZ, S., LION, U., and HEIL, M. 2008. Trade-offs between direct and indirect defences of lima bean (*Phaseolus lunatus*). *J. Ecol.* 96(5):971–980. doi:10.1111/j.1365-2745.2008.01404.x.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248–254.

HEIL, M. 2004. Direct defense or ecological costs: responses of herbivorous beetles to volatiles released by wild lima bean (*Phaseolus lunatus*). *J. Chem. Ecol.* 30:1289–1295.

HEIL, M. 2008. Indirect defence via tritrophic interactions. *New Phytol.* 178:41–61.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants: to grow or defend. *Q. Rev. Biol.* 67:283–335.

MATTSON, W. J. 1980. Herbivory in relation to plant nitrogen content. *Annu. Rev. Ecol. Syst.* 11:119–161.

MAURICIO, R. 1998. Costs of resistance to natural enemies in field populations of the annual plant *Arabidopsis thaliana*. *Am. Nat.* 151:20–28.

side chain, are characteristically accumulated as the major secondary metabolite. Together with endogenous myrosinase (β-thioglucoside glucohydrolase, 3.2.1.147) glucosinolates serve a central role in defense against herbivores and pathogens (Wink 1988; Jander et al. 2001; Kliebenstein et al. 2005a, b). Tissue damage brings together myrosinase and glucosinolates, which are otherwise spatially separated (Kelly et al. 1998; Koroleva et al. 2000) yielding a variety of hydrolysis products such as isothiocyanates, epithionitriles, thiocyanates, and nitriles (Bones and Rossiter 1996, 2006; Halkier and Gershenzon 2006).

The final outcome of myrosinase-catalyzed hydrolysis of glucosinolates is determined by the epithiospecifier protein (ESP) and related proteins. The most common products are isothiocyanates, which are formed after myrosinase-catalyzed glucosinolate hydrolysis in the absence of ESP, by a spontaneous 'Lossen' like rearrangement (Bones and Rossiter 2006). By contrast, alkenylglucosinolates are converted to thiiranylalkylnitriles, commonly known as epithionitriles, and nitriles when ESP is present together with ferrous/ferric ions, while non-alkenylglucosinolates and indoleglucosinolates form simple nitriles. While epithionitrile formation is dependent on the presence of both ESP and iron, formation of nitriles may occur in the absence of ESP if iron is present (Zabala et al. 2005). Figure 1 shows the structures of the alkenylglucosinolate-derived isothiocyanates, epithionitriles, and nitriles. Despite this range of hydrolysis products, the toxicity of glucosinolates to pathogens and herbivores can be attributed mainly to the isothiocyanates and compounds derived from them (Halkier and Gershenzon 2006). Indeed, in studies of insect herbivory, *Arabidopsis thaliana* (L.) plants that express ESP and, therefore produce nitriles, were apparently less well defended against two species of generalist insect herbivore compared with plants that produced isothiocyanates (Lambrix et al. 2001; Burow et al. 2006). However, as short chain nitriles are volatile compounds, it has been speculated that they may serve as signals in indirect defense responses (Wittstock and Burow 2007). By contrast, little is known about the function of epithionitriles, as few bioassays have been carried out with epithionitriles derived from glucosinolates. This is, in part, due to their perceived instability, but also because they require either chemical synthesis or preparative isolation.

The braconid wasp, *Diaeretiella rapae* (McIntosh), is a solitary endoparasitoid that characteristically attacks aphids

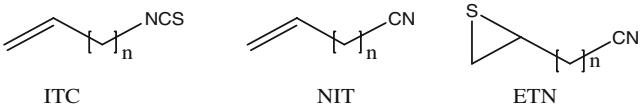

Fig. 1 Diagram showing the possible hydrolysis products from 2-propenyl- or 3-butenylglucosinolate; *ITC* isothiocyanate, *NIT* nitrile, *ETN* epithionitrile

that feed on crucifers, including crucifer specialists *Brevicoryne brassicae* (L.) and *Lipaphis pseudobrassicae* (= *L. erysimi*) (Kaltenbach). In several olfactometry studies, female *D. rapae* discriminated between aphid infested and uninfested crucifers of agricultural importance (Read et al. 1970; Reed et al. 1995; Blande et al. 2007), as well as the model crucifer, *A. thaliana* (Girling et al. 2006). Air entrainments completed by Blande (2004) confirmed that higher levels of isothiocyanates were produced by aphid infested turnip plants compared with uninfested plants. In addition, isothiocyanates also have been indicated as signaling chemicals by a number of olfactometry studies in which female *D. rapae* orient toward pure compounds, which were presented in place of aphid-infested plants (Read et al. 1970; Vaughn et al. 1996; Blande et al. 2007).

A number of studies demonstrated that host finding behavior of some species of aphid parasitoids may be determined by the plant on which the insect develops (Wickremasinghe and van Emden 1992; Storeck et al. 2000). From these studies, it is suggested that conditioning of the parasitoid occurs as it chews its way out of the aphid mummy. Blande et al. (2007) similarly suggest that *D. rapae* acquires chemical cues, probably glucosinolates, as it emerges from the mummy case, although it was proposed that these cues play a role in host recognition rather than host-finding behavior. In an olfactometry study by Bradburne and Mithen (2000), experienced *D. rapae* oriented into the air stream containing the odor from ground plant material of one of two near-isogenic *Brassica oleracea* L. lines, one of which primarily produced 3-isothiocyanatoprop-1-ene (3-PROP-ITC) and the other 4-isothiocyanatobut-1-ene, (4-BUT-ITC) when presented opposite a blank control. However, when the two lines were presented opposite each other, significantly more parasitoids oriented to the air stream containing the odor of the 3-butenylglucosinolate-producing line. This response was recorded regardless of the line on which the insects were reared and, therefore, suggests an innate preference for 4-BUT-ITC over 3-PROP-ITC, although the role of other plant volatiles in this study cannot be excluded.

Given that the chemical functionalities of the epithionitriles, isothiocyanates, and nitriles are different, it might be expected that the electrophysiological and behavioral responses of *D. rapae* would be affected. Thus, we used naïve *D. rapae* females to test their responses to a range of 2-propenyl and 3-butenylglucosinolate hydrolysis products.

Methods and Materials

Insects Two cultures of the crucifer specialist aphid *B. brassicae* (L.) were maintained at 18°C, with a 16/8 hr, L/D photoperiod. Aphids were cultured either on individual

4 wk-old *B. nigra* (L.) plants or similar age *B. rapa* L. var *rapifera* plants, each plant being enclosed within a perforated bread bag. Aphids were transferred to fresh plants to establish new colonies every 1–2 wk.

The aphid parasitoid *D. rapae* (obtained from Rothamsted Research) was maintained at 21°C and 18°C (16:8 hr, L/D). Standardized cohorts were produced by allowing mated 2- to 3-d-old adults to parasitize mixed-age *B. brassicae* in a Petri dish for approximately 2 hr. Parasitized aphids were transferred to a fresh *B. nigra* or *B. rapa* var *rapifera* plant on which they continued to feed. Aphid mummies were removed approximately 8 d later. Adult parasitoids were provided with a dilute honey solution on emergence.

Synthesis of Isothiocyanates and Epithionitriles Hydrolysis products derived from 2-propenylglucosinolate and 3-butenylglucosinolate were prepared for use in this study. For 2-propenylglucosinolate, 3-isothiocyanatoprop-1-ene (3-PROP-ITC) and but-3-enenitrile (3-BUT-NIT) were purchased from Sigma-Aldrich (>95% purity), while 2-(thiiran-2-yl)acetonitrile (2-ACETO-ETN) was synthesized according to the method of Luthy and Benn (1979). For 3-butenylglucosinolate, 4-isothiocyanatobut-1-ene (4-BUT-ITC) was purchased from Sigma-Aldrich (>95% purity), pent-4-enenitrile (4-PENT-NIT) was synthesized from 3-butenylbromide and sodium cyanide, and purified by distillation and 3-(thiiran-2-yl)propanenitrile (3-PROP-ETN) again synthesized according to the method of Luthy and Benn (1979).

Electroantennogram (EAG) Recordings To prepare *D. rapae* females for EAG recordings, insects were anaesthetized with CO_2 before the head was separated from the thorax and the distal portion of the last antennal segment of one antenna removed. Glass electrodes (borosilicate glass capillary, 1.5 mm O.D.×1.17 mm I.D. with filament; Clark Electromedical Instruments®, UK) were filled with 0.1 M KCl. The base of the head was mounted on the reference electrode, while the cut end of the antenna was inserted a short way into the recording electrode. Chlorided-silver wire connected the recording electrode to a probe with an internal amplifier (Syntech®, the Netherlands). Responses were recorded by using a PC fitted with an IDAC interface board (Syntech®, the Netherlands) and running EAG software (Syntech®, the Netherlands).

Test Compounds and Stimulation Each compound tested was dissolved in paraffin liquid (Fisher Scientific, UK, density 0.83–0.86 g/ml) to make a 1% (*v*/*v*) solution. Twenty-five microliters of each solution were applied to a piece (8×60 mm) of filter paper (Whatman®, No. 2 ashless) immediately before each insect preparation was tested. The filter paper was inserted into a glass Pasteur pipette. The same amount of paraffin liquid was used as a blank control. The tip of the glass Pasteur pipette was inserted about 3 mm into a small hole of a glass tube (9 mm diam., 120 mm long) directed at the antennal preparation. An air stimulus controller (model CS-05, Syntech®, the Netherlands) was used for air and odor delivery. A constant flow (1.8 l/min) of charcoal-filtered and humidified air passed over the antenna through the open end of the glass tube, positioned 15 mm from the antenna. During odor stimulation, 60 ml/min of air was applied through the Pasteur pipette into the main air flow for 2 sec, with a 1-min interval between stimulations. In each case, EAG peak amplitude in response to a compound was determined and then normalized against the mean of two responses to (*E*)-2-hexenal (the standard stimulus, purchased from Sigma-Aldrich) presented before and after the test stimulus.

The EAG responses to 2-propenyl- and 3-butenylglucosinolate hydrolysis products were recorded from naïve female *D. rapae*. Eight insect preparations (replicates) were tested against each of the compounds, which were presented in random order. Differences between mean EAG responses to each compound were assessed by Fisher's least significant difference tests (LSDs). Additional analysis of recovery rates of antennal receptors after maximum depolarization was completed for each compound tested by using the method of Baker & Roelofs (1976).

Olfactometry A glass Y-tube olfactometer, of the design previously described by Du et al. (1996), was used to record behavioral responses of female *D. rapae* to pure compounds (glucosinolate hydrolysis products). The olfactometer had 12 mm internal diam, 100 mm stem, and 100 mm arms at 60° angle. Air was pumped through Teflon tubing by a Dymax30 pump (Charles Austen Pumps Ltd., Byfleet, Surrey, UK) through an activated charcoal filter before being regulated by a flowmeter to 800 ml/min. The airflow was split by a brass T-junction (Swagelok, OH, USA), each flow of 400 ml/min then passing into an airtight glass chamber (T. J. Adams—Artistic & Scientific Glassblower, Kidlington, Oxfordshire, UK) into which the volatile source was placed. From the two glass chambers, air flowed through additional Teflon tubing into the arms of the olfactometer via modified glass quick fit sockets. A white cardboard screen was placed around the olfactometer to exclude visual cues, and diffused lighting was provided by a fluorescent strip held 300 mm above and 100 mm in front of the branches of the olfactometer. A single female was introduced into the stem of the olfactometer and then given 5 min to make a choice. During this period, if the parasitoid failed to move more than 50 mm up the stem of the olfactometer, it was excluded from the experiment. Insects were recorded as having selected an odor if they moved more than 50 mm up one of the arms and remained beyond this

point for more than 30 sec. Insects that moved more than 50 mm up the stem but did not select an arm of the olfactometer were recorded as not having selected an odor. After each individual was tested, the position of the odor sources was swapped in order to account for any directional bias by the parasitoids. These criteria are similar to those previously described (Girling et al. 2006; Blande et al. 2007). Between bioassay sets, glassware was washed with acetone and distilled water and then baked overnight at 200°C.

Pure compounds were prepared as 1 mg/ml solutions in diethyl ether. Test solutions were presented in 50-µl aliquots applied to a piece of filter paper (Whatman No. 1), which was changed after every fifth individual. Naïve female parasitoids were used in bioassays. Aphid mummies were removed from plants so that on emergence parasitoids were not provided with cues that might influence behavioral responses. Adult male and female *D. rapae* were kept together, and mating was assumed. Parasitoids were provided with a dilute honey solution, and females were used in the bioassays approximately 72 hr after emergence.

The following olfactometry experiments were completed with *D. rapae* females. In each case, responses of 30 naïve wasps were recorded:

1. Responses of naïve *D. rapae* females to (a) 2-propenylglucosinolate hydrolysis products, and (b) 3-butenylglucosinolate hydrolysis products. Naïve *D. rapae* were reared on *B. brassicae*, which in turn were reared on *B. nigra*.
2. Responses of naïve *D. rapae* females to 3-PROP-ITC and 4-BUT-ITC when parasitoids were reared on *B. brassicae*, which in turn were reared on (a) *B. nigra* or on (b) *B. rapa* var *rapifera*.

Results

EAG Responses EAG responses of *D. rapae* females to 2-propenyl- and 3-butenylglucosinolate hydrolysis products were recorded (Fig. 2). Responses are presented normalized against a standard stimulus, (*E*)-2-hexenal. However, mean absolute responses recorded to isothiocyanates, 3-PROP-ITC and 4-BUT-ITC, were 0.53 and 0.57 mV, respectively.

Diaeretiella rapae females responded significantly to all 3-butenylglucosinolate hydrolysis compounds tested, with normalized EAG responses 3.2, 2.0, and 1.9 times larger than that of the paraffin control for the isothiocyanate, nitrile, and epithionitrile, respectively (Fig. 2). By contrast, when compounds derived from 2-propenylglucosinolate were tested, females responded significantly only to 3-PROP-ITC, with a normalized EAG response 2.7 times larger than that of the paraffin control. Similarly, EAG responses to the 3-butenylglucosinolate derived 4-PENT-NIT and 3-PROP-ETN were significantly larger than responses to the corresponding 2-propenylglucosinolate derived nitrile and epithionitrile. There was no significant

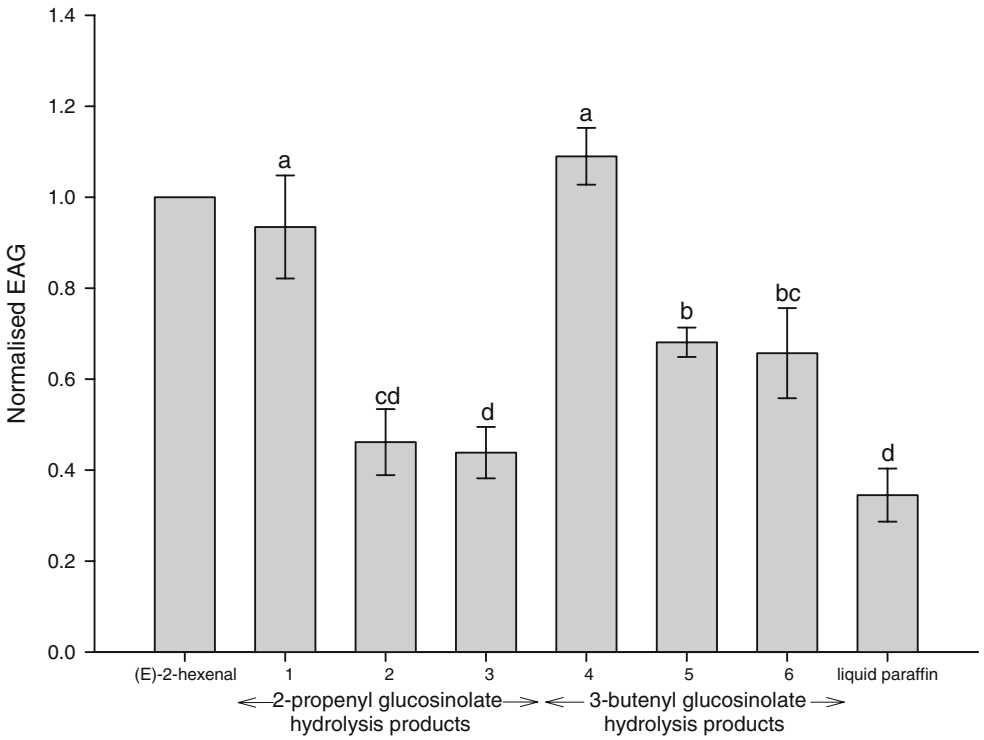

Fig. 2 EAG responses of *Diaeretiella rapae* females to 2-propenyl- and 3-butenylglucosinolate hydrolysis products (mean ± SE, *N*=8). Stimuli: 1 = 3-isothiocyanatoprop-1-ene; 2 = but-3-enenitrile; 3 = 2-(thiiran-2-yl)acetonitrile; 4 = 4-isothiocyanatobut-1-ene; 5 = pent-4-enenitrile; 6 = 3-(thiiran-2-yl)propanenitrile. Different letters indicate significant differences (Fisher's LSD, $P<0.05$)

difference in EAG response size to 4-BUT-ITC and 3-PROP-ITC. Although *D. rapae* responded significantly to all 3-butenylglucosinolate hydrolysis products, the EAG response to 4-BUT-ITC was significantly larger than to the nitrile or to the epithionitrile. EAG response size was, however, similar for 4-PENT-NIT compared with 3-PROP-ETN (Fig. 2).

Receptor recovery rates were affected by the compound with which they were stimulated (F=2.64, P=0.02). Individual contrasts revealed that the recovery rate following stimulation by (*E*)-2-hexenal (x=0.27 ± 0.06) was faster, between 1.7 and 2.9 times, than recovery rates following stimulation by the other compounds tested. Recovery rates following stimulation by the glucosinolate hydrolysis products were similar.

Olfactometry Naïve *D. rapae* females reared on *B. brassicae* in turn feeding on *B. nigra* were presented with 3-butenylglucosinolate hydrolysis products. When each compound was presented opposite a solvent control (diethyl ether), more parasitoids oriented toward 4-BUT-ITC (χ^2= 4.17, P<0.05), but the insects did not respond to either the corresponding nitrile or epithionitrile (Fig. 3a). In comparisons between these compounds, more females selected the arm containing 4-BUT-ITC than either the nitrile or epithionitrile. However, in both cases differences were not significant. The comparison between the 3-butenylglucosinolate derived 4-PENT-NIT and 3-PROP-ETN was also not significant. A second experiment was completed in which similarly reared naïve *D. rapae* females were presented with a range of 2-propenylglucosinolate hydrolysis products.

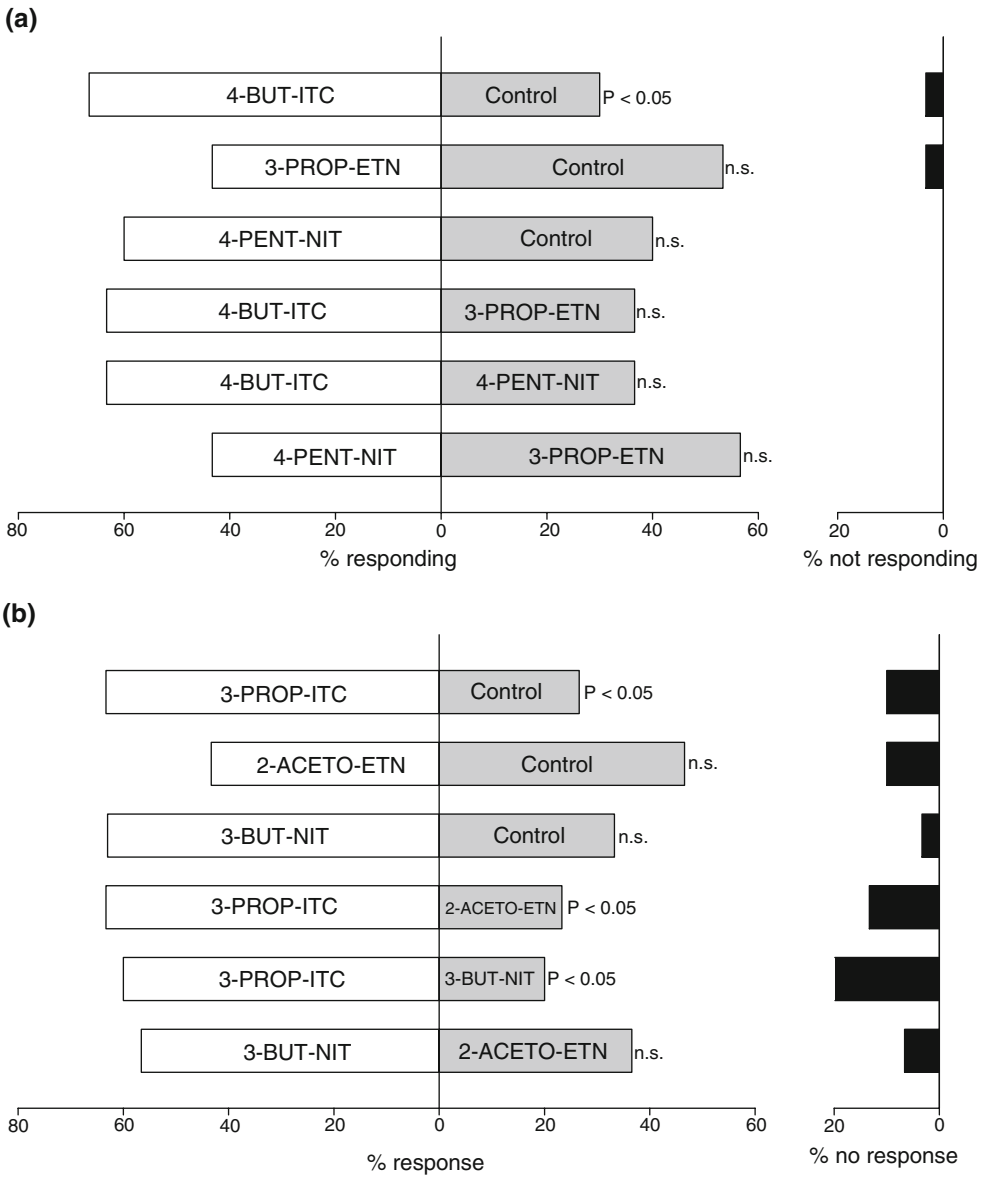

Fig. 3 Responses of naïve *Diaeretiella rapae* females in a Y-tube olfactometer to (**a**) 3-butenylglucosinolate hydrolysis products, where 4-BUT-ITC = 4-isothiocyanatobut-1-ene; 4-PENT-NIT = pent-4-enenitrile; 3-PROP-ETN = 3-(thiiran-2-yl) propanenitrile and (**b**) 2-propenylglucosinolate hydrolysis products, where 3-PROP-ITC = 3-isothiocyanatoprop-1-ene; 3-BUT-NIT = but-3-enenitrile; 2-ACETO-ETN = 2-(thiiran-2-yl) acetonitrile. A total of N= 30 naïve *D. rapae* females were tested for each comparison

More parasitoids responded to the isothiocyanate, in this case 3-PROP-ITC (χ^2=4.48, P<0.05) when presented opposite a solvent control (Fig. 3b). In contrast, although more *D. rapae* females oriented toward 3-BUT-NIT compared to the control, this difference was not significant. Similar numbers of insects moved toward 2-ACETO-ETN and the solvent control. From comparisons between 2-propenylglucosinolate derived hydrolysis products, more *D. rapae* responded to 3-PROP-ITC compared with either the corresponding nitrile (χ^2=6.00, P<0.05) or epithionitrile (χ^2=5.54, P<0.05). Similar numbers of insects moved toward 3-BUT-NIT and 2-ACETO-ETN when they were presented opposite each other.

Naïve *D. rapae* females reared on *B. brassicae*, which in turn were feeding on *B. nigra*, were presented with 4-BUT-ITC or 3-PROP-ITC. Each compound was presented opposite a solvent control and also opposite each other. More parasitoids moved into the air stream that contained the 4-BUT-ITC (χ^2=9.85, P<0.01) or 3-PROP-ITC (χ^2=7.76, P<0.05) compared with the control (Fig. 4a). When 4-BUT-ITC was presented opposite 3-PROP-ITC, more *D. rapae* responded to 3-PROP-ITC (χ^2=4.48, P<0.05). A second similarly designed experiment was completed that used naïve *D. rapae* females reared on *B. brassicae*, which in turn were feeding on *B. rapa* var *rapifera*. Again, more parasitoids moved into the air stream containing the 4-BUT-ITC (χ^2=5.14, P<0.05) or 3-PROP-ITC (χ^2=10.80, P<0.01) compared with the control (Fig. 4b). However, when 4-BUT-ITC was presented opposite 3-PROP-ITC similar numbers of insects responded to each compound.

In each of these experiments, the number of insects not responding was low. In the majority of cases, less than 10% of parasitoids did not respond with a maximum of 20%.

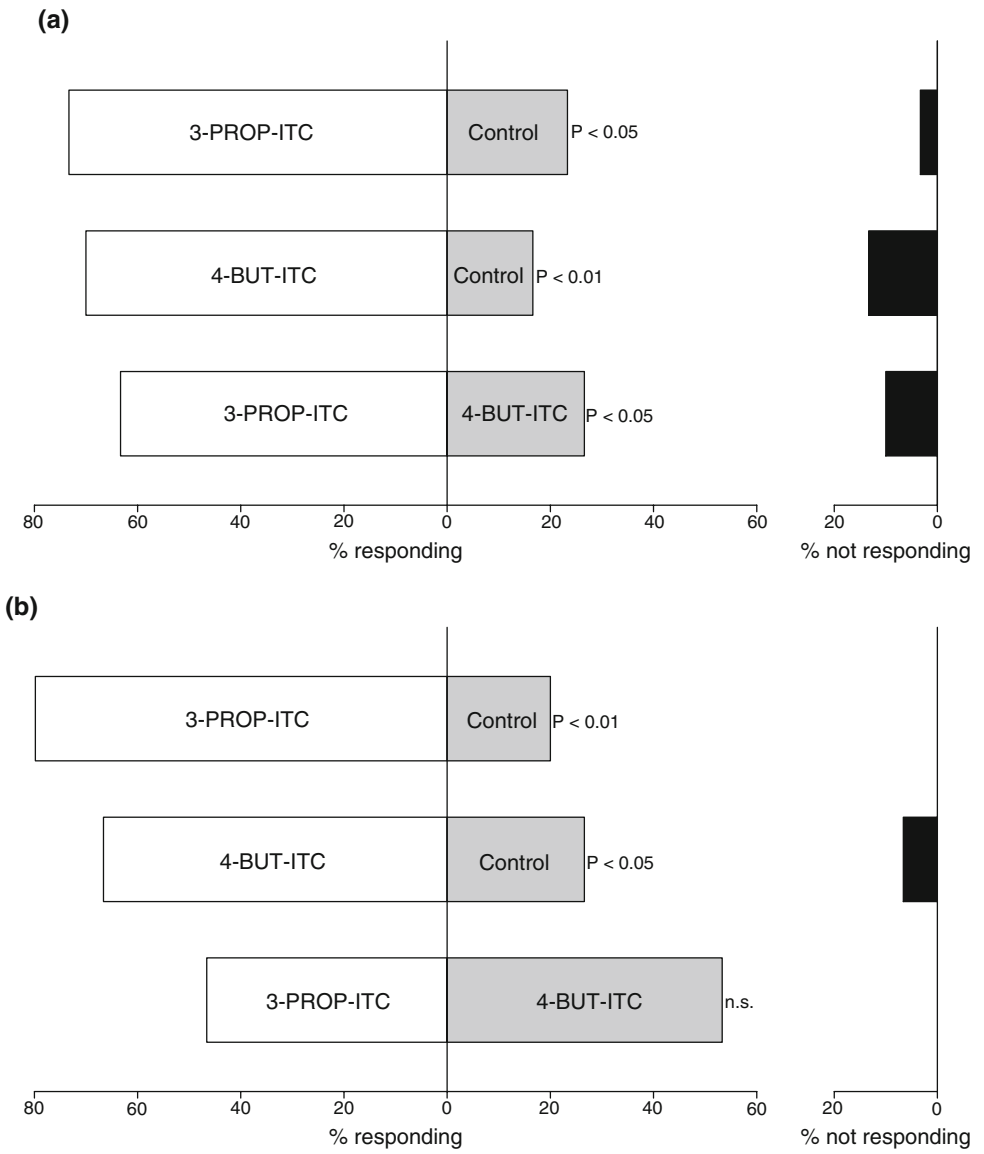

Fig. 4 Responses of naïve *Diaeretiella rapae* females to 3-PROP-ITC = 3-isothiocyanato-prop-1-ene and 4-BUT-ITC = 4-isothiocyanatobut-1-ene, having previously been reared on *Brevicoryne brassicae* feeding either on (**a**) *Brassica nigra* or (**b**) *Brassica rapa* var *rapifera*. A total of N= 30 naïve *D. rapae* females were tested for each comparison

Discussion

Research that has investigated the importance of the myrosinase–glucosinolate defense against insect herbivores has so far largely focused on the role of isothiocyanates (Halkier and Gershenzon 2006; Wittstock and Burow 2007). Many studies have investigated the direct effects of isothiocyanates against herbivores, while others have focused on indirect effects of isothiocyanates that act as cues for parasitoids, including *D. rapae*. However, little is currently known about the peripheral odor perception of *D. rapae* to glucosinolate hydrolysis products. Indeed, *D. rapae* has previously been tested only against, and shown to respond to, 3-PROP-ITC (Vaughn et al. 1996).

Here, we confirmed this earlier result while also recording EAGs from naïve *D. rapae* females in response to 3-butenyl- and 2-propenylglucosinolate hydrolysis products, including isothiocyanates and (for the first time) nitriles and epithionitriles. Although significant EAG responses were recorded to 3-PROP-ITC, parasitoids did not respond to the corresponding nitrile or epithionitrile, reflecting either the lower volatility of these compounds or a lack of olfactory receptors. By contrast, *D. rapae* females showed peripheral odor perception to all of the 3-butenylglucosinolate hydrolysis products tested, including 4-BUT-ITC, 4-PENT-NIT, and 3-PROP-ETN, although again the largest response was to the isothiocyanate. Large EAG responses were also recorded in response to (*E*)-2-hexenal, and *D. rapae* females have previously been shown to respond to another green leaf volatile, (*Z*)-3-hexenol (Vaughn et al. 1996).

Vaughn et al. (1996) noted that although *D. rapae* females responded to both (*Z*)-3-hexenol and to 3-PROP-ITC; there were clear differences in the recovery rates following stimulation by each compound, suggesting possible differences in receptor type. Similarly, in the present study, antennal response recovery rates were significantly faster for (*E*)-2-hexenal than to the alkenylglucosinolate hydrolysis products tested. This result further supports the idea that, like its aphid hosts, *L. pseudobrassicae* and *B. brassicae*, female *D. rapae* possess more than one receptor type (Dawson et al. 1987; Nottingham et al. 1991). Slower recovery rates previously have been attributed to chemical-specific receptors (Baker and Roelofs 1976). Results presented here suggest that *D. rapae* females possess receptors specific for isothiocyanates, as previously identified by (Vaughn et al. 1996), and in addition for certain nitriles and epithionitriles. The physico-chemical properties of the isothiocyanates are quite different from that of the nitrile group, being more polar and electrophilic and with more potential for interaction with proteins. The thiirane ring of the epithionitriles, while less polar than that of the isothiocyanate group, is also electrophilic, and has the potential for ring opening with biological nucleophiles to form conjugates. Although no specific work has been carried out on isothiocyanate receptors in insects, a recent report describes the effect of mustard oil (3-PROP-ITC) on endogenous porcine transient receptor potential V1 (pTRPV1) channels (Ohta et al. 2007). It was found that 3-PROP-ITC acts as an agonist for porcine TRPV1. Other notable receptors of this type are the vanilloid and capsaicin receptors present in *Drosophila* that have been well characterized (Caterina et al. 1997, 1999). Another class of receptors is the odorant receptors (OR), which are undergoing characterization in *Drosophila* and are potential candidates for interactions with glucosinolate hydrolysis products (Benton 2006). It would be of interest to explore the action of glucosinolate-derived isothiocyanates as chemical cues and repellents at a molecular level in order to understand at the physiological level the behavior of insects.

There is considerable evidence that isothiocyanates function as signals in indirect plant defense responses, thus enhancing attraction of beneficial parasitoids (Read et al. 1970; Vaughn et al. 1996; Bradburne and Mithen 2000; Blande et al. 2007), and it has been speculated that simple nitriles may serve also as similar signals induced by herbivory (Wittstock and Burow 2007). Indeed, female *Cotesia rubecula* Marshall, a specialist parasitoid of *Pieris rapae* L. caterpillars, may use nitriles to discriminate between infested and uninfested *A. thaliana* plants (van Poecke et al. 2001). Air entrainments of these plants implicated two nitriles, 5-(methylthio) pentanenitrile and 6,7-dithiooctanenitrile, along with the monoterpene myrcene and higher levels of methyl salicylate, as potential cues used by this parasitoid. In the present study, there was a lack of orientation behavior to the 2-propenylglucosinolate-derived nitrile and epithionitrile, which supports the apparent lack of peripheral perception of these compounds indicated by the EAG results. By contrast, although EAG results indicated peripheral odor perception to all of the 3-butenylglucosinolate hydrolysis products tested, olfactometry results indicated only a significant behavioral response to 4-BUT-ITC. However, when 4-BUT-ITC was presented opposite either the corresponding nitrile or epithionitrile, there was no significant difference in the numbers of *D. rapae* orienting to each compound. This result suggests either a weak response to the 3-butenylglucosinolate-derived 4-PENT-NIT and 3-PROP-ETN or that these compounds in some way disrupt the response to 4-BUT-ITC. A similar result was recorded by Blande et al. (2007) who noted that there was no difference in the number of *D. rapae* females orienting to 4-BUT-ITC and 5-isothiocyanatopent-1-ene when the compounds were presented opposite each other. This result was recorded despite the fact that when each compound was presented opposite a solvent

control, parasitoids oriented toward the 4-BUT-ITC but not toward the 5-isothiocyanatopent-1-ene.

The results from this study provide no evidence for naïve *D. rapae* females responding behaviorally to either the nitriles or epithionitriles tested. However, subtle effects such as potential interactions between compounds cannot be excluded. Indeed, the nitriles implicated as host-finding cues utilized by *C. rubecula* were present against a background of plant odors (van Poecke et al. 2001). In contrast with the lack of behavioral response to either the nitriles or epithionitriles, results from the olfactometry experiment indicated that naïve *D. rapae* females did orient toward both 3-PROP-ITC and 4-BUT-ITC. This result supports a number of earlier studies in suggesting that these two isothiocyanates provide an indirect defense by acting as host finding cues for *D. rapae*, a natural enemy of aphids feeding on crucifers (Read et al. 1970; Vaughn et al. 1996; Bradburne and Mithen 2000; Blande et al. 2007). In the present study, naïve *D. rapae* females responded positively to both 3-PROP-ITC and 4-BUT-ITC, whether the parasitoids were reared on *B. nigra* or *B. rapa* var *rapifera*. This is despite the fact that *B. nigra* is characterized by the accumulation of 2-propenylglucosinolate (Cole 1976), while *B. rapa* var *rapifera* accumulates 3-butenylglucosinolate (Carlson et al. 1981). In both cases, the glucosinolates are hydrolyzed to the corresponding isothiocyanates (unpublished data).

When the two compounds were presented opposite each other in the olfactometer, the responses of naïve *D. rapae* females were, however, affected by the host plant on which the insects were reared. When reared on *B. nigra*, more parasitoids oriented toward 3-PROP-ITC, but when reared on *B. rapa* var *rapifera* there was no difference in the numbers of females responding to the two isothiocyanates. This result contrasts with that of Bradburne and Mithen (2000) who also used a Y-tube olfactometer to record *D. rapae* behavior but utilized two near-isogenic *B. oleracea* lines. Hydrolysis analysis, rather than air entrainments of aphid-infested plants such as that used by Blande et al. (2007), was used to confirm that one of these isogenic lines produced predominantly 4-BUT-ITC and the other mainly 3-PROP-ITC. Bradburne and Mithen (2000) recorded more *D. rapae* orienting toward the 4-BUT-ITC line as opposed to the 3-PROP-ITC producing line, regardless of the line on which the insects were reared. It is unclear why these two studies produced contrasting results, although this may reflect differences in the way that the parasitoids interpret individual volatiles as opposed to a bouquet of different compounds. This point is particularly relevant, as Bradburne and Mithen (2000) presented hydrated ground plant material, which is likely to have presented the insects with many volatile compounds that may or may not be associated with aphid infested plants. In contrast, here, individual compounds were presented as cues in the olfactometry experiments.

Host plant chemistry plays a role in both host-finding and host recognition in other species of aphid parasitoids (Wickremasinghe and van Emden 1992; Storeck et al. 2000). The cereal aphid specialist *Aphidius rhopalosiphi* De Stefani Perez discriminated between different cultivated varieties of winter wheat in a Y-tube olfactometer, but showed a preference for the cultivar on which it was reared (Wickremasinghe and van Emden 1992). Similarly, the generalist aphid parasitoid *Aphidius colemani* Viereck also showed a preference for the host-plant on which it was reared (Storeck et al. 2000). In both cases, this preference was associated with chemical cues present on the mummy case, which the adult parasitoid comes into contact with at the time of emergence.

Interestingly, Blande et al. (2007) also suggest that *D. rapae* encounters chemical cues, probably glucosinolates, as the wasp emerges from the aphid mummy. Indeed, when naïve *D. rapae* females were presented with *B. rapa* var *rapifera* plants infested either with *L. pseudobrassicae* (which characteristically accumulates glucosinolates) or *Myzus persicae* (Sulzer) (not known to accumulate glucosinolates), parasitoids attacked a higher proportion of the *L. pseudobrassicae* regardless of the aphid species on which the wasps were reared (Blande et al. 2004). However, subsequent olfactometry experiments, again using experienced *D. rapae* reared on *L. pseudobrassicae* or *M. persicae* feeding on *B. rapa* var *rapifera*, found no evidence for parasitoids preferentially orienting toward either plant/aphid complex (Blande et al. 2007). From these results, the authors suggest that glucosinolate cues accumulated as the parasitoid emerges from the aphid mummy are important in host acceptance but that similar cues appear to be utilized for locating *L. pseudobrassicae* or *M. persicae* feeding on *B. rapa* var *rapifera*. Here, by using one species of aphid and altering the species of host plant rather than, in the case of Blande et al. (2007), using two aphid species reared on the same species of host plant, we recorded changes in orientation behavior. This result raises interesting questions about the type of chemical cue that may be acquired as the wasp emerges from the aphid mummy and in particular whether these results are explained by non-volatile glucosinolates and/or the volatile isothiocyanates.

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References

BAKER, T. C., and ROELOFS, W. L. 1976. Electroantennogram responses of the male moth, *Argyrotaenia velutinana* to mixtures

of sex pheromone components of the female. *J. Insect Physiol.* 22:1357–1364.

BENTON, R. 2006. On the origin of smell: odorant receptors in insects. *Cell. Mol. Life Sci.* 63:1579–1585.

BLANDE, J. D. 2004. Differential signaling from specialist and generalist *Brassica* feeding aphids to differentially adapted aphid parasitoids. Ph.D. thesis, University of Southampton, UK.

BLANDE, J. D., PICKETT, J. A., and POPPY, G. M. 2004. Attack rate and success of the parasitoid *Diaeretiella rapae* on specialist and generalist feeding aphids. *J. Chem. Ecol.* 30:1781–1795.

BLANDE, J. D., PICKETT, J. A., and POPPY, G. M. 2007. A comparison of semiochemically mediated interactions involving specialist and generalist *Brassica*-feeding aphids and the braconid parasitoid *Diaeretiella rapae*. *J. Chem. Ecol.* 33:767–779.

BONES, A. M., and ROSSITER, J. T. 1996. The myrosinase-glucosinolate system, its organisation and biochemistry. *Physiol. Plant.* 97:194–208.

BONES, A. M., and ROSSITER, J. T. 2006. The enzymic and chemically induced decomposition of glucosinolates. *Phytochemistry* 67:1053–1067.

BRADBURNE, R. P., and MITHEN, R. 2000. Glucosinolate genetics and the attraction of the aphid parasitoid *Diaeretiella rapae* to *Brassica*. *Proc. R. Soc. Lond., Ser. B Biol. Sci.* 267:89–95.

BUROW, M., MULLER, R., GERSHENZON, J., and WITTSTOCK, U. 2006. Altered glucosinolate hydrolysis in genetically engineered Arabidopsis thaliana and its influence on the larval development of *Spodoptera littoralis*. *J. Chem. Ecol.* 32:2333–2349.

CARLSON, D. G., DAXENBICHLER, M. E., VANETTEN, C. H., and TOOKEY, H. L. 1981. Glucosinolates in crucifer vegetables—turnips and rutabagas. *J. Agric. Food Chem.* 29:1235–1239.

CATERINA, M. J., SCHUMACHER, M. A., TOMINAGA, M., ROSEN, T. A., LEVINE, J. D., and JULIUS, D. 1997. The capsaicin receptor: a heat-activated ion channel in the pain pathway. *Nature* 389:816–824.

CATERINA, M. J., ROSEN, T. A., TOMINAGA, M., BRAKE, A. J., and JULIUS, D. 1999. A capsaicin-receptor homologue with a high threshold for noxious heat. *Nature* 398:436–441.

COLE, R. 1976. Isothiocyanates, nitriles and thiocyanates as products of autolysis of glucosinolates in Cruciferae. *Phytochemistry* 15:759–762.

DAWSON, G. W., GRIFFITHS, D. C., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1987. Plant-derived synergists of alarm pheromone from turnip aphid, Lipaphis (Hyadaphis) erysimi (Homoptera, Aphididae). *J. Chem. Ecol.* 13:1663–1671.

DU, Y. J., POPPY, G. M., and POWELL, W. 1996. Relative importance of semiochemicals from first and second trophic levels in host foraging behavior of Aphidius ervi. *J. Chem. Ecol.* 22:1591–1605.

GIRLING, R. D., HASSALL, M., TURNER, J. G., and POPPY, G. M. 2006. Behavioural responses of the aphid parasitoid *Diaeretiella rapae* to volatiles from *Arabidopsis thaliana* induced by *Myzus persicae*. *Entomol. Exp. Appl.* 120:1–9.

HALKIER, B. A., and GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* 57:303–333.

JANDER, G., CUI, J. P., NHAN, B., PIERCE, N. E., and AUSUBEL, F. M. 2001. The TASTY locus on chromosome 1 of Arabidopsis affects feeding of the insect herbivore *Trichoplusia ni*. *Plant Physiol.* 126:890–898.

KELLY, P. J., BONES, A., and ROSSITER, J. T. 1998. Sub-cellular immunolocalization of the glucosinolate sinigrin in seedlings of *Brassica juncea*. *Planta* 206:370–377.

KLIEBENSTEIN, D. J. 2004. Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant Cell Environ.* 27:675–684.

KLIEBENSTEIN, D. J., KROYMANN, J., and MITCHELL-OLDS, T. 2005a. The glucosinolate-myrosinase system in an ecological and evolutionary context. *Curr. Opin. Plant Biol.* 8:264–271.

KLIEBENSTEIN, D. J., ROWE, H. C., and DENBY, K. J. 2005b. Secondary metabolites influence *Arabidopsis*/*Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant J.* 44:25–36.

KOROLEVA, O. A., DAVIES, A., DEEKEN, R., THORPE, M. R., TOMOS, A. D., and HEDRICH, R. 2000. Identification of a new glucosinolate-rich cell type in Arabidopsis flower stalk. *Plant Physiol.* 124:599–608.

LAMBRIX, V., REICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D. J., and GERSHENZON, J. 2001. The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13:2793–2807.

LUTHY, J., and BENN, M. 1979. Conversion of potassium allylglucosinolate to 3,4-epithiobutanenitrile by crambe-abyssinica seed flour. *Phytochemistry* 18:2028–2029.

NOTTINGHAM, S. F., HARDIE, J., DAWSON, G. W., HICK, A. J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1991. Behavioral and electrophysiological responses of aphids to host and nonhost plant volatiles. *J. Chem. Ecol.* 17:1231–1242.

OHTA, T., IMAGAWA, T., and ITO, S. 2007. Novel agonistic action of mustard oil on recombinant and endogenous porcine transient receptor potential V1 (pTRPV1) channels. *Biochem. Pharmacol.* 73:1646–1656.

READ, D. P., FEENY, P. P., and ROOT, R. B. 1970. Habitat selection by the aphid parasite *Diaeretiella rapae* (Hymenoptera: Braconidae) and hyperparasite *Charips brassicae* (Hymenoptera: Cynipidae). *Can. Entomol.* 102:1567–&.

REED, H. C., TAN, S. H., HAAPANEN, K., KILLMON, M., REED, D. K., and ELLIOTT, N. C. 1995. Olfactory responses of the parasitoid *Diaeretiella rapae* (Hymenoptera: Aphidiidae) to odor of plants, aphids, and plant–aphid complexes. *J. Chem. Ecol.* 21:407–418.

STORECK, A., POPPY G. M., VAN EMDEN, H. F., and POWELL, W. 2000. The role of plant chemical cues in determining host preference in the generalist aphid parasitoid Aphidius colemani. *Entomol. Exp. Appl.* 97:41–46.

VAN, POECKE, R. M. P., POSTHUMUS, M. A., and DICKE, M. 2001. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. *J. Chem. Ecol.* 27:1911–1928.

VAUGHN, T. T., ANTOLIN, M. F., and BJOSTAD, L. B. 1996. Behavioral and physiological responses of *Diaeretiella rapae* to semiochemicals. *Entomol. Exp. Appl.* 78:187–196.

WICKREMASINGHE, M. G. V., and VAN, EMDEN, H. F. 1992. Reactions of adult female parasitoids, particularly *Aphidius rhopalosiphi*, to volatile chemical cues from the host plants of their aphid prey. *Physiol. Entomol.* 17:297–304.

WINK, M. 1988. Plant breeding—importance of plant secondary metabolites for protection against pathogens and herbivores. *Theor. Appl. Gen.* 75:225–233.

WITTSTOCK, U., and BUROW, M. 2007. Tipping the scales—Specifier proteins in glucosinolate hydrolysis. *IUBMB Life* 59:744–751.

ZABALA, M. D., GRANT, M. BONES, A. M., BENNETT, R., LIM, Y. S., KISSEN, R., and ROSSITER, J. T. 2005. Characterisation of recombinant epithiospecifier protein and its over-expression in *Arabidopsis thaliana*. *Phytochemistry* 66:859–867.

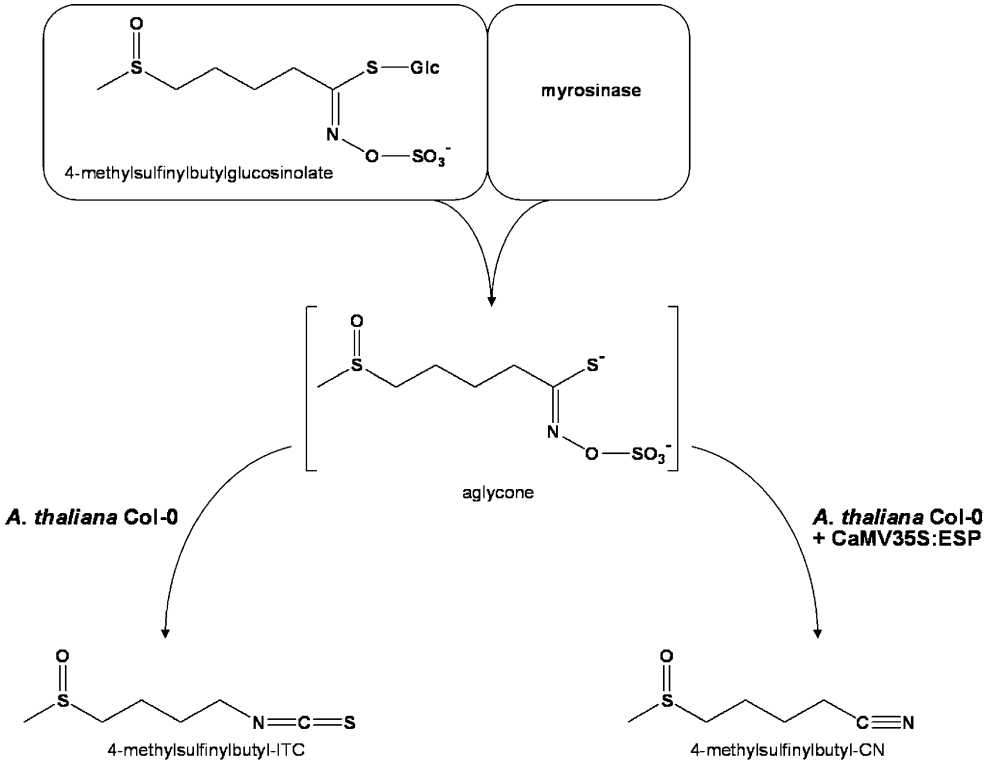

Fig. 1 Glucosinolate hydrolysis in *A. thaliana* Col-0 wild-type and 35S:*ESP* plants as shown for 4-methylsulfinylbutylglucosinolate, the main glucosinolate found in the Col-0 ecotype. Glucosinolates and their hydrolytic enzymes, the myrosinases, are stored in different cells in intact plant tissue. Damage to the tissue results in myrosinase-catalyzed hydrolysis of glucosinolates yielding glucose and unstable aglucones. In the ecotype Col-0, these aglucones predominantly rearrange into isothiocyanates due to the absence of a functional epithiospecifier protein. Expression of ESP from the *Arabidopsis* ecotype L*er* under the control of the constitutive CaMV35S promoter results in nitrile formation upon leaf damage

(Fig. 1) (Rask et al. 2000). Spontaneous rearrangement of the aglucone then leads to the formation of an isothiocyanate. Under the influence of specifier proteins that are present in many glucosinolate-containing plants including *Brassica napus* and some *Arabidopsis* accessions, alternative products such as simple nitriles and epithionitriles are formed at the expense of isothiocyanates (Bernardi et al. 2000; Foo et al. 2000; Lambrix et al. 2001; Wittstock and Burow 2007). Glucosinolate hydrolysis in intact plant tissue is prevented by spatial separation of glucosinolates and myrosinases through storage in different cells and thus occurs only upon tissue disruption, e.g., by a feeding herbivore (Andréasson and Jørgensen 2003).

The glucosinolate–myrosinase system is involved in defense responses against herbivorous insects of different feeding guilds (Kliebenstein et al. 2002; Agrawal and Kurashige 2003; Wittstock et al. 2003; Kliebenstein 2004; Mewis et al. 2005; Barth and Jander 2006; Kim and Jander 2007). Its role in direct defense has been attributed mainly to the isothiocyanates, which are the predominant hydrolysis products in many plant species. Isothiocyanates are toxic upon ingestion, contact, or when present in the gas phase (Agrawal and Kurashige 2003; Wittstock et al. 2003). However, the glucosinolate–myrosinase system appears to differentially affect specialist and generalist herbivores (Giamoustaris and Mithen 1995; Li et al. 2000). Several specialist insects have developed counteradaptations to circumvent the toxic effects of glucosinolates and their hydrolysis products (Müller et al. 2001; Alibadi et al. 2002; Ratzka et al. 2002; Wittstock et al. 2004; Agerbirk et al. 2006; Vergara et al. 2006; Wheat et al. 2007). Moreover, glucosinolates and isothiocyanates often serve as attractants or oviposition and feeding stimulants for these insects (Van Loon et al. 1992; Huang and Renwick 1994; Wittstock et al. 2003; Miles et al. 2005; Schoonhoven et al. 2005; Renwick et al. 2006; Barth and Jander 2006; Smallegange et al. 2007). Still, several studies have shown negative correlations between the glucosinolate content of the diet and the larval performance of herbivorous insects specialized on Brassicaceae (Mewis et al. 2005, 2006; Gols et al. 2007, 2008; Kim and Jander 2007).

Little is known about the biological activities of hydrolysis products other than isothiocyanates. In general, simple nitriles are considered less toxic than isothiocyanates (Lambrix et al. 2001; Wittstock et al. 2003; Burow et al. 2006b). For example, larvae of the generalist *Tricho-*

plusia ni (cabbage looper, Lepidoptera, Plusiinae) feed more, and larvae of the generalist *Spodoptera littoralis* (Egyptian cotton leafworm, Lepidoptera, Noctuidae) perform better on nitrile-producing *Arabidopsis* plants than on isothiocyanate-producing plants (Lambrix et al. 2001; Burow et al. 2006b; Zhang et al. 2006). Given that insect herbivores seem to suffer less from simple nitriles than from isothiocyanates, the ecological rationale for nitrile formation in the Brassicaceae is not easy to understand. Different scenarios can be drawn in which a plant may benefit from producing nitriles. For example, nitrile formation could be advantageous in direct and indirect defense against specialized herbivores. Direct plant defense negatively affects the feeding stages or the egg deposition of the herbivore, whereas indirect defense acts by recruiting natural enemies of the herbivore, such as predators and parasitoids (Hilker and Meiners 2002). Since isothiocyanates are often exploited as attractants, oviposition, or feeding stimulants by specialized herbivores (Rask et al. 2000; Renwick 2002; Wittstock et al. 2003; Renwick et al. 2006), plants that do not produce isothiocyanates upon damage may become less apparent to specialists. Alternatively, carnivorous insects such as predators and parasitoids may be differentially attracted by isothiocyanates and nitriles. A few studies have demonstrated that parasitoids are attracted to isothiocyanates (Titayavan and Altieri 1990; Pivnick 1993; Murchie et al. 1997; Bradburne and Mithen 2000; Reddy et al. 2002; Blande et al. 2007). However, parasitoid and predator performance is also known to correlate negatively with glucosinolate content (Harvey et al. 2003; Kazana et al. 2007; Gols et al. 2008). This negative relationship might favor a behavioral response of predators and parasitoids to plant volatiles that contain simple nitriles instead of isothiocyanates.

Because the glucosinolate–myrosinase system is an activated plant defense, the role of specific glucosinolate hydrolysis products is difficult to study in experimental settings. Plants that differ in the type of hydrolysis products they form often also differ in other properties such as glucosinolate content or myrosinase activity. The use of synthetic compounds alone or in mixtures to compare the defensive role of certain hydrolysis products also can be misleading due to differences in volatility. Moreover, many of them cannot be obtained as pure compounds. However, the availability of molecular tools to genetically manipulate model plants such as *Arabidopsis* provide an opportunity to overcome these difficulties (e.g., Degenhardt et al. 2003; Snoeren et al. 2007). The use of transgenic *Arabidopsis* lines that have been modified in glucosinolate hydrolysis provide a more natural system to compare the effects of naturally occurring relative and absolute amounts of members of different types of glucosinolate hydrolysis products.

Arabidopsis accessions differ in the type of glucosinolate hydrolysis products they produce upon tissue damage. This is due to allelic variation at the locus that encodes the epithiospecifier protein (ESP, Lambrix et al. 2001). For example, plants of the Landsberg *erecta* (L*er*) accession possess a functional *ESP* gene and produce predominantly simple nitriles upon tissue disruption, while isothiocyanates are the major glucosinolate hydrolysis products of the Columbia-0 (Col-0) accession that lacks functional ESP (Lambrix et al. 2001). We used a transgenic line of *Arabidopsis* Col-0, which expresses the *ESP* cDNA from the L*er* accession under the control of the CaMV35S promoter (35S:*ESP* plants; Burow et al. 2006b). In the 35S:*ESP* plants, glucosinolates are predominantly hydrolyzed to simple nitriles, whereas Col-0 wild-type plants mainly produce isothiocyanates upon damage (Burow et al. 2006b). For example, in Col-0 wild-type plants, the hydrolysis of 4-methylsulfinylbutylglucosinolate, the most abundant glucosinolate in the rosette leaves of this ecotype, leads to the formation of 4-methylsulfinylbutyl isothiocyanate. If ESP is overexpressed, however, the corresponding nitrile is produced (Fig. 1). However, neither the glucosinolate profile, the myrosinase activity levels, nor the morphology is altered in 35S:*ESP* plants compared with Col-0 wild type (Burow et al. 2006b).

In the present study, we used the 35S:*ESP* plants to investigate the role of simple nitriles in the interaction between *Arabidopsis*, the specialist cabbage white butterfly *Pieris rapae* L. (Lepidoptera, Pieridae), and its specialist larval endoparasitoid *Cotesia rubecula* (Marshall) (Hymenoptera, Braconidae). *P. rapae* is one of the most abundant butterflies in Northern and Central Europe and is neurophysiologically and biochemically adapted to using glucosinolate-containing plants as its sole hosts (Renwick 2002; Wittstock et al. 2004; Schoonhoven et al. 2005; Braby and Trueman 2006, and references therein). Most interestingly, caterpillars of *P. rapae* excrete simple nitriles in their feces (Agelopoulos et al. 1995) due to the action of a midgut nitrile-specifier protein that directs the hydrolysis of ingested glucosinolates from isothiocyanate towards simple nitrile formation (Wittstock et al. 2004; Burow et al. 2006a). *P. rapae* is able to complete its development on *Arabidopsis* (Van Loon et al. 2000; Harvey et al. 2007).

Volatiles of different plant species, including *Arabidopsis*, that are infested with *P. rapae* caterpillars are known to attract *C. rubecula* females (Agelopoulos and Keller 1994a; Geervliet et al. 1994, 1996; Van Poecke et al. 2001). The headspace of *P. rapae*-infested *Arabidopsis* plants contains nitriles that may originate from the frass of the caterpillars or the wounded plant tissue (Van Poecke et al. 2001; Van Poecke 2007; Wittstock et al. 2004). The feces of *P. rapae* caterpillars are attractive to *C. rubecula* from a distance, and their headspace contains predominantly nitriles (Agelopoulos

et al. 1995; Wittstock et al. 2004). Electrophysiological studies have demonstrated that antennae of *C. rubecula* respond to certain isothiocyanates and nitriles (J.J.A. van Loon, unpublished results; Smid et al. 2002). Therefore, we used wind tunnel experiments to test whether *Arabidopsis* plants that overexpress ESP are more attractive to *C. rubecula* than the isothiocyanate-producing Col-0 wild-type plants.

Isothiocyanates are involved in long-distance host recognition by a number of specialized insect herbivores (reviewed in Wittstock et al. 2003). Therefore, we investigated whether the higher proportion of nitriles formed in 35S:*ESP* plants as compared to wild-type plants influences the oviposition behavior of *P. rapae* butterflies. Glucosinolates stimulate egg deposition by *P. rapae* upon contact, but in nature butterflies might also be exposed to glucosinolate hydrolysis products, e.g., when host plants are already infested with feeding caterpillars (e.g., Huang et al. 1993, 1994). Additionally, we tested whether the feeding preference and performance of *P. rapae* differs between 35S:*ESP* plants and Col-0 wild-type plants. Despite the known glucosinolate detoxification mechanism in the midgut of *P. rapae* caterpillars, high concentrations of isolated isothiocyanates reduce *P. rapae* caterpillar growth and survival (Agrawal and Kurashige 2003). Combining these diverse bioassays, we aimed at obtaining a better insight into the contribution of nitriles to the relationships of *P. rapae* with its host plant and its enemies.

Methods and Materials

Plants *A. thaliana* Col-0 and the transgenic line 35S:*ESP* (2.6, T4; in the Col-0 background; Burow et al. 2006b) used for wind tunnel and oviposition experiments were grown from seed in soil (Lentse Potgrond, Cuijk, the Netherlands) in a controlled-climate room at 21 ± 1°C, a L8:D16-h photoperiod, 80–110μmol m^{-2} s^{-1} photosynthetically active radiation (PAR), and 55 ± 5% relative humidity (RH). Two-week-old seedlings were transferred to pots (Teku, Pöppelmann, Lohne, Germany) containing the same soil. Plants used for the experiments were 4–6-week-old and in the vegetative stage. Plants used to test the feeding preference and larval performance of *P. rapae* were grown under similar conditions for 4–5weeks except that the photoperiod was a L10:D14-h photoperiod and the relative humidity was 60 ± 5%.

Insects A continuous rearing of *P. rapae* for oviposition preference tests was maintained on Brussels sprouts plants in a climatized room at 21 ± 1°C, 60 ± 10% RH and an L16:D8-h photoperiod. *P. rapae* larvae used for feeding preference tests and larval performance tests were raised under similar conditions except that relative humidity was 75 ± 5%. The parasitoid *C. rubecula* was reared on *P. rapae* caterpillars feeding on Brussels sprouts plants in a greenhouse at 24 ± 4°C, 60 ± 20% RH, and a L16:D8-h photoperiod. For experiments, *C. rubecula* pupae were collected and kept in a cage in a climate cabinet (23 ± 1°C, 60 ± 10% RH, and L16:D8-h photoperiod). Emerging wasps were provided with water and honey. Male and female wasps were kept together until the experiment.

Plant Treatments for Parasitoid and Oviposition Experiments Plants were either artificially damaged or infested with ten first-instar *P. rapae* caterpillars. Plants were infested for 24 h and were kept in a climate chamber at 21 ± 1°C, a L8:D16-h photoperiod, 80–110μmol m^{-2} s^{-1} photosynthetically active radiation, and 55 ± 5% RH until the experiments. Artificially damaged plants were obtained by punching a ~7-mm^2 hole into each of six leaves of a plant right before the experiment. Intact *Arabidopsis* plants served as controls.

Wind Tunnel Experiments Behavioral choice experiments with the parasitoid *C. rubecula* were done with a wind tunnel setup (25 ± 1°C, 60 ± 10% RH, 35μmol m^{-2} s^{-1} PAR) as described by (Geervliet et al. 1994). The wind speed was adjusted to 0.2m s^{-1}. Two-choice experiments were conducted by placing two odor sources each consisting of six *Arabidopsis* plants at the upwind end of the tunnel as described by Van Poecke et al. (2001). Naïve *C. rubecula* females (without oviposition experience) were separated from males 3h prior to the experiment and transferred to another cage that was placed in the experimental room to acclimatize the parasitoids to the new environment. The wasps were individually introduced into the wind tunnel on *Arabidopsis* leaves of the treatments that they were also exposed to in the wind tunnel. These leaves were used in an alternating way, i.e., in the case that *P. rapae*-infested Col-0 wild-type was tested against caterpillar-infested 35S:*ESP* plants, the first, third, fifth, etc. wasp was introduced on an Col-0 wild-type leaf from which the caterpillars and their products had been carefully removed. Consequently, the second, fourth, sixth, etc. tested parasitoid was introduced on an 35S:*ESP* leaf from which the caterpillars and their products had been carefully removed as well. This increases the general behavioral response of the parasitoids to plant cues but likely does not induce a shift of preference (Kaiser and Cardé 1992; Bleeker et al. 2006; Smid 2006). Wasps were allowed to walk onto the leaves themselves. The leaf with the wasp was placed at the middle of the release cylinder, which was 60cm downwind from the two odor sources. As soon as the parasitoid had left the leaf for a few seconds, the leaf was carefully removed with tweezers without

disturbing the parasitoid. The flight behavior of the wasps was observed. Flights that resulted in a landing on one of the two odor sources were recorded as a "choice." Parasitoids that did not leave the release cylinder or landed on other parts of the wind tunnel within 10min were recorded as "no choice." Every parasitoid was used once. In order to correct for unforeseen asymmetry in the setup, the position of the odor sources was swapped after five tested parasitoids. After testing ten parasitoids, the odor sources were replaced with new ones. Every experiment was repeated at least five times over the course of several days. In order to check whether parasitoid discrimination was influenced by the amount of the caterpillar feeding, the leaf area removed by caterpillars from three infested Col-0 wild-type plants and three 35S:*ESP* plants from three experimental days was analyzed using the program ImageJ 1.37v (http://rsb.info.nih.gov/ij/).

Oviposition Preference Test Freshly emerged *P. rapae* adults were transferred to a large cage (67 × 100 × 75cm) in a greenhouse compartment at 24 ± 4°C, 60 ± 20% RH, and an L16:D8-h photoperiod. Butterflies were provided with 10% sucrose solution. Three to 5 days after emergence, one male and one female butterfly were transferred to each of the oviposition cages (67 × 50 × 75cm) in the same greenhouse compartment. Each butterfly couple was also provided with a 10% sucrose solution. In addition to natural daylight, the cages were illuminated by sodium vapor lamps (SON-T, 500W, Philips, the Netherlands) from 10:00a.m. to 4:00p.m. At 48h prior to the experiment, a single untreated Brussels sprouts leaf was placed in each cage as an oviposition substrate. After 6h, the leaf was removed. On the experimental day between 10:00a.m. and 11:00a.m., a transgenic 35S:*ESP* plant and a Col-0 wild-type plant were placed approximately 40cm apart from each other in each cage. In one experiment, both plants were intact, and in a second experiment both plants were artificially damaged. *P. rapae* was allowed to lay eggs on the two plants for 5 h. The plants were then removed, and the eggs laid were counted. The experiments were conducted in several cages at the same time and on several days per treatment, each replicate with new plants and butterflies.

P. rapae *Feeding Preference Test* Feeding choice tests were carried out in a climate chamber with an L16:D8-h photoperiod, 75 ± 5% RH, and a temperature of 21 ± 1°C. For each replicate, three Col-0 plants and three 35S:*ESP* plants were alternately arranged in a circle. A single *P. rapae* third instar was released in the center of each arena at the level of the leaf rosettes, and the larvae were allowed to feed for 24 h. A total of 18 replicates was carried out on three experimental days with independently grown sets of plants. Leaf areas removed from the three wild-type plants and the three 35S:*ESP* plants in each arena were measured. Leaf rosettes were digitally photographed with a reference mark before and after the experiments to calculate the removed leaf area using the program ImageJ 1.37v (http://rsb.info.nih.gov/ij/).

P. rapae *Performance and Developmental Studies* To compare the performance of *P. rapae* on the 35S:*ESP* plants and Col-0 wild-type plants, larvae were reared from emergence to pupation on 5-week-old 35S:*ESP* plants in a climate chamber (21 ± 1°C, L16:D8-h photoperiod, 75 ± 5% relative humidity). Single newly emerged larvae (<24 h old) were placed on individual plants that were then covered with perforated plastic bread bags to prevent insects from escaping. The weight of each larva was recorded on day 7 after hatching, when the larvae were transferred to fresh plants for the first time, and again on day 10. The larvae were transferred to new plants three to four times depending on their consumption rate. One to 2 days after pupation, the pupae were removed from the plants and placed individually in small ventilated plastic vessels. The emergence of adults was monitored every day for a period of 30 days.

Statistical Analysis In the wind tunnel experiments, a *binomial test* was used to analyze whether the behavioral choices of the parasitoids differed from a 50:50 distribution between the two odor sources. Parasitoids that did not make a choice were excluded from the statistical analysis. To check whether introducing the parasitoids on different leaves influenced the subsequent choice of the parasitoids, a *McNemar test* for marginal homogeneity was applied. In the oviposition choice tests, most individuals of *P. rapae* laid eggs on both the Col-0 wild-type and the 35S:*ESP* plant. The number of eggs on each treatment per individual were considered as a paired sample and were analyzed with the nonparametric *Wilcoxon signed ranks test*. In addition, we also analyzed the egg incidence by using a *sign test*. For the feeding choice experiments with *P. rapae*, the removed leaf area from each of the three Col-0 wild-type and the three 35S:*ESP* plants of each replicate were pooled, and a mean was calculated. A *Wilcoxon signed ranks test* was applied to test whether means were statistically different. *Mann–Whitney U tests* were applied to test for differences in performance-related parameters between Col-0 wild-type and 35S:*ESP* plants. All tests are described by Glantz (2005), and for some tests the statistical software package SPSS 12.0.1 (SPSS Inc., Chicago, IL, USA) was used.

Results

Behavioral Response of C. rubecula *to Volatiles of ESP Overexpressing and Wild-Type Arabidopsis Plants. P.*

rapae-caterpillar-infested 35S:*ESP* plants attracted more *C. rubecula* parasitoids than caterpillar-infested Col-0 plants in a two-choice wind tunnel setup (*binomial test*, $p < 0.05$; Fig. 2). However, when artificially damaged before the experiment, 35S:*ESP* plants attracted significantly fewer parasitoids than caterpillar-infested Col-0 wild-type plants (*binomial test*, $p < 0.05$; Fig. 2). When caterpillar-infested 35S:*ESP* plants were compared with artificially damaged ones, more parasitoids landed on the caterpillar-infested plants (*binomial test*, $p < 0.005$; Fig. 2). However, artificially damaged 35S:*ESP* plants were more attractive to *C. rubecula* than intact plants of the same line (*binomial test*, $p < 0.001$; Fig. 2). The introduction of the parasitoids to the wind tunnel on leaves of either Col-0 or 35S:*ESP* plants did not bias their subsequent choice but stimulated the general response of the wasps to plant volatiles (data not shown, *McNemar test*, $p > 0.05$). The leaf area removed by the caterpillars did not differ between 35S:*ESP* and Col-0 wild-type plants (35S:*ESP*: $1.62 \pm 0.11 cm^2$, Col-0: $1.61 \pm 0.22 cm^2$; mean ± SE, $N = 18$; *Mann–Whitney U test*, $p > 0.4$).

Oviposition Preference of P. rapae *P. rapae* lays individual eggs that are usually distributed over the plants. In general, *P. rapae* females began to lay eggs soon after being exposed to the experimental plants, and they deposited eggs on both the transgenic and the wild-type plant in more than 99.5% of all replicates. When plants were undamaged, ovipositing butterflies did not discriminate between 35S:*ESP* and Col-0 wild-type plants (Fig. 3): the average number of eggs laid per female did not differ between plant genotypes (*Wilcoxon signed ranks test*, $p > 0.05$, $N = 18$, Fig. 3). In 56% of the cases, the butterflies laid more eggs on the wild-type plant, while in 44% they deposited more eggs on the 35S:*ESP* plant (*sign test*, $p > 0.05$, $N = 18$). However, the situation changed when plants were artificially damaged before being exposed to the butterflies. In this case, the butterflies significantly preferred to lay eggs on wild-type plants. In 81% of the cases, *P. rapae* laid more eggs on Col-0 plants than on the 35S:*ESP* plants (*sign test*, $p < 0.01$, $N = 22$, Fig. 3). This trend is also reflected in the significantly larger number of eggs laid per female on wild-type plants (Fig. 3). Overall, when plants were damaged, the nitrile-producing 35S:*ESP* plants were significantly less attractive for egg deposition by *P. rapae* than the isothiocyanate-producing wild-type Col-0.

Feeding Choice Experiments and Performance of P. rapae The next step was to test whether caterpillars show the same preference as adult female butterflies. In a multiple-choice experiment, young *P. rapae* caterpillars were allowed to choose between three 35S:*ESP* plants and three Col-0 wild-type plants for 24h. All tested caterpillars fed on both the transgenic and the wild-type plants. There was no significant difference in the mean leaf area removed between 35S:*ESP* and wild-type plants (35S:*ESP*: 7.0 ± 4.7 cm^2, Col-0: 6.1 ± 3.6 cm^2; $N = 18$; *Mann–Whitney U test*, $p > 0.05$). Furthermore, we analyzed the performance of *p. rapae* caterpillars on the 35S:*ESP* line and on wild-type plants. We recorded the larval weight after only 7 and 10

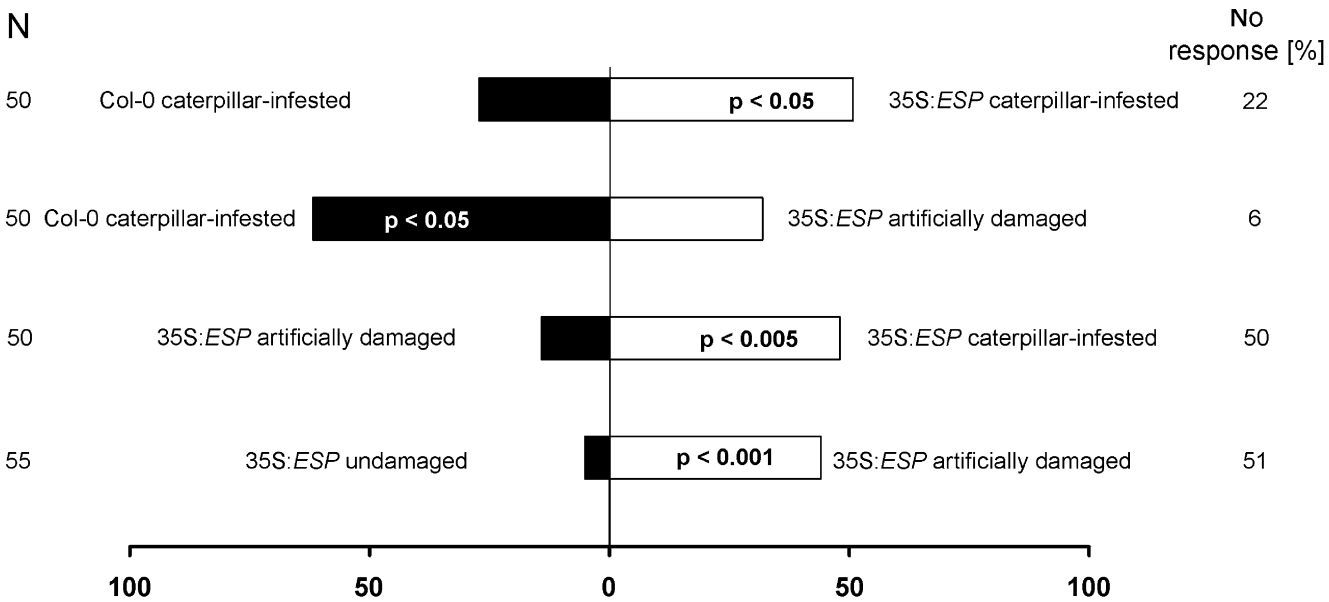

Fig. 2 Response of *C. rubecula* to odors of wild-type *Arabidopsis* Col-0 and transgenic 35S:*ESP* plants in a two-choice wind tunnel setup. Plants were either intact, artificially damaged right before the experiment, or infested with *P. rapae* caterpillars for 24 h. Parasitoids that did not make a choice were not included in the analysis. Choices between odor sources were analyzed using a *binomial test*

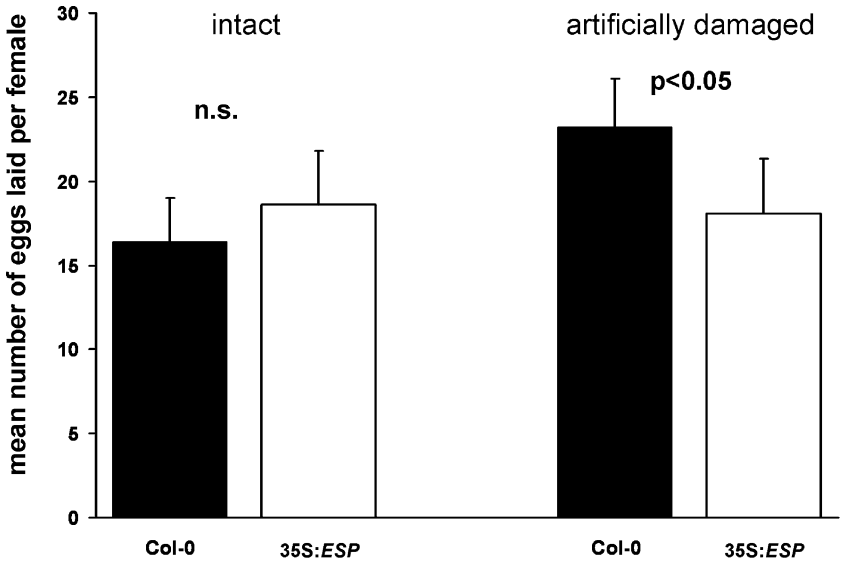

Fig. 3 Oviposition by *P. rapae* on wild-type *Arabidopsis* Col-0 (*black bars*) and transgenic 35S:*ESP* plants (*white bars*). Plants were either intact (*left*) or artificially damaged (*right*) before the experiment. Depicted is the mean number of eggs laid per female+standard error (analyzed using the *Wilcoxon signed ranks test* (N=18 with intact plants, N=22 with damaged plants)

days as differences in food quality at this early stage have a particularly strong effect on caterpillar growth and development (J.J.A. van Loon, personal communication). However, larval weight of caterpillars did not significantly differ between the plant genotypes at day 7 or 10 (Table 1). Likewise, pupal weight, developmental time until pupation, and the total developmental time were not significantly different between *P. rapae* caterpillars that fed on either 35S:*ESP* or Col-0 wild-type plants (Table 1).

Discussion

Our study demonstrates that glucosinolate-containing plants can benefit from producing predominantly simple nitriles instead of isothiocyanates upon herbivore damage despite the fact that simple nitriles are less toxic than isothiocyanates to insect herbivores. The experiments described employed a transgenic 35S:*ESP* line that upon tissue maceration hydrolyzes glucosinolates to simple nitriles rather than to isothiocyanates as in Col-0 wild-type plants (Burow et al. 2006b). Despite the differences in glucosinolate hydrolysis, there were no significant alterations between 35S:*ESP* and wild-type plants in glucosinolate content and composition and myrosinase activity (Burow et al. 2006b). The nitrile-producing 35S:*ESP* plants attracted significantly more *C. rubecula* parasitoids than Col-0 wild-type plants when both were infested by *P. rapae* (Fig. 2).

Simple nitriles are not only released by plants but are also the predominant volatiles emitted by the frass of *P. rapae* caterpillars, which is known to attract *C. rubecula* from a distance (Agelopoulos and Keller 1994b; Geervliet et al. 1994; Agelopoulos et al. 1995; Wittstock et al. 2004). Hence, nitrile-producing plants such as 35S:*ESP* plants may indicate the presence of feeding and defecating host larvae to reinforce the response of the parasitoids to *P. rapae*-induced volatiles (Fig. 2). This explanation is supported by a previous study showing that a combination of volatiles from *P. rapae*-infested cabbage and *P. rapae* feces was more attractive to *C. rubecula* than those of caterpillar-infested plants alone depending on the infestation level (Agelopoulos et al. 1995). In the present study, significantly more parasitoids were attracted to infested 35S:*ESP* and infested Col-0 plants compared to artificially damaged 35S:*ESP* plants. This demonstrates that the volatile blend emitted upon herbivory, including plant volatiles and volatiles derived from the herbivore, is more attractive to *C. rubecula* than volatiles emitted from the plant after mechanical damage (Fig. 2). Volatile blends of mechanically damaged 35S:*ESP* plants are most likely dominated by green leaf volatiles and simple nitriles, whereas the blend of caterpillar-infested plants is more complex, including also terpenoids and methyl salicylate (Van Poecke et al. 2001; Burow et al. 2006b). Indeed, *C. rubecula* parasitoids show electroantennogram responses to terpenoids and methyl salicylate in addition to responses to green leaf volatiles (Smid et al. 2002), indicating that these compounds have an important role in attracting the parasitoids as well. That the attraction of parasitoids is beneficial to the reproductive fitness of *A. thaliana* was demonstrated previously (Van Loon et al. 2000).

Many parasitoids change their behavioral preference to certain infochemicals by associative learning of these odors after a rewarding experience, e.g., an oviposition (Turlings et al. 1993; Vet et al. 1995). Also, *C. rubecula* can learn associatively to respond to certain plant odors either after an oviposition experience or by simply having contact with

Table 1 Performance of *P. rapae* on *Arabidopsis* Col-0 and 35S:*ESP*

	Col-0 wt (*N*=39)	35S:*ESP* (*N*=37)	Mann–Whitney *U* test (*p* value)
Larval weight on day 7 [mg]	24±2.1[a]	27±1.8	>0.05
Larval weight on day 10 [mg]	150±7.7	157±5.8	>0.05
Pupal weight [mg]	146±2.1	141±3.0	>0.05
Time until pupation [d]	12.3±0.2	12.2±0.2	>0.05
Total developmental time [d]	19.4±0.2	19.2±0.2	>0.05

[a] Data are presented as means±standard error

host products. However, unlike the situation in the closely related parasitoid species *Cotesia glomerata*, learning in *C. rubecula* results in an increased behavioral response towards the learned odor but does not shift an innate preference towards another odor (Bleeker et al. 2006; Smid 2006). This is confirmed by results of our study because introducing the parasitoids into the wind tunnel on a host-damaged leaf, alternating between a wild-type and a transgenic leaf, did not bias the subsequent choice to the leaf type they previously experienced. Moreover, giving *C. rubecula* females an oviposition experience in the presence of the odor of one of the two plant types would likely not result in a preference for that learned odor (Bleeker et al. 2006; Smid 2006).

P. rapae females laid significantly fewer eggs on mechanically damaged 35S:*ESP* plants than on mechanically damaged Col-0 wild-type plants. However, there was no preference when plants were undamaged suggesting that glucosinolate hydrolysis products rather than the glucosinolates themselves influenced plant acceptance by *P. rapae* (Fig. 3). De Vos et al. (2008) showed that ovipositing *P. rapae* preferred isothiocyanate-producing *Arabidopsis* Col-0 plants over 35S:*ESP* plants, even when the plants were not damaged. Numerous studies have shown that the nonvolatile glucosinolates serve as oviposition stimulants for *P. rapae* and closely related species (overview given by Chew and Renwick 1995; Hern et al. 1996; Schoonhoven et al. 2005). However, given that most glucosinolate hydrolysis products are volatile, while the parent glucosinolates are not, the hydrolysis products might be important cues for *P. rapae* at an earlier stage of the host location process. In fact, isothiocyanates in particular are known to attract many specialist herbivorous species (reviewed by Wittstock et al. 2003) and even to stimulate oviposition (Renwick et al. 2006). On the other hand, only a few studies have demonstrated that nitriles attract herbivores, and in general these were less attractive than isothiocyanates (Pivnick et al. 1992; Bartlet et al. 1997; Smart and Blight 2000; De Vos et al. 2008). Whether the preference of *P. rapae* to oviposit on isothiocyanate-emitting wild-type plants is due to the repellent effect of nitriles or to the stimulating effect of isothiocyanates or both remains to be investigated. However, oviposition preference of *P. rapae* for isothiocyanate-emitting wild-type plants is likely to benefit the butterflies either because isothiocyanates would indicate the presence of a (glucosinolate-containing) host plant or because nitrile emission could indicate a host plant that is already infested with conspecific larvae. Butterflies that avoid laying eggs on already infested host plants should reduce intraspecific competition and parasitism (Thompson and Pellmyr 1991). In fact, *P. rapae* is known to avoid laying eggs on plants that are infested with conspecific larvae or that carry conspecific eggs (Rothschild and Schoonhoven 1977; Schoonhoven et al. 1990; Sato et al. 1999). Nitriles, acting as indicators of feeding and defecating larvae, may not only signify the presence of competitors but may also hint at increased levels of glucosinolates and other defense compounds in the host plant as a result of induction that have a negative influence on the performance of the offspring (e.g., Agrawal and Kurashige 2003; Mewis et al. 2005, 2006; Gols et al. 2008). Accordingly, cabbage plants that were treated with jasmonic acid, a key hormone involved in induced plant defense, received significantly fewer eggs from *P. rapae* than respective control plants (Bruinsma et al. 2007).

The preference of *P. rapae* to oviposit on plants emitting isothiocyanates is likely due to the fact that isothiocyanate release indicates a functioning glucosinolate–myrosinase system. As many herbivores are not as well adapted to the glucosinolate–myrosinase system, the preference for a glucosinolate-containing plant by *P. rapae* butterflies is one key to avoid interspecific competition. Our present results show that *P. rapae* performed just as well on the isothiocyanate-producing Col-0 wild-type as on the nitrile-producing 35S:*ESP* line. In contrast, generalist herbivores are known to perform significantly better on nitrile-producing plants vs. isothiocyanate-producing plants (Lambrix et al. 2001; Burow et al. 2006b). *P. rapae* butterflies thus may be able to use isothiocyanates to select competitor-sparse food plants for their offspring (De Vos et al. 2008). To recap, gravid *P. rapae* females foraging for suitable host plants may have a number of reasons to prefer isothiocyanate-producing vs. nitrile-producing plants as an oviposition site. The release of nitriles upon damage may signal a higher risk of interspecific and intraspecific competition, an increased chance of parasitism, and a poorer substrate for larval

development. Alternatively, nitrile-producing plants might receive fewer eggs due to the lack of isothiocyanates as oviposition stimulus.

In summary, this paper has demonstrated that *A. thaliana* plants producing simple nitriles rather than isothiocyanates are better defended against the specialist herbivore, *P. rapae*, because of reduced oviposition and increased attraction of a specialist larval parasitoid. In contrast, isothiocyanate-emitting plants appear to be better defended against generalist herbivores. These findings provide a rationale for the existence of a polymorphism among *A. thaliana* ecotypes (Lambrix et al. 2001). The selective balance between these glucosinolate hydrolysis phenotypes may shift depending on the proportion of specialist to generalist herbivores present.

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References

AGELOPOULOS, N. G., and KELLER, M. A. 1994a. Plant–natural enemy association in the tritrophic system *Cotesia rubecula–Pieris rapae*–Brassicaceae (Cruciferae): II. Preference of *C. rubecula* for landing and searching. *J. Chem. Ecol.* 20:1735–1748.

AGELOPOULOS, N. G., and KELLER, M. A. 1994b. Plant–natural enemy association in the tritrophic system, *Cotesia rubecula–Pieris rapae*–Brassicaceae (Cruciferae): I. Sources of infochemicals. *J. Chem. Ecol.* 20:1725–1734.

AGELOPOULOS, N. G., DICKE, M., and POSTHUMUS, M. A. 1995. Role of volatile infochemicals emitted by feces of larvae in host-searching behavior of parasitoid *Cotesia rubecula* (Hymenoptera: Braconidae): a behavioral and chemical study. *J. Chem. Ecol.* 21:1789–1811.

AGERBIRK, N., MÜLLER, C., OLSEN, C. E., and CHEW, F. S. 2006. A common pathway for metabolism of 4-hydroxybenzylglucosinolate in *Pieris* and *Anthocaris* (Lepidoptera: Pieridae). *Biochem. Syst. Ecol.* 34:189–198.

AGRAWAL, A. A., and KURASHIGE, N. S. 2003. A role for isothiocyanates in plant resistance against the specialist herbivore *Pieris rapae*. *J. Chem. Ecol.* 29:1403–1415.

ALIBADI, A., RENWICK, J. A. A., and WHITMAN, D. W. 2002. Sequestration of glucosinolates by harlequin bug *Murgantia histrionica*. *J. Chem. Ecol.* 29:1749–1762.

ANDRÉASSON, E., and JØRGENSEN, L. B. 2003. Localization of plant myrosinases and glucosinolates, pp. 79–99, in J. T. Romeo (ed.). Integrative Phytochemistry: From Ethnobotany to Molecular Ecology. Pergamon, Amsterdam.

BARTH, C., and JANDER, G. 2006. *Arabidopsis* myrosinases TGG1 and TGG2 have redundant function in glucosinolate breakdown and insect defense. *Plant J.* 46:549–562.

BARTLET, E., BLIGHT, M. M., LANE, P., and WILLIAMS, I. H. 1997. The responses of the cabbage seed weevil *Ceutorhynchus assimilis* to volatile compounds from oilseed rape in a linear track olfactometer. *Entomol. Exp. Appl.* 85:257–262.

BERNARDI, R., NEGRI, A., RONCHI, S., and PALMIERI, S. 2000. Isolation of the epithiospecifier protein from oil-rape (*Brassica napus* ssp *oleifera*) seed and its characterization. *FEBS Lett.* 467:296–298.

BLANDE, J. D., PICKETT, J. A., and POPPY, G. M. 2007. A comparison of semiochemically mediated interactions involving specialist and generalist *Brassica*-feeding aphids and the braconid parasitoid *Diaeretiella rapae*. *J. Chem. Ecol.* 33:767–779.

BLEEKER, M. A. K., SMID, H. M., STEIDLE, J. L. M., KRUIDHOF, H. M., VAN LOON, J. J. A., and VET, L. E. M. 2006. Differences in memory dynamics between two closely related parasitoid wasp species. *Anim. Behav.* 71:1343–1350.

BRABY, M. F., and TRUEMAN, J. W. H. 2006. Evolution of larval host plant associations and adaptive radiation in pierid butterflies. *J. Evol. Biol.* 19:1677–1690.

BRADBURNE, R. P., and MITHEN, R. 2000. Glucosinolate genetics and the attraction of the aphid parasitoid *Diaeretiella rapae* to *Brassica*. *Proc. R. Soc. Lond. Ser. B* 267:89–95.

BRUINSMA, M., VAN DAM, N. M., VAN LOON, J. J. A., and DICKE, M. 2007. Jasmonic acid-induced changes in *Brassica oleracea* affect oviposition preference of two specialist herbivores. *J. Chem. Ecol.* 33:655–668.

BUROW, M., MARKERT, J., GERSHENZON, J., and WITTSTOCK, U. 2006a. Comparative biochemical characterization of nitrile-forming proteins from plants and insects that alter myrosinase-catalysed hydrolysis of glucosinolates. *FEBS J.* 273:2432–2446.

BUROW, M., MÜLLER, R., GERSHENZON, J., and WITTSTOCK, U. 2006b. Altered glucosinolate hydrolysis in genetically engineered *Arabidopsis thaliana* and its influence on the larval development of *Spodoptera littoralis*. *J. Chem. Ecol.* 32:2333–2349.

CHEW, F. S., and RENWICK, J. A. A. 1995. Host plant choice in *Pieris* butterflies, pp. 214–238, in R. T. Cardé, and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman & Hall, New York.

DEGENHARDT, J., GERSHENZON, J., BALDWIN, I. T., and KESSLER, A. 2003. Attracting friends to feast on foes: engineering terpene emission to make crop plants more attractive to herbivore enemies. *Curr. Opin. Biotechnol.* 14:169–176.

DE VOS, M., KRIKSUNOV, K. L., and JANDER, G. 2008. Indole-3-acetonitrile production from indole glucosinolates deters oviposition by *Pieris rapae* (white cabbage butterfly). *Plant Physiol.* 146:916–926.

FAHEY, J. W., ZALCMANN, A. T., and TALALAY, P. 2001. The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. *Phytochemistry* 56:5–51.

FOO, H. L., GRONNING, L. M., GOODENOUGH, L., BONES, A. M., DANIELSEN, B. E., WHITING, D. A., and ROSSITER, J. T. 2000. Purification and characterisation of epithiospecifier protein from *Brassica napus*: enzymic intramolecular sulphur addition within alkenyl thiohydroximates derived from alkenyl glucosinolate hydrolysis. *FEBS Lett.* 468:243–246.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1994. Volatiles from damaged plants as major cues in long-range host-searching by the specialist parasitoid *Cotesia rubecula*. *Entomol. Exp. Appl.* 73:289–297.

GEERVLIET, J. B. F., VET, L. E. M., and DICKE, M. 1996. Innate responses of the parasitoids *Cotesia glomerata* and *C. rubecula*

(Hymenoptera: Braconidae) to volatiles from different plant–herbivore complexes. *J. Insect Behav.* 9:525–538.

GIAMOUSTARIS, A., and MITHEN, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape (*Brassica napus* ssp *oleifera*) on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126:347–363.

GLANTZ, S. A. 2005. Primer of Biostatistics. McGraw-Hill, New York.

GOLS, R., RAAIJMAKERS, C. E., VAN DAM, N. M., DICKE, M., BUKOVINSZKY, T., and HARVEY, J. A. 2007. Temporal changes affect plant chemistry and tritrophic interactions. *Basic Appl. Ecol.* 8:421–433.

GOLS, R., WAGENAAR, R., BUKOVINSZKY, T., VAN DAM, N. M., DICKE, M., BULLOCK, J. M., and HARVEY, J. A. 2008. Genetic variation in the defense chemistry of wild cabbage populations and its effects on native herbivores and their endoparasitoids. *Ecology* 89:1616–1626.

GRUBB, C. D., and ABEL, S. 2006. Glucosinolate metabolism and its control. *Trends Plant Sci.* 11:89–100.

HALKIER, B. A., and GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant Biol.* 57:303–333.

HARVEY, J. A., VAN DAM, N. M., and GOLS, R. 2003. Interactions over four trophic levels: foodplant quality affects development of a hyperparasitoid as mediated through a herbivore and its primary parasitoid. *J. Anim. Ecol.* 72:520–531.

HARVEY, J. A., WITJES, L. M. A., BENKIRANE, M., DUYTS, H., and WAGENAAR, R. 2007. Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, *Pieris rapae*. *Plant Ecol.* 189:117–126.

HERN, A., EDWARDS-JONES, G., and MCKINLAY, R. G. 1996. A review of the pre-oviposition behaviour of the small cabbage white butterfly, *Pieris rapae* (Lepidoptera: Pieridae). *Ann. Appl. Biol.* 128:349–371.

HILKER, M., and MEINERS, T. 2002. Induction of plant responses to oviposition and feeding by herbivorous arthropods: a comparison. *Entomol. Exp. Appl.* 104:181–192.

HUANG, X. P., and RENWICK, J. A. A. 1994. Relative activities of glucosinolates as oviposition stimulants for *Pieris rapae* and *P. napi oleracea*. *J. Chem. Ecol.* 20:1025–1037.

HUANG, X. P., RENWICK, J. A. A., and SACHDEV-GUPTA, K. 1993. Oviposition stimulants and deterrents regulating differential acceptance of *Iberis amara* by *Pieris rapae* and *P. napi oleracea*. *J. Chem. Ecol.* 19:1645–1663.

HUANG, X. P., RENWICK, J. A. A., and SACHDEVGUPTA, K. 1994. Oviposition stimulants in *Barbarea vulgaris* for *Pieris rapae* and *P. napi oleracea*—isolation, identification and differential activity. *J. Chem. Ecol.* 20:423–438.

KAISER, L., and CARDÉ, R. T. 1992. In-flight orientation to volatiles from the plant–host complex in *Cotesia rubecula* (Hym.: Braconidae): increased sensitivity through olfactory experience. *Physiol. Entomol.* 17:62–67.

KAZANA, E., POPE, T. W., TIBBLES, L., BRIDGES, M., PICKETT, J. A., BONES, A. M., POWELL, G., and ROSSITER, J. T. 2007. The cabbage aphid: a walking mustard oil bomb. *Proc. R. Soc. Lond. Ser. B* 274:2271–2277.

KIM, J. H., and JANDER, G. 2007. *Myzus persicae* (green peach aphid) feeding on *Arabidopsis* induces the formation of a deterrent indole glucosinolate. *Plant J.* 49:1008–1019.

KLIEBENSTEIN, D. J. 2004. Secondary metabolites and plant/environment interactions: a view through *Arabidopsis thaliana* tinged glasses. *Plant Cell Environ.* 27:675–684.

KLIEBENSTEIN, D., PEDERSEN, D., BARKER, B., and MITCHELL-OLDS, T. 2002. Comparative analysis of quantitative trait loci controlling glucosinolates, myrosinase and insect resistance in *Arabidopsis thaliana*. *Genetics* 161:325–332.

KLIEBENSTEIN, D. J., KROYMANN, J., and MITCHELL-OLDS, T. 2005. The glucosinolate–myrosinase system in an ecological and evolutionary context. *Curr. Opin. Plant Biol.* 8:264–271.

LAMBRIX, V., REICHELT, M., MITCHELL-OLDS, T., KLIEBENSTEIN, D. J., and GERSHENZON, J. 2001. The *Arabidopsis* epithiospecifier protein promotes the hydrolysis of glucosinolates to nitriles and influences *Trichoplusia ni* herbivory. *Plant Cell* 13:2793–2807.

LI, Q., EIGENBRODE, S. D., STRINGAM, G. R., and THIAGARAJAH, M. R. 2000. Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. *J. Chem. Ecol.* 26:2401–2419.

MEWIS, I., APPEL, H. M., HOM, A., RAINA, R., and SCHULTZ, J. C. 2005. Major signaling pathways modulate *Arabidopsis* glucosinolate accumulation and response to both phloem-feeding and chewing insects. *Plant Physiol.* 138:1149–1162.

MEWIS, I., TOKUHISA, J. G., SCHULTZ, J. C., APPEL, H. M., ULRICHS, C., and GERSHENZON, J. 2006. Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. *Phytochemistry* 67:2450–2462.

MILES, C. I., DEL CAMPO, M. L., and RENWICK, J. A. A. 2005. Behavioral and chemosensory responses to a host recognition cue by larvae of *Pieris rapae*. *J. Comp. Physiol. A* 191:147–155.

MÜLLER, C., AGERBIRK, N., OLSEN, C. E., BOEVÉ, J. L., SCHAFFNER, U., and BRAKEFIELD, P. M. 2001. Sequestration of host plant glucosinolates in the defensive hemolymph of the sawfly *Athalia rosae*. *J. Chem. Ecol.* 27:2505–2516.

MURCHIE, A. K., SMART, L. E., and WILLIAMS, I. H. 1997. Responses of *Dasineura brassicae* and its parasitoids *Platygaster subuliformis* and *Omphale clypealis* to field traps baited with organic isothiocyanates. *J. Chem. Ecol.* 23:917–926.

PIVNICK, K. A. 1993. Response of *Meteorus leviventris*, (Hymenoptera: Braconidae) to mustard oils in field trapping experiments. *J. Chem. Ecol.* 19:2075–2079.

PIVNICK, K. A., LAMB, R. J., and REED, D. 1992. Response of flea beetles, *Phyllotreta spp*, to mustard oils and nitriles in field trapping experiments. *J. Chem. Ecol.* 18:863–873.

RASK, L., ANDREASSON, E., EKBOM, B., ERIKSSON, S., PONTOPPIDAN, B., and MEIJER, J. 2000. Myrosinase: gene family evolution and herbivore defense in Brassicaceae. *Plant Mol. Biol.* 42:93–113.

RATZKA, A., VOGEL, H., KLIEBENSTEIN, D. J., MITCHELL-OLDS, T., and KROYMANN, J. 2002. Disarming the mustard oil bomb. *Proc. Natl. Acad. Sci. U. S. A.* 99:11223–11228.

REDDY, G. V. P., HOLOPAINEN, J. K., and GUERRERO, A. 2002. Olfactory responses of *Plutella xylostella* natural enemies to host pheromone, larval frass, and green leaf cabbage volatiles. *J. Chem. Ecol.* 28:131–143.

RENWICK, J. A. A. 2002. The chemical world of crucivores: lures, treats and traps. *Entomol. Exp. Appl.* 104:35–42.

RENWICK, J. A. A., HARIBAL, M., GOUINGUENE, S., and STADLER, E. 2006. Isothiocyanates stimulating oviposition by the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* 32:755–766.

ROTHSCHILD, M., and SCHOONHOVEN, L. M. 1977. Assessment of egg load by *Pieris brassicae* (Lepidoptera: Pieridae). *Nature* 266:352–355.

SATO, Y., YANO, S., TAKABAYASHI, J., and OHSAKI, N. 1999. *Pieris rapae* (Lepidoptera: Pieridae) females avoid oviposition on *Rorippa indica* plants infested by conspecific larvae. *Appl. Entomol. Zool.* 34:333–337.

SCHOONHOVEN, L. M., BEERLING, E. A. M., BRAAKSMA, R., and VANVUGT, Y. 1990. Does the imported cabbageworm, *Pieris rapae*, use an oviposition deterring pheromone? *J. Chem. Ecol.* 16:1649–1655.

SCHOONHOVEN, L. M., VAN LOON, J. J. A., and DICKE, M. 2005. Insect–Plant Biology. Oxford University Press, Oxford.

SMALLEGANGE, R. C., VAN LOON, J. J. A., BLATT, S. E., HARVEY, J. A., AGERBIRK, N., and DICKE, M. 2007. Flower versus leaf feeding by *Pieris brassicae*: glucosinolate-rich flower tissues are preferred and sustain higher growth rate. *J. Chem. Ecol.* 33:1831–1844.

SMART, L. E., and BLIGHT, M. M. 2000. Response of the pollen beetle, *Meligethes aeneus*, to traps baited with volatiles from oilseed rape, *Brassica napus*. *J. Chem. Ecol.* 26:1051–1064.

SMID, H. M. 2006. Variation in learning of herbivory-induced plant odours by parasitic wasps, pp 89–103, in M. Dicke, and W. Takken (eds.). Chemical Ecology: From Gene to Ecosystem, Springer, Dordrecht.

SMID, H. M., VAN LOON, J. J. A., POSTHUMUS, M. A., and VET, L. E. M. 2002. GC–EAG-analysis of volatiles from Brussels sprouts plants damaged by two species of *Pieris* caterpillars: olfactory receptive range of a specialist and a generalist parasitoid wasp species. *Chemoecology* 12:169–176.

SNOEREN, T. A. L., DE JONG, P. W., and DICKE, M. 2007. Ecogenomic approach to the role of herbivore-induced plant volatiles in community ecology. *J. Ecol.* 95:17–26.

THOMPSON, J. N., and PELLMYR, O. 1991. Evolution of oviposition behavior and host preference in Lepidoptera. *Annu. Rev. Entomol.* 36:65–89.

TITAYAVAN, M., and ALTIERI, M. A. 1990. Synomone-mediated interactions between the parasitoid *Diaeretiella rapae* and *Brevicoryne brassicae* under field conditions. *Entomophaga* 35:499–507.

TURLINGS, T. C. J., WÄCKERS, F. L., VET, L. E. M., LEWIS, W. J., and TUMLINSON, J. H. 1993. Learning of host-finding cues by hymenopterous parasitoids, pp. 51–78, in D. R. Papaj, and A. C. Lewis (eds.). Insect Learning: Ecological and Evolutionary PerspectivesChapman & Hall, New York.

VAN LOON, J. J. A., BLAAKMEER, A., GRIEPINK, F. C., VAN BEEK, T. A., SCHOONHOVEN, L. M., and DE GROOT, A. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3:39–44.

VAN LOON, J. J. A., DE BOER, J. G., and DICKE, M. 2000. Parasitoid–plant mutualism: parasitoid attack of herbivore increases plant reproduction. *Entomol. Exp. Appl.* 97:219–227.

VAN POECKE, R. M. P. 2007. *Arabidopsis*-insect interactions, pp. 1–34, in C. Somerville, and E. M. Meyerowitz (eds.). The *Arabidopsis* BookAmerican Society of Plant Biologists, Rockville http://www.aspb.org/publications/Arabidopsis/.

VAN POECKE, R. M. P., POSTHUMUS, M. A., and DICKE, M. 2001. Herbivore-induced volatile production by *Arabidopsis thaliana* leads to attraction of the parasitoid *Cotesia rubecula*: chemical, behavioral, and gene-expression analysis. *J. Chem. Ecol.* 27:1911–1928.

VERGARA, F., SVATOS, A., SCHNEIDER, B., REICHELT, M., GERSHENZON, J., and WITTSTOCK, U. 2006. Glycine conjugates in a lepidopteran insect herbivore—the metabolism of benzylglucosinolate in the cabbage white butterfly, *Pieris rape*. *Chembiochem* 7:1982–1989.

VET, L. E. M., LEWIS, W. J., and CARDÉ, R. T. 1995. Parasitoid foraging and learning, pp. 65–101, in R. T. Cardé, and W. J. Bell (eds.). Chemical Ecology of Insects 2. Chapman and Hall, London.

WHEAT, C. W., VOGEL, H., WITTSTOCK, U., BRABY, M. F., UNDERWOOD, D., and MITCHELL-OLDS, T. 2007. The genetic basis of a plant insect coevolutionary key innovation. *Proc. Natl. Acad. Sci. U. S. A.* 104:20427–20431.

WITTSTOCK, U., and BUROW, M. 2007. Tipping the scales—specifier proteins in glucosinolate hydrolysis. *IUBMB Life* 59:744–751.

WITTSTOCK, U., and HALKIER, B. A. 2002. Glucosinolate research in the *Arabidopsis* era. *Trends Plant Sci.* 7:263–270.

WITTSTOCK, U., KLIEBENSTEIN, D. J., LAMBRIX, V., REICHELT, M., and GERSHENZON, J. 2003. Glucosinolate hydrolysis and its impact on generalist and specialist insect herbivores, pp 101–125, in J. T. Romeo (ed.). Integrative Phytochemistry: From Ethnobotany to Molecular Ecology. Pergamon, Amsterdam.

WITTSTOCK, U., AGERBIRK, N., STAUBER, E. J., OLSEN, C. E., HIPPLER, M., MITCHELL-OLDS, T., GERSHENZON, J., and VOGEL, H. 2004. Successful herbivore attack due to metabolic diversion of a plant chemical defense. *Proc. Natl. Acad. Sci. U. S. A.* 101:4859–4864.

ZHANG, Z. Y., OBER, J. A., and KLIEBENSTEIN, D. J. 2006. The gene controlling the quantitative trait locus *EPITHIOSPECIFIER MODIFIER1* alters glucosinolate hydrolysis and insect resistance in *Arabidopsis*. *Plant Cell* 18:1524–1536.

addition, mycorrhizal fungi can alter host plant responses to herbivory in many ways, i.e., by inducing changes in host plant chemical quality, host plant defense (Bezemer and van Dam 2005; Bennett et al. 2006), and host plant tolerance (Bennett et al. 2006).

To better understand the effects of mycorrhiza on aboveground herbivory, Jones and Last (1991) constructed a conceptual model that predicts the effects of ectomycorrhizal (ECM) infection on plant defense and growth in different environmental conditions. In that model, ECM should increase antiherbivore defense of host plants aboveground through carbon allocation under low soil nutrients and high light conditions. Bennett et al. (2006) used mostly arbuscular mycorrhiza (AM) as a model organism to predict how mycorrhizal fungi could affect plant enemies by improving plant nutrition, modifying plant tolerance, or modifying plant defenses for the long-term dynamics of the plant–enemy interactions. However, experimental evidence to support the predictions of these model systems is still limited. Earlier studies with ECM have shown positive (Gehring et al. 1997; Manninen et al. 1999, 2000), negative (Gehring et al. 1997; Manninen et al. 1998a; Rieske 2001; Gange et al. 2005b), or no effects (Manninen et al. 1998a, 2000; Palermo et al. 2003) on insect herbivore performance compared to performance on nonmycorrhizal or low-ectomycorrhizal conspecific plants. Similarly, the few earlier studies with AM plants show that fungal inoculation either improves (Borowicz 1997; Gange and West 1994; Gange et al. 2005a; Bennett and Bever 2007), reduces (Rabin and Pacovsky 1985; Gange and West 1994; Gange 2001; Bennett and Bever 2007), or does not affect (Gange 2001; Bennett and Bever 2007) the performance of aboveground herbivores. The responses of herbivores on certain plant species could be dependent on the AM species associated in the root system (Bennett and Bever 2007).

The effects of mycorrhizas on the performance of aboveground insect herbivores seem to be dependent on the insect feeding guild and also on the degree of specialization of the insect studied. Specialist insects, both sucking (Manninen et al. 1999; Gange et al. 2002) and chewing (Gange et al. 2002), have performed better on mycorrhizal plants (ECM in Manninen et al. 1999; AM in Gange et al. 2002), whereas generalist sucking (Manninen et al. 2000) and generalist chewing (Gange et al. 2002) have shown lowered performance. Insect responses to plants that live in association with mycorrhizal fungi may be due to host plant chemistry affected by mycorrhizae (Manninen 1999; Gange et al. 2002), but chemical changes of a plant as such have not been clearly linked with the mycorrhizal status of host plants (Manninen 1999). Often, generalist insects are deterred by high concentrations of defense compounds, whereas specialists in contrast use them to identify potential hosts (van der Meijden 1996).

In our laboratory study, we compared how a generalist and specialist insect herbivore, with either sucking or chewing feeding behavior, perform on silver birch with different ECM infection levels in their roots. Different ECM infection levels were achieved by artificially inoculating already naturally infected plantlets with two different species of ECM fungi or by reducing the natural infection with a fungicide. Our hypotheses were, following the allocation model of Bennett et al. (2006), that the ectomycorrhizal infection level would influence aboveground insect herbivore performance on deciduous tree plantlets either positively (1) by increasing available plant biomass, (2) by enhancing availability of nutrients, or negatively (3) by modifying the concentration of defense compounds, i.e., phenolics, in birch foliage.

Methods and Materials

Plant Material Tissue-cultured clonal silver birch plantlets (clone KL-5-M) grown in the Research Garden of Kuopio University were transplanted into mini-greenhouses in a mixture (2:1, *v*/*v*) of prefertilized (Vapo *Sphagnum* peat, 12:9:18, N/P/K) and γ-sterilized (Kolmi-Set Ltd. Ilomantsi, Finland) peat and autoclaved sand (particle size 0.5–1.2 mm). This clone was chosen because we already had some background information, e.g., it is fast growing (Pääkkönen et al. 1993). Plantlets (N=140, 8-week old) were transplanted into 0.8-L plastic pots in the same growth medium as mentioned above and transported to the growth chamber at the University. Growth conditions in the chamber simulated weather conditions of June in Finland (day–night temperature 19°C:12°C, photoperiod 22 L:2 h/day, and maximum daytime irradiance about 500 μE m^{-2} s^{-1}).

Plantlets were divided randomly into four mycorrhizal treatments (35 plantlets per treatment): (1) C = control (natural mycorrhizal infection from the growth medium); (2) PI+ = *Paxillus involutus*-inoculated (our own collection, strain number 62); (3) LV+ = *Leccinum versipelle*-inoculated (our own collection, strain number 58); and (4) F− = fungicide-treated plantlets. Artificial inoculations were done once by adding 10 ml of fungal suspension into each pot, whereas fungicide propiconazole (TILT 250 EC, Syngenta Crop Protection, Ltd.) was applied twice at intervals of 1 week, so that the amount of active ingredient reached 0.025 g m^{-2} in each pot. Plantlets were watered when needed. Because quartz sand and *Sphagnum* peat are so poor in nutrients that reduced ectomycorrhizal growth can be expected (Kainulainen et al. 1996), plantlets were fertilized once a week at the age of 10–18 weeks with 0.1% 9-Superex (19:5:20, N/P/K, Kekkilä Oy), yielding 95 mg nitrogen per plantlet (corresponds approximately 148 kg N ha^{-1}).

All insect bioassays were performed under laboratory conditions during summer when these particular insects also are naturally feeding on their host plants in Finland (i.e., the age of plantlets during the insect bioassays was between 12 and 18 weeks). Final sampling for all chemical analyses was conducted when plantlets were 20-week old.

Lygus rugulipennis Experiment The phloem-feeding generalist, the tarnished plant bug, *Lygus rugulipennis*, Popp., (Heteroptera: Miridae) causes damage especially in forest nurseries by damaging the apical meristem and causing growth disturbance. Adults were collected from silver birch stocks in a forest nursery, kept at +12°C, and transferred to +20°C 1 day before the oviposition test started. For the oviposition test, 12-week-old silver birch plantlets were evenly distributed among 12 plastic containers. Thus, each container (360× 260 mm with a height of 220 mm) had one plantlet with each mycorrhizal treatment (C, PI+, LV+, F−; N=12 per treatment). Containers were covered with nylon gauze, and two adult females plus one male *L. rugulipennis* were released into each container for mating and egg laying (+21°C). After 12 days, adults were removed and nymphs were allowed to hatch from eggs in chamber conditions described above. Every second day during the following 24-day period, plantlets were shaken gently to access the new emerged nymphs. Afterwards, these plantlets were maintained under the same chamber conditions described above and used in the following *L. rugulipennis* feeding test.

For the feeding test, *L. rugulipennis* nymph (first to fourth instar; the same colony as mentioned above and maintained on barley, *Hordeum vulgare*) was weighed and enclosed in a clip cage (one nymph per seedling) on the upper surface of the second or third leaf of a side branch of the same plants used in the oviposition test (12 plantlets/treatment, age of plants 18 weeks). The clip cage was made of a pair of plastic cylinders (diam. 1.5 cm, length of each 1.2 cm) and fixed on the leaf with a curtain clip. To prevent leaf damage by the clip cage, plastic foam was attached to the cylinder edges. Nylon mesh at both ends of the cylinders prevented escape of the nymphs. Nymphs from different instars were distributed evenly among the four treatments. Three days later, nymphs were reweighed, and their relative growth rate (RGR; [ln(final fresh mass) − ln (initial fresh mass)]/number of days) was calculated (Waldbauer 1968).

Calaphis flava and Epirrita autumnata Experiment *C. flava* Mordvilko (Homoptera: Drepanosiphidae) is a phloem-feeding specialist aphid that causes damage both in forest nurseries and forests in Finland. Damage may be harmful especially for young seedlings. RGR was determined by using 5-day-old aphid nymphs (laboratory culture, age less than 1 year). Sets of plantlets (N=11 per treatment) different from those in the previous *Lygus* experiment were selected randomly from the original plantlet material. Four weeks after fungal inoculation, two weighed nymphs per 13-week-old plantlet were individually enclosed inside clip cages as described earlier on the lower side of two distinct leaves. For determination of RGR, nymphs were reweighed after 3 day and, thereafter, allowed to develop into adults on the same plantlets. The cumulative number of nymphs produced during 15 days was counted (age of plantlets approximately 15 weeks at the end of the experiment).

Epirrita autumnata Bkh (Lepidoptera: Geometridae) is a chewing generalist that causes damage especially to mountain birches in northern Finland. This herbivore is ecologically important at the timber line area. RGR of fifth instars, originating from Kevo Subarctic Research Station in northern Finland (one brood), was determined during a 24-h feeding period (N=12 per treatment, same plantlets as in *C. flava* test above plus one more selected from the original plantlet material, age of plants approximately 16 weeks). A weighed larva and the fourth birch leaf from the top of a plantlet were enclosed in a moistened gypsum container for 24 h at room temperature (+21°C). The initial and final mass of larvae and the dry weight of leaf material eaten were used to determine the efficiency of conversion of ingested material (ECI=100 × larval dry weight gain/dry weight of plant material eaten). RGR of larvae was calculated as described above.

Host Plant Parameters From the birches used in *L. rugulipennis* tests, two leaves were collected at the end of the oviposition test from 18-week-old plantlets, and air-dried for phenolic analyses by high performance liquid chromatography (HPLC; Lavola et al. 1994) to detect possible ectomycorrhiza-induced compounds. Two weeks later at the final harvest, when the plants were 20-week-old, separate leaves from the same birches were put into liquid nitrogen, stored in a deep-freezer (−80°C), and later freeze-dried for the enzymatic starch analysis (Boehringer starch kit for food analysis, Mannheim, Germany). Also at the final harvest, leaves from all plantlets used in the *L. rugulipennis* and *C. flava* tests described above were collected for nutrient analyses (N=92; 23 samples per treatment). Major nutrient (N, P, K, Ca, Mg) concentrations of oven-dried (+60°C) and wet-digested (Allen 1989) leaves were analyzed as described earlier (Manninen et al. 1999). The length of the main stems and the fresh and dry masses (oven drying at +70°C, 2 days) of the leaves were determined as well (N=92). All roots were washed with tap water and stored at −24°C for microscopic and biomass analysis, except sub-samples of roots taken from *C. flava* plantlets; these were stored at −80°C before being freeze-dried for ergosterol analysis. HPLC analysis of roots to determine ergosterol quantities was conducted as

described earlier (Nylund and Wallander 1992; Markkola 1996; Manninen et al. 1998b). For the determination of mycorrhizal infection and number of short roots, frozen roots were allowed to thaw, and a 1-m long sample from fine roots (∅≤2 mm) was randomly collected individually from each plantlet, stained with Ponceau S (Daughtridge et al. 1986), and analyzed visually under a stereomicroscope. Thereafter, roots were oven-dried, and the total dry weight was measured.

Statistical Analyses The data were checked for normal distribution and homogeneity of variances. The effects of different mycorrhizal treatments on parameters were tested either with one-way ANOVA (Tukey's or Dunnett's T3 a-posteriori test, $P<0.05$) or Kruskal–Wallis (Mann–Whitney U a-posteriori test, $P<0.05$). Before testing with ANOVA, data were log ($n+1$)- or arcsin-transformed when necessary. Correlation analyses were done with pooled data only ($N=44$–48) using Spearman's correlation coefficient. All analyses were done with the SPSS Software package.

Results

In the plantlets used in *L. rugulipennis* and *C. flava* experiments, both PI+ and LV+ treatments increased mycorrhizal infection level compared to the control treatment. Fungicide (F−) treatment reduced mycorrhizal infection significantly in the plantlets of both experiments (Fig. 1a,b), but it was not possible to produce totally nonmycorrhizal birch plantlets. Correspondingly, ergosterol concentration of roots, determined from *C. flava* plantlets only, increased significantly after PI+ and LV+ treatment but decreased after F− treatment (Fig. 1c). However, the total amount of short roots did not vary between treatments (data not shown).

Silver birch plantlets inoculated with PI+ had the lowest leaf and root dry mass (Table 1). The main stem dry mass of the plantlets used in *C. flava* experiment clearly decreased after both PI+ and LV+ inoculations compared to C and F− treatments. The total height of plantlets was also significantly reduced after PI+ inoculation compared to C and F− treatments (Table 1).

The nutrient concentrations of leaves responded to mycorrhizal manipulations quite variably. In the *C. flava* experiment, N concentrations of leaves were lowest in LV+ plantlets and highest in PI+ plantlets; phosphorous (P) concentration was highest in LV+ plantlets differing significantly from F− treatment (Table 2). F− treatment of plantlets used in the *C. flava* experiment led to a significant decrease of leaf calcium (Ca) concentration only compared to all other treatments. When considering plant samples

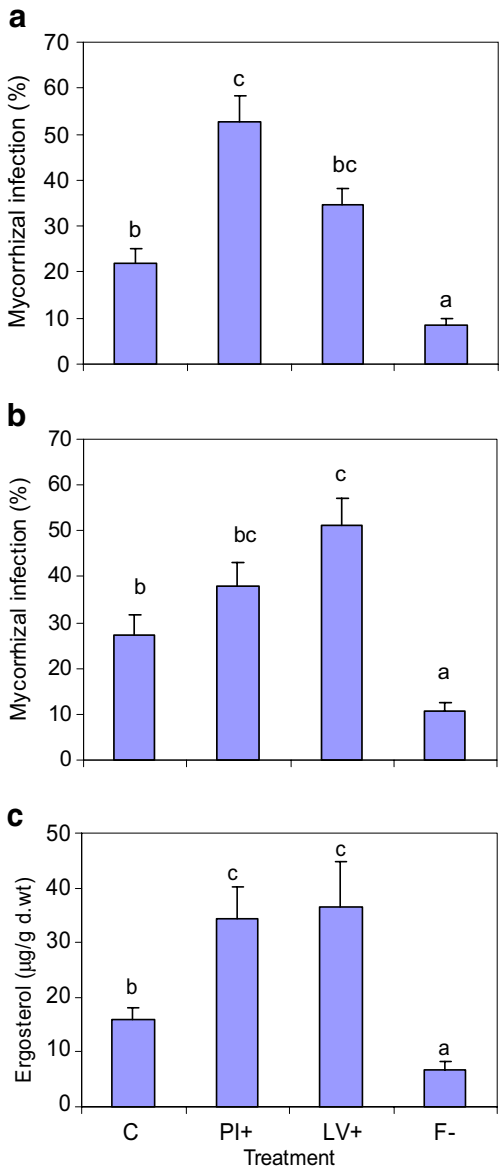

Fig. 1 Mycorrhizal infection (%) (+SE) of birch roots in different mycorrhizal treatments (**a**) in *Lygus rugulipennis* experiment ($N=12$) and (**b**) in *Calaphis flava* experiment ($N=11$), and (**c**) root ergosterol concentration (+SE) in plantlets used in *Calaphis flava* experiment ($N=11$). Treatments: *C* control, natural mycorrhizal infection, *PI+ Paxillus involutus*-inoculated, *LV+ Leccinum versipelle*-inoculated, and *F−* fungicide treated. Data tested with one-way ANOVA, and means followed by different letter are significantly different ($P<0.05$) according to Dunnett T3 (**a**, **b**) or Tukey's test (**c**)

used in the *L. rugulipennis* experiment and pooled plantlets from both type of experiments, leaf magnesium (Mg) concentrations significantly increased after F− treatment compared to C and LV+ treatments (Table 2). Starch concentration did not vary significantly between mycorrhizal treatments (Table 2). Altogether, 11 phenolics (Table 3) were analyzed from the leaves of the plantlets used in *L. rugulipennis* experiment, myricitrin, and quercitrin being

Table 1 The mean total height of plant (cm) and leaf and root dry mass (g) in differently treated birch plantlets used in experiments with *Lygus rugulipennis* (*N*=12) and *Calaphis flava* (*N*=11)

Experiment and parameter	C	PI+	LV+	F−	*P*-value
L. rugulipennis experiment					
Height of plant	26.53(3.12)a	23.02(5.64)a	26.13(3.60)a	25.72(4.96)a	0.220
Leaf dry mass	1.43(0.50)b	0.90(0.31)a	1.32(0.24)b	1.54(0.51)b	0.001
Root dry mass	2.63(0.67)b	1.99(0.56)a	2.42(0.51)ab	2.24(0.44)ab	0.043
C. flava experiment					
Height of plant	32.19(2.74)bc	27.74(2.83)a	29.40(3.47)ab	33.62(3.06)c	0.001
Leaf dry mass	2.62(0.36)b	1.81(0.43)a	2.20(0.32)ab	2.29(0.44)b	<0.001
Root dry mass	3.13(0.73)b	1.53(0.83)a	2.56(0.45)b	2.53(0.46)b	<0.001
Main stem dry mass	1.89(0.22)c	1.09(0.34)a	1.51(0.19)b	1.82(0.17)c	<0.001
Root:shoot ratio	68.73(10.48)a	50.74(19.32)a	69.72(13.16)a	61.57(8.09)a	0.007
Pooled plantlets					
Height of plant	29.24(4.08)b	25.27(5.04)a	27.70(3.84)ab	29.50(5.73)b	0.012
Leaf dry mass	2.00(0.74)b	1.33(0.59)a	1.74(0.53)ab	1.90(0.61)b	0.003
Root dry mass	2.87(0.73)b	1.77(0.72)a	2.49(0.47)b	2.38(0.46)b	<0.001

SD in parentheses. Pooled plantlets (*N*=23). *P* values according to Kruskal-Wallis or One-way ANOVA. Different letters after the mean and SD indicate statistically significant differences between the treatments ($P<0.05$). The mean main stem dry mass (g) and root/shoot ratio (%) (SD in parentheses) are determined from *C. flava* experiment plantlets only.
C Control, natural mycorrhizal infection, *PI+ Paxillus involutus*-inoculated, *LV+ Leccinum versipelle*-inoculated, *F−* fungicide treated.

the major compounds. We did not find evidence of ectomycorrhiza-induced phenolic production in the foliage.

RGR (Table 4) and fecundity (Fig. 2a) of *L. rugulipennis* did not differ among treatments, whereas *E. autumnata* had significantly higher RGR on PI+ plantlets than on LV+ plantlets (Table 4). ECI of *E. autumnata* larvae was not different among the treatments (data not shown). *C. flava* performed poorly on plantlets with enhanced ECM level: the cumulative number of nymphs decreased remarkably when feeding took place on PI+ and LV+ treated plants

Table 2 Main nutrient (N, P, Ca, Mg and K) concentrations (mg g^{-1} of leaf dry mass) of birch leaves in differently treated plantlets (treatments as in Table 1) used in experiments with *Lygus rugulipennis* (*N*=12) and *Calaphis flava* (*N*=11)

Insect species and parameter	C	PI+	LV+	F−	*P* value
L. rugulipennis exp.					
N	10.2(1.0)a	10.15(2.51)a	9.66(1.75)a	10.75(1.34)a	0.476
P	2.41(0.76)a	2.86(0.56)a	2.58(0.27)a	2.34(0.64)a	0.051
Ca	5.97(0.73)b	5.00(0.80)a	4.64(0.48)a	5.56(1.16)ab	0.002
Mg	3.70(0.41)a	4.03(0.62)ab	3.72(0.41)a	4.35(0.43)b	0.005
K	9.06(1.06)a	10.10(3.66)a	10.05(1.12)a	9.85(1.17)a	0.618
Starch	145.83(29.35)a	135.5(43.1)a	135.73(40.41)a	130.56(42.61)a	0.811
C. flava exp.					
N	9.66(0.65)ab	11.10(1.03)c	9.43(0.57)a	10.64(1.32)bc	<0.001
P	2.20(0.52)ab	2.62(0.46)ab	2.67(0.49)b	2.11(0.45)a	0.015
Ca	5.92(0.64)b	6.48(0.75)b	6.34(0.34)b	4.29(0.85)a	<0.001
Mg	3.33(0.42)a	3.43(0.34)a	3.28(0.12)a	3.50(0.27)a	0.262
K	9.38(0.69)a	10.21(1.40)a	8.93(0.50)a	9.58(1.10)a	0.152
Pooled plantlets					
N	9.93(0.90)ab	10.60(1.97)ab	9.55(1.30)a	10.70(1.30)b	0.015
P	2.31(0.65)a	2.74(0.52)b	2.63(0.38)ab	2.23(0.56)a	0.003
Ca	5.94(0.67)b	5.71(1.07)ab	5.45(0.96)ab	4.95(1.19)a	0.015
Mg	3.52(0.45)a	3.74(0.58)ab	3.51(0.37)a	3.94(0.56)b	0.012
K	9.21(0.90)a	10.15(2.75)a	9.51(1.03)a	9.72(1.12)a	0.445

SD in parentheses. *P* values according to Kruskal–Wallis or one-way ANOVA. Different letters after the mean and SD indicate statistically significant differences between the treatments ($P<0.05$). Pooled plantlets (*N*=23). Mean starch concentration (mg g^{-1} of leaf dry mass) of leaves was analyzed from plantlets used in the *L. rugulipennis* experiment only.

Table 3 Mean birch leaf phenolic concentrations (mg g^{-1} dry mass) of differently treated plantlets (treatments as in Table 1) used in the *Lygus rugulipennis* experiment

Phenolic compound	C	PI+	LV+	F−	*P* value
DHPPG	1.82 (0.55)	2.31 (0.86)	1.98 (0.55)	2.09 (0.59)	0.481
Chlorogenic acid	4.34 (1.78)	3.97 (0.82)	3.88 (1.75)	4.33 (1.04)	0.864
Cinnamic acid derivative 1	0.60 (0.30)	0.67 (0.30)	0.63 (0.31)	0.39 (0.30)	0.275
Cinnamic acid derivative 2	1.74 (0.26)	1.94 (0.24)	1.65 (0.47)	1.85 (0.26)	0.291
Cinnamic acid derivative 3	1.01 (0.12)	0.94 (0.19)	0.94 (0.13)	0.93 (0.10)	0.706
Myricetin 3-galactoside	1.70 (0.70)	2.30 (0.95)	2.15 (1.04)	2.41 (1.04)	0.456
Myricitrin	13.03 (4.00)	15.42 (4.98)	15.75 (4.23)	16.70 (3.99)	0.645
Hyperin	0.80 (0.31)	0.97 (0.37)	0.80 (0.43)	1.04 (0.37)	0.456
Avicularin	1.22 (0.59)	1.53 (0.64)	1.35 (0.40)	1.63 (0.54)	0.445
Quercitrin	9.69 (4.09)	9.76 (2.29)	8.83 (2.98)	10.44 (1.46)	0.714
Kaempferol 3-rhamnoside	3.22 (1.05)	2.79 (0.46)	2.58 (0.96)	3.06 (0.80)	0.422
Total	39.15 (9.02)	42.60 (7.70)	40.54 (2.47)	44.88 (7.08)	0.875

SD in parentheses. N=8–9, DHPPG; 3,4′-dihydroxypropiophenone-3-glucoside; P values according to Kruskal–Wallis or one-way ANOVA.

(Fig. 2b). RGR was lowest after feeding on PI+-treated plantlets, but it did not differ significantly from RGR of nymphs that were offered the other treatments (Table 4).

RGR of *C. flava* aphids (N=44) correlated negatively with foliar P (r=−0.401, P<0.001), foliar N (r=−0.307, P=0.001), and root ergosterol concentrations (r=−0.415, P=0.0001). Similarly, the fecundity of aphids on the plantlets decreased when the amount of foliar P (r=−0.386, P<0.001) and Ca (r=−0.331, P<0.001) as well as root ergosterol concentration increased (r=−0.358, P<0.001). Interestingly, increasing avicularin concentration of leaves (r=−0.361, P=0.083, N=24) seemed to be marginally related to the reduced RGR of *L. rugulipennis*, while the RGR of *E. autumnata* correlated positively with leaf N concentration (r=0.409, P=0.006, N=44).

Discussion

Host Plant Quality We hypothesized according to the allocation model of Bennett et al. (2006) that mycorrhizal plants with better nutrient uptake would have more foliar biomass and a higher carrying capacity of herbivores than nonmycorrhizal plants. Our findings do not support this hypothesis. PI+-inoculated plantlets showed reduced foliar biomass, and LV+ inoculation did not significantly affect leaf dry mass. The result that growth responses of host plants may depend on the ectomycorrhizal fungal species was demonstrated earlier with *Pinus sylvestris* (Manninen et al. 1999; Jonsson et al. 2001) and birch (Jonsson et al. 2001) seedlings, and also with host plants having AM fungi (Bennett and Bever 2007). Plantlets in our experiments were young; the initial establishment of ECM on roots of these young plants may have demanded more plant resources then were available, thus, leading to the observed suppressive effects on host plant growth. At a later developmental stage, ECM might become beneficial for the plants. Corrêa et al. (2006) have found that ectomycorrhiza formation had detrimental effects on plant's productivity during the establishment stage, and they suggested that the age of host plants determines whether the impact of mycorrhizas on host plants is negative or not.

According to our second hypothesis, elevated ECM colonization should increase the nutritional quality of foliage by enhancing nutrient uptake. Consequently, depletion of mycorrhizae with fungicide should reduce nutrient uptake. There was some evidence of increased macronutrient concentration in plantlets with enhanced ECM colonization, i.e.,

Table 4 Relative growth rate (mg mg^{-1} day^{-1}) of *Lygus rugulipennis* (N=12), *Calaphis flava* (N=11) and *Epirrata autumnata* (N=12) feeding on birch leaves of differently treated plantlets (treatments explained in Table 1)

Insect species	C	PI+	LV+	F−	*P* value
L. rugulipennis	0.081(0.072)a	0.064(0.064)a	0.075(0.063)a	0.068(0.047)a	0.933
C. flava	0.203(0.092)a	0.138(0.065)a	0.204(0.066)a	0.192(0.042)a	0.092
E. autumnata	0.296(0.145)ab	0.443(0.223)b	0.204(0.259)a	0.342(0.200)ab	0.059

SD in parentheses. P values according to one-way ANOVA. Different letters after the mean and SD indicate statistically significant differences between the treatments according to Tukey's test (P<0.05).

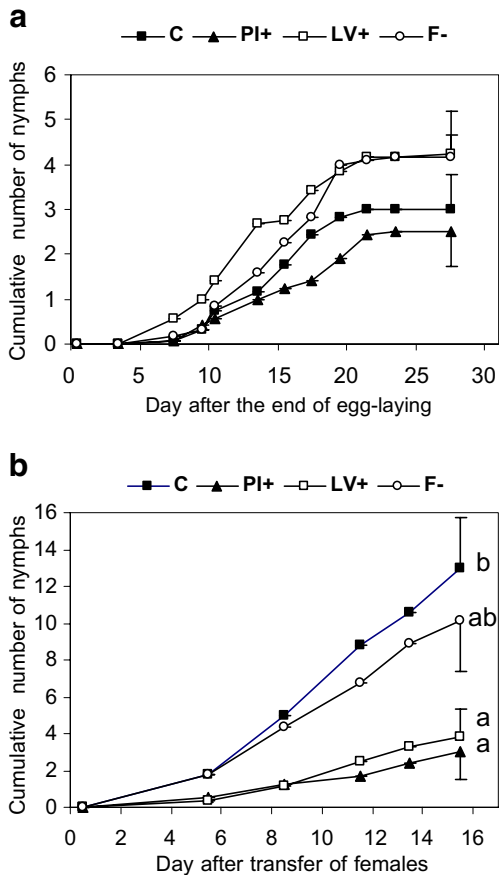

Fig. 2 (**a**) Cumulative number of *Lygus rugulipennis* nymphs hatched on birches in different mycorrhizal treatments (explained in Fig. 1) after the end of oviposition tests. Earlier hatching indicates earlier oviposition preference of females. (**b**) Cumulative number of nymphs produced by *Calaphis flava* females on birches in different mycorrhizal treatments. Standard error bars are shown only at the end of the experiment. Means in **b** followed by different letter are significantly different according to Tukey's test ($P<0.05$)

higher foliar P (PI+-pooled plantlets and LV+ plantlets in *C. flava* experiment) and N (PI+ plantlets in *C. flava* experiment) concentration, but at the same time, PI+ plantlets were considerably smaller than plantlets of other treatments. On the other hand, experimental depletion of ECM-reduced concentration of foliar micronutrient (Ca in *C. flava* experiment), and F− plantlets also had the lowest P concentration in the *C. flava* experiment (see also pooled data, Table 2). These results are in line with an earlier study where ectomycorrhizal pine seedlings had reduced P uptake after fungicide treatment (Manninen et al. 2000). Thus, our results confirm the importance of mycorrhizas in facilitating nutrient uptake even in a relatively early phase of plant development (Smith and Read 1997; Read and Perez-Moreno 2003).

The formation of ECM in roots activates a sequence of gene expression typical to different morphological and developmental stages, such as preinfection and adhesion, and formation of mantle and Hartig net (Le Quere et al. 2005). During mantle and Hartig net development in a birch root system, several genes normally involved in defense responses against pathogenic fungi are upregulated (Le Quere et al. 2005). Thus, it is obvious that the production of defensive plant chemicals in plant tissues and probably also in aboveground parts of the plant may vary in different stages of ECM formation. The plantlets studied in our experiment were young but had well-established ectomycorrhiza at the end of the experiment. There were no treatment effects, however, on major leaf phenolic compounds. Thus, our results did not support the third hypothesis of altered concentration of defense compounds in the foliage of mycorrhizal plants. Possibly, sampling of phenolic compounds at several time points (Peltonen et al. 2005) might have revealed some temporal foliar responses related to the dynamic process of mycorrhizal colonization. Since plant genotype is known to determine silver birch leaf phenolics (Keinänen et al. 1999), future studies of several clones are needed to interpret the relationships between leaf phenolic concentrations and mycorrhizal colonization.

Insect Performance Many herbivorous insect species show preference for fast-growing plant shoots because the capacity of these plants to regrow and maintain herbivore population is better (Price 1991). For example, large Scots pine shoots (Manninen et al. 1998c) have been more attractive to the generalist *L. rugulipennis* as an oviposition site than small ones. Bennett et al. (2006) predicted that plants with well-developed mycorrhizae should produce more foliage through improved plant nutrition, but we were unable to demonstrate this with the relatively young birch plantlets, even though the ectomycorrhiza was already well developed. Chewing larvae of the generalist *E. autumnata* grew better on PI+ plants than on LV+ plants, but PI+ plantlets had significantly reduced leaf dry mass compared to controls. Thus, the result with the chewing generalist was the opposite of that expected. The phloem-feeding specialist aphid *C. flava*, in contrast, behaved according to the "foliar quantity hypothesis" (Bennett et al. 2006): it showed a significantly reduced reproduction rate on PI+ plants. Additionally, the performance of the phloem-feeding generalist, *L. rugulipennis*, was lowest on PI+ plantlets, which had lower leaf dry mass than plantlets of other treatments and also had the lowest root dry mass, thus, supporting our first hypothesis.

Nitrogen is the most important limiting nutrient and can explain insect herbivore performance (Holopainen et al. 1995). In earlier studies, the performance of aphids (Kainulainen et al. 1996; Manninen et al. 1999) and *L. rugulipennis* (Manninen et al. 1998a) has been better on conifer seedlings with high N concentration, but performance

of these herbivores has not been associated with the mycorrhizal status of Scots pine roots as such. In the current study, a poor nitrogen status of LV+ plantlets might explain the reduced reproduction of the specialist *C. flava* aphids. However, the low reproduction rate of *C. flava* on the nitrogen-rich PI+ plants and the negative correlation of aphid RGR with leaf N concentration are not easy to explain. The present results suggest that PI+ treatment of plantlets may have a retarding effect on aphid growth that is not directly related to plant N status but to some other factor, such as the ratio of free amino acids in the phloem of inoculated plantlets. For instance, the ratio of, e.g., glutamine and other amino acids in phloem is an important factor for aphid performance (Karley et al. 2002). This ratio may have changed in the amino acid pool of *P. involutus*-inoculated birch seedlings (Blaudez et al. 1998) in a suboptimal way for this aphid species (Day et al. 2004). On the other hand, RGR of the generalist *E. autumnata* was highest on PI+ plants that had significantly higher N concentration than LV+ plants, and RGR of *E. autumnata* also correlated positively with leaf N in accordance with our second hypothesis. The importance of host plant nitrogen for performance of *E. autumnata* was observed also earlier (Kaitaniemi et al. 1998; Kause et al. 1999).

Many flavonoids are assumed to be associated with insect herbivore performance, both as determinants of a particular feeding choice and as feeding deterrents (Harborne 1991). However, experiments have shown that birch leaf phenolics poorly explain performance (Nurmi et al. 1996; Ossipov et al. 2001) or host plant selection (Peltonen et al. 2006) of insect herbivores. In our experiment, only high avicularin concentration of birch leaves was marginally inversely related to the growth of generalist *L. rugulipennis*, but it was not related to any mycorrhizal treatment. Furthermore, our finding that growth rates of the specialist insect herbivores were not affected by birch leaf phenolics did not support our third hypothesis. In earlier studies larval growth of *E. autumnata* has been reduced by high soluble proanthocyanidin concentration of mountain birch leaves (Kause et al. 1999) and high condensed tannin concentration (Mutikainen et al. 2000), but not by gallotannins (Kause et al. 1999).

The simplistic models that expect insect herbivore performance on ectomycorrhizal plants as a result merely of variation in carbon or nitrogen balance (Jones and Last 1991) or plant biomass (Bennett et al. 2006) were not supported by this study, since insect herbivore response was dependent on the fungal species forming the ectomycorrhiza. Our results indicate further that the interaction between host plant mycorrhizal status and above-ground insect herbivore performance is multifaceted, particularly with respect to the degree of specialization and feeding guild of insects. Thus, a generalization as to the effects of mycorrhizal colonization of plants on insect herbivores is not currently possible. Continuous monitoring of the dynamic balance of nutrients and secondary metabolites in host plant foliage might help us construct more precise mechanistic models for further understanding of the ectomycorrhizal effects on above-ground herbivory.

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References

ALLEN, S. E. 1989. Chemical Analysis of Ecological Materials. Blackwell, Oxford.

BENNETT, A. E., ALERS-GARCIA, J., and BEVER, J. D. 2006. Three-way interactions among mutualistic mycorrhizal fungi, plants, and plant enemies: hypotheses and synthesis. *Am. Nat.* 167:141–152.

BENNETT, A. E., and BEVER, J. D. 2007. Mycorrhizal species differentially alter plant growth and response to herbivory. *Ecology* 88:210–218.

BEZEMER, T. M., and VAN DAM, N. M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.* 20:617–624.

BLAUDEZ, D., CHALOT, M., DIZENGREMEL, P., and BOTTON, B. 1998. Structure and function of the ectomycorrhizal association between *Paxillus involutus* and *Betula pendula* - II. Metabolic changes during mycorrhiza formation. *New Phytol.* 138:543–552.

BOROWICZ, V. A. 1997. A fungal root symbiont modifies plant resistance to an insect herbivore. *Oecologia* 112:534–542.

CORRÊA, A., STRASSER, R. J., and MARTINS-LOUÇÃO, M. A. 2006. Are mycorrhiza always beneficial? *Plant Soil* 279:65–73.

DAUGHTRIDGE, A. D., BOESE, S. R., PALLARDY, S. G., and GARRETT, H. E. 1986. A rapid staining technique for assessment of ectomycorrhizal infection of oak roots. *Can. J. Bot.* 64:1101–1103.

DAY, K. R., ARMOUR, H., and DOCHERTY, M. 2004. Population responses of a conifer-dwelling aphid to seasonal changes in its host. *Ecol. Entomol.* 29:555–565.

GANGE, A. C. 2001. Species-specific responses of a root- and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. *New Phytol.* 150:611–618.

GANGE, A. C., and WEST, H. M. 1994. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytol.* 128:79–87.

GANGE, A. C., STAGG, P. G., and WARD, L. K. 2002. Arbuscular mycorrhizal fungi affect phytophagous insect specialism. *Ecol. Lett.* 5:11–15.

GANGE, A. C., BROWN, V. K., and APLIN, D. M. 2005a. Ecological specificity of arbuscular mycorrhizae: evidence from foliar- and seed-feeding insects. *Ecology* 86:603–611.

GANGE, A. C., GANE, D. R. J., CHEN, Y. L., and GONG, M. Q. 2005b. Dual colonization of *Eucalyptus urophylla* ST Blake by arbuscular and ectomycorrhizal fungi affects levels of insect herbivore attack. *Agric. For. Entomol.* 7:253–263.

GEHRING, C. A., COBB, N. S., and WHITHAM, T. G. 1997. Three-way interactions among ectomycorrhizal mutualists, scale insects and resistant and susceptible pinyon pines. *Am. Nat.* 149:824–841.

HARBORNE, J. B. 1991. Flavonoid pigments, pp. 389–429, in G.A. Rosenthal, and M.R. Berenbaum (eds.). Herbivores: Their Interactions with Secondary Plant Metabolites. Academic Press, New York.

HOLOPAINEN, J. K., RIKALA, R., KAINULAINEN, P., and OKSANEN, J. 1995. Resource partitioning to growth, storage and defence in nitrogen-fertilized Scots pine and susceptibility of the seedlings to the tarnished plant bug *Lygus rugulipennis*. *New Phytol.* 131:521–532.

JOHNSON, N. C., GRAHAM, J. H., and SMITH, F. A. 1997. Functioning of mycorrhizal associations along the mutualism-parasitism continuum. *New Phytol.* 135:575–586.

JONES, C. G., and LAST, F. T. 1991. Ectomycorrhizae and trees: implications for aboveground herbivory, pp. 65–103, in B. Barbosa, V.A. Krischik, and C.G. Jones (eds.). Microbial Mediation of Plant-Herbivore Interactions. Wiley, New York.

JONSSON, L. M., NILSSON, M. -C., WARDLE, D. A., and ZACKRISSON, O. 2001. Context dependent effects of ectomycorrhizal species richness on tree seedling productivity. *Oikos* 93:353–364.

KAINULAINEN, P., HOLOPAINEN, J., PALOMÄKI, V., and HOLOPAINEN, T. 1996. Effects of nitrogen fertilization on secondary chemistry and ectomycorrhizal state of Scots pine seedlings and on growth of grey pine aphid. *J. Chem. Ecol.* 22:617–636.

KAITANIEMI, P., RUOHOMÄKI, K., OSSIPOV, V., HAUKIOJA, E., and PIHLAJA, K. 1998. Delayed induced changes in the biochemical composition of host plant leaves during an insect outbreak. *Oecologia* 116:182–190.

KARLEY, A. J., DOUGLAS, A. E., and PARKER, W. E. 2002. Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. *J. Exp. Biol.* 205:3009–3018.

KAUSE, A., OSSIPOV, V., HAUKIOJA, E., LEMPA, K., HANHIMÄKI, S., and OSSIPOVA, S. 1999. Multiplicity of biochemical factors determining quality of growing birch leaves. *Oecologia* 120:102–112.

KEINÄNEN, M., JULKUNEN-TIITTO, R., MUTIKAINEN, P., WALLS, M., OVASKA, J., and VAPAAVUORI, E. 1999. Trade-offs in phenolic metabolism of silver birch: effects of fertilization, defoliation, and genotype. *Ecology* 80:1970–1986.

LAVOLA, A., JULKUNEN-TIITTO, R., and PÄÄKKÖNEN, E. 1994. Does ozone stress change the primary and secondary metabolites of birch (*Betula pendula* Roth.)? *New Phytol.* 126:637–642.

LE QUERE, A., WRIGHT, D. P., SODERSTROM, B., TUNLID, A., and JOHANSSON, T. 2005. Global patterns of gene regulation associated with the development of ectomycorrhiza between birch (*Betula pendula* Roth.) and *Paxillus involutus* (Batsch) fr. *Mol. Plant Microbe Interact.* 18:659–673.

MANNINEN, A. -M. 1999. Susceptibility of Scots pine seedlings to specialist and generalist insect herbivores – Importance of plant defence and mycorrhizal status. PhD dissertation. University of Kuopio, Finland.

MANNINEN, A. -M., HOLOPAINEN, T., and HOLOPAINEN, J. K. 1998a. Susceptibility of ectomycorrhizal and nonmycorrhizal Scots pine (*Pinus sylvestris*) seedlings to a generalist insect herbivore, *Lygus rugulipennis*, at two nitrogen availability levels. *New Phytol.* 140:55–63.

MANNINEN, A. -M., LAATIKAINEN, T., and HOLOPAINEN, T. 1998b. Condition of Scots pine fine roots and mycorrhiza after fungicide application and low-level ozone exposure in a 2-year field experiment. *Trees* 12:347–355.

MANNINEN, A. -M., VUORINEN, M., and HOLOPAINEN, J. K. 1998c. Variation in growth, chemical defense, and herbivore resistance in Scots pine provenances. *J. Chem. Ecol.* 24:1315–1331.

MANNINEN, A. -M., HOLOPAINEN, T., and HOLOPAINEN, J. K. 1999. Performance of grey pine aphid, *Schizolachnus pineti*, on ectomycorrhizal and nonmycorrhizal Scots pine seedlings at different levels of nitrogen availability. *Entomol. Exp. Appl.* 93:117–120.

MANNINEN, A. -M., HOLOPAINEN, T., and HOLOPAINEN, J. K. 2000. The role of low-level ozone exposure and mycorrhizas in chemical quality and insect herbivore performance on Scots pine seedlings. *Glob. Chang. Biol.* 6:111–121.

MARKKOLA, A. -M. 1996. Resource allocation in ectomycorrhizal symbiosis in Scots pine affected by environmental changes. PhD dissertation. University of Oulu, Finland.

MUTIKAINEN, P., WALLS, M., OVASKA, J., KEINÄNEN, M., JULKUNEN-TIITTO, R., and VAPAAVUORI, E. 2000. Herbivore resistance in *Betula pendula*: effect of fertilization, defoliation, and plant genotype. *Ecology* 81:49–65.

NURMI, K., OSSIPOV, V., HAUKIOJA, E., and PIHLAJA, K. 1996. Variation of total phenolic content and individual low-molecular-weight phenolics in foliage of mountain birch trees (*Betula pubescens* ssp. *tortuosa*). *J. Chem. Ecol.* 22:2023–2040.

NYLUND, J. -E., and WALLANDER, H. 1992. Ergosterol analysis as a means of quantifying mycorrhizal biomass. *Method Microbiol.* 24:77–88.

OSSIPOV, V., HAUKIOJA, E., OSSIPOVA, S., HANHIMÄKI, S., and PIHLAJA, K. 2001. Phenolic and phenolic-related factors as determinants of suitability of mountain birch leaves to an herbivorous insect. *Biochem. Syst. Ecol.* 29:223–240.

PALERMO, B. L., CLANCY, K. M., and KOCH, G. W. 2003. The potential role of ectomycorrhizal fungi in determining Douglas-fir resistance to defoliation by the western spruce budworm (Lepidoptera: Tortricidae). *J. Econ. Entomol.* 96:783–791.

PÄÄKKÖNEN, E., PAASISALO, S., HOLOPAINEN, T., and KÄRENLAMPI, L. 1993. Growth and stomatal responses of birch (*Betula pendula* Roth.) clones to ozone in open-air and chamber fumigations. *New Phytol.* 125:615–623.

PELTONEN, P. A., VAPAAVUORI, E., and JULKUNEN-TIITTO, R. 2005. Accumulation of phenolic compounds in birch leaves is changed by elevated carbon dioxide and ozone. *Glob. Chang. Biol.* 11:1305–1324.

PELTONEN, P. A., VAPAAVUORI, E., JULKUNEN-TIITTO, R., and HOLOPAINEN, J. K. 2006. effects of elevated carbon dioxide and ozone on aphid oviposition preference and birch bud exudate phenolics. *Glob. Chang. Biol.* 12:1670–1679.

PRICE, P. W. 1991. The plant vigor hypothesis and herbivore attack. *Oikos* 62:244–251.

RABIN, L. B., and PACOVSKY, R. S. 1985. Reduced larva growth of two lepidoptera (Noctuidae) on excised leaves of soybean infected with a mycorrhizal fungus. *J. Econ. Entomol.* 78:1358–1363.

READ, D. J., and PEREZ-MORENO, J. 2003. Mycorrhizas and nutrient cycling in ecosystems - a journey towards relevance. *New Phytol.* 157:475–492.

RIESKE, L. K. 2001. Influence of symbiotic fungal colonization on oak seedling growth and suitability for insect herbivory. *Environ. Entomol.* 30:849–854.

SMITH, S. E., and READ, D. J. 1997. Mycorrhizal Symbiosis. Academic, Cambridge.

VAN DER MEIJDEN, E. 1996. Plant defense, an evolutionary dilemma: contrasting effects of (specialist and generalist) herbivores and natural enemies. *Entomol. Exp. Appl.* 80:307–310.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect. Physiol.* 5:229–288.

In plants, AO appears to be located in the cell wall, where it is believed to help regulate cell expansion (Gonzalez-Reyes et al. 1995; Kato and Esaka 2000) and the redox status of the apoplastic space during growth or stress signaling (Pignocchi et al. 2003, 2006; Sanmartin et al. 2003, 2007). In some plants, AO is also induced by herbivory or wounding and has been proposed to act as an antiherbivore defense (Felton and Summers 1993; Bi and Felton 1995; Bi et al. 1997; Garcia-Pineda et al. 2004). For example, AO in soybean leaves was believed to be responsible for the complete loss of ascorbate from the midgut contents of *Helicoverpa zea* caterpillars (Felton and Summers 1993). However, demonstration of a direct impact of foliar AO on insect herbivores has been confounded by the many other changes that occur when plants are stressed or induced by herbivory. For example, levels of essential nutrients, secondary chemicals, and many defensive proteins are all commonly altered in herbivore-damaged leaves (e.g., Duffey and Stout 1996; Karban and Baldwin 1997; Hermsmeier et al. 2001; Major and Constabel 2006). Therefore, in this study, AO-overexpressing transgenic poplar trees were produced in order to perform controlled experiments on the potential role of AO as a plant defense against herbivorous insects.

Tree-feeding caterpillars often ingest high levels of phenolic compounds, and an ascorbate-based antioxidant system is central to the defense of caterpillars against the oxidation of these phenolics caused by the high pH of the midgut (Felton and Duffey 1992; Barbehenn et al. 2001). In the absence of sufficient ascorbate in the midgut contents, phenolic oxidation produces elevated levels of reactive oxygen species (ROS), including semiquinone radicals, peroxides, and hydroxyl radicals (Barbehenn et al. 2003, 2005a,b). The production of ROS is associated with oxidative damage to nutrients in the gut lumen and oxidative stress in gut tissues (Summers and Felton 1994; Barbehenn et al. 2005a). Thus, AO might act synergistically with phenolics to increase their pro-oxidant effects on herbivores. Oxidative stress in the midgut lumen was examined with electron paramagnetic resonance (EPR) spectrometry to measure changes in free radical levels. The potential for synergism between AO and phenolics was examined by comparing semiquinone radical levels in larvae that fed on either low or high AO leaves coated with tannins. This work tested the hypothesis that elevated foliar AO activity (1) decreases levels of reduced ascorbate in the midgut contents of *Lymantria dispar* L. caterpillars, (2) produces oxidative stress in the gut lumen, and (3) decreases insect performance. To increase the generality of our results, the potential impact of AO-overexpressing poplar on ascorbate levels in the acidic guts of *Melanoplus sanguinipes* (Fabricius) grasshoppers was also examined.

Methods and Materials

Transgenic Poplars Hybrid poplar was chosen for this study because it is an acceptable host plant for *L. dispar*, is easily transformed, grows rapidly, and has been widely studied as a model tree in plant molecular biology and in studies of insect–plant interactions. AO-overexpressing poplars were produced by introducing the cucumber AO gene into *Populus tremula*×*Populus alba* (INRA 717I-B4) via *Agrobacterium*-mediated transformation using the method of Leplé et al. (1992). The complete cucumber AO-coding sequence (GenBank accession number J04494), including the signal peptide sequence, was inserted into the binary plasmid pGA643 (An et al. 1988) between the cauliflower mosaic virus 35S promoter and the T7-5 terminator, giving rise to pGA-CAO, as described previously (Sanmartin et al. 2003). Putative transformed shoots were first tested by rooting ability on kanamycin, and the presence of the transgene was confirmed by using polymerase chain reaction. Independently transformed plantlets were assayed for AO activity as described below, and plants with the highest AO levels were selected and moved to a greenhouse. The high expression of the AO transgene and the size of the AO transcript were confirmed in greenhouse-grown plants by Northern analysis by using a fragment of the cucumber AO gene as a probe (Sambrook and Russell 2001). Plantlets were propogated *in vitro* or as green cuttings and rooted in potting mix under high humidity in a mist chamber. Of the more than 10 independent transformants, three lines that showed high AO activities (designated genotype AO1, AO2, and AO3) were selected for further analysis. Untransformed parental stock plants served as the control (wild type) genotype. Seedlings were shipped from the University of Victoria to the University of Michigan.

Saplings were grown in a greenhouse in 8-l "Treepots" (Hummert International, Earth City, MO, USA) in Sunshine potting mix (Type 4), with Osmocote slow-release fertilizer (14–14–14) (Scotts, Marysville, OH, USA) and watered as needed. Growth lights with 400 W high-pressure sodium bulbs (PL Light Systems; Beamsville, ON, Canada) were used from September to May (L16/D8). Saplings were grown to a height of ≥1 m over a period of 3–4 mo before their use in experiments. Leaves were numbered sequentially from the shoot apex using the leaf plastochron index (LPI; Larson and Isebrands 1971). The first leaf lamina with a length greater than 2 cm was defined as LPI 0. Leaves for all experiments were excised with a sterile razor blade from LPI 10–20 from at least three trees of each genotype. Two to three leaves per day from each tree were excised, beginning with the upper leaves. After washing for 20 min in a water bath, leaf surfaces were blotted dry with paper towels and allowed to air dry with their petioles placed in tubes of water. Leaf disks were cut with a cork

borer (23 mm diameter), and all disks within each genotype were mixed to randomize potential effects of leaf position and individual tree.

Insects L. dispar is a polyphagous tree-feeding species that has been used widely as a model herbivore in studies of plant–insect interactions. It has a mildly acidic to neutral foregut (pH 5.2–7.2) and a basic midgut ranging from pH 8.0 to 11.0 (Schultz and Lechowicz 1986; Appel and Maines 1995). Eggs of *L. dispar* were obtained from the US Department of Agriculture (Otis Air Force Base, Massachusetts, USA). Third- and fourth-instar larvae were reared on an artificial diet prepared as described previously (Barbehenn et al. 2001), with the exceptions that linseed oil was used instead of wheat germ oil and sodium alginate (2.6%) was added to the dry ingredients. Larvae were reared in Petri dishes in incubators at either 18°C or 23°C to regulate developmental rates. Newly molted larvae (containing empty midguts) were switched from the rearing diet to an experimental food.

M. sanguinipes is a polyphagous grasshopper that normally feeds on a wide variety of forbs and grasses but will readily accept hybrid poplar leaves. It was chosen for comparison with *L. dispar* because it has both an acidic foregut (pH 5.5) and midgut (pH 6.8; Barbehenn et al. 1996), which could permit high AO reaction rates. Eggs of a non-diapause strain were obtained from the US Department of Agriculture (Sidney, MT, USA). Nymphs were reared on romaine lettuce and wheat bran in a wire mesh cage with an adjacent 100 W light bulb on a L16/D8 photoperiod.

Chemical Analyses Ascorbate oxidase was analyzed in leaf disks that were stored frozen (−80°C) in airtight plastic bags. Samples for analysis were collected on five dates across 2 mo during feeding experiments on first and fourth instars. Groups of three frozen disks were weighed quickly and ground with a chilled pestle and mortar in a mixture of 1.0 ml of pH 5.6 sodium phosphate buffer (0.1 M) containing 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mg polyvinyl polypyrolidone, and approximately 15 mg sand. Leaf homogenates were centrifuged for 3 min (4°C; 8,000×*g*), and the supernatant solutions were used immediately for AO analysis. One-milliliter reaction mixtures contained 960 µl of pH 5.6 buffer, 20 µl of ascorbate in buffer (37.5 mM), and 20 µl of the supernatant solution. The AO reaction was measured as the rate of decrease in absorbance at 290 nm. An extinction coefficient of 2.8 mM^{-1} cm^{-1} was used to express change in absorbance units on a molar basis (Yoshimura et al. 1998). Boiled control supernatant solutions showed no AO activity. Comparisons of AO activity at pH 5.6 and 10 (70 mM carbonate–bicarbonate buffer) were made by using fresh leaf disks from each of three trees per AO genotype (LPI 15). Rates of ascorbate autoxidation at pH 10 were subtracted from the overall oxidation rates to measure the enzymatic component. AO rates were also compared by using commercial AO prepared from a *Cucurbita* species (Calbiochem, San Diego, CA, USA). AO activities provided by the manufacturer were 325 U/mg solid or 1,625 U/mg protein, where 1 U was defined as the amount of enzyme that will oxidize 1.0 µmol of ascorbic acid per minute at 30°C (pH 5.6). The reaction mixtures were prepared as described above with the exception that the pH 10 solution was prepared by adjusting the phosphate buffer with sodium hydroxide.

Ascorbate in each genotype was measured in the same collections of leaf disks used for measuring AO activity. Samples (approximately 100 mg) were weighed quickly and ground in liquid nitrogen to avoid artifacts caused by thawing. Frozen ground samples were transferred to 15-ml plug-seal cap centrifuge tubes and extracted in 1.0 ml 5% metaphosphoric acid (containing 1 mM EDTA). Following a 30-min extraction with continuous mixing, samples were centrifuged (1,000×*g*, 4°C, 5 min), the supernatant solutions removed, and the extraction repeated. Supernatant solutions were pooled within samples, mixed, and stored frozen (−80°C) until analysis with high performance liquid chromatography (HPLC; Barbehenn 2003).

To examine the impact of ingested AO on ascorbate levels in caterpillars, ascorbate was measured in the midgut contents of fourth instars that fed for 2 d on leaf disks from one of the four genotypes. Midgut contents were dissected (N=11–13 replicate larvae/genotype), dispersed in tared centrifuge tubes containing 300 µl of 5% metaphosphoric acid, and weighed to the nearest 0.1 mg. Ascorbate was measured with HPLC as described above.

Ascorbate was measured in adult *M. sanguinipes* after they fed on either a mixture of leaf disks from control genotype trees (N=15 insects) or a mixture of leaf disks from all three AO-overexpressing genotypes (N=15 insects). Grasshoppers were placed in individual containers in a 26°C incubator and were starved overnight to clear their guts and stimulate rapid feeding. Insects were dissected soon after they had eaten one or more leaf disks to ensure that their foreguts and midguts were full. Sample collection and ascorbate analysis were done as described above, with the exception that the foregut and midgut contents were extracted in 400 and 250 µl of metaphosphoric acid, respectively. Ascorbate was measured in a haphazard subset of the collected samples (N=8–10 per genotype and site), since this was sufficient to determine the effects of AO on ascorbate levels in *M. sanguinipes*.

Oxidative Stress in the Midgut To test the hypothesis that AO-overexpressing leaves have a synergistic effect on the oxidation of phenolics, leaf disks from the control genotype

and AO2 (LPI 13 and 14) were prepared as described above and treated with 20-µl aliquots of a tannin solution, producing an increase of 7.5% dry weight of pedunculagin and 7.5% pentagalloyl glucose. Fourth instars (N=9) fed on the leaf disks for 18–22 hr (18°C), following which the levels of semiquinone radicals in midgut contents, were measured with EPR spectrometry. Sample preparation and EPR analyses were performed as described previously (Barbehenn et al. 2005a). Briefly, midgut contents were dissected, weighed in a tared centrifuge tubes containing 300 µl of pH 10 carbonate buffer (containing 10% dimethylsulfoxide), dispersed, and then centrifuged. Supernatant solutions (200 µl) were placed directly in a flat cell in the EPR sample cavity, and the resulting first derivative spectra were integrated with WinEPR software. Radical levels were calculated in the midgut fluid volumes (approximately 90% of the mass) using 2,2,6,6-tetramethyl-1-piperidinyloxy radical standards. A synergistic effect would be indicated by the presence of higher semiquinone concentrations in the midgut contents of larvae that fed on the AO-overexpressing genotypes. At the time of dissection, all larvae were found to have produced dark frass pellets and to have full midguts, indicating that they fed well on the treated disks.

The potential for commercial AO (Calbiochem; purified from *Cucurbita* sp.) to produce oxidative stress in caterpillars was examined by measuring free radicals in the midgut contents of fourth instars that fed on AO-treated leaf disks from the control genotype. AO was suspended in 50% acetone (containing 5.7 mg/ml sucrose) at 0.33 mg/ml (low level) and 1.6 mg/ml (high level). Leaves were cut and prepared from LPI 10–20 as described above. Leaf disks were treated with 20-µl aliquots of either the high or low AO suspensions or the AO-free control solution. Based on a recovery of AO activity from treated leaves of 33% (±2%, N=6), we calculated that the AO activity of control genotype leaves was increased by fourfold (low level) or 20-fold (high level). Newly molted larvae were placed at random on each of the three treatment groups and fed poplar leaf disks for a 2-d period. Freshly treated disks were provided daily, and a moist filter paper was placed in each plastic cup to maintain leaf turgidity. Larvae were fed fresh disks prior to examination of their gut contents on the third day with EPR spectrometry. Between eight and 10 larvae were examined in each treatment group. This experiment was repeated 1 wk later with a different set of saplings.

Larval Performance To confirm that the performance of *L. dispar* larvae is affected by the absence of ascorbic acid from their food, performance was measured in fourth instars on artificial diet containing either 2.5% dry weight ascorbic acid or diet containing no ascorbic acid. The ascorbate-free diet contained an additional 2.5% DW cellulose instead of ascorbate. Newly molted fourth instars were assigned at random to the two diets. Larvae were fed with freshly prepared diets daily and kept in individual 35-ml plastic cups in a 23°C incubator (L16/D8). Upon molting to the fifth instar, larvae were frozen and then dried to determine their final weights. Initial larval dry weights were determined by using a fresh weight/dry weight ratio from newly molted fourth instars. Frass was collected and weighed after drying to completion (70°C). Previous work found no effects of ascorbate concentration on the approximate digestibility of an artificial diet (Lindroth and Weiss 1994), and therefore, frass production was measured to compare the amounts of each diet consumed. Relative egestion rate (RER) was defined as milligram frass per milligram initial body mass per day. Relative growth rate (RGR) was measured as milligram growth per milligram initial body mass per day. RER and RGR were measured on a dry mass basis.

The performance of first instars was measured on the control and transgenic genotypes. Newly hatched larvae were randomly assigned to each genotype (N=15 larvae/genotype). Larval fresh weights were measured to the nearest 0.01 mg on a Cahn 25 Electobalance (Cahn Instruments, Cerritos, CA, USA) and converted to dry weights based on fresh weight/dry weight ratios of five killed larvae. Larvae were kept in individual plastic cups, as described above. Leaf disks were prepared daily, also as described above. A moist filter paper was placed in each cup to maintain the water content of the leaf disks. To estimate the amount consumed by each larva, frass pellets were collected and dried daily (70°C) during the first instar. Upon molting to the second instar, larvae were frozen, dried, and weighed.

This experiment was repeated using newly molted fourth instars. To measure consumption, fresh weights of leaf disks fed to each larva were recorded, and fresh weight/dry weight ratios of representative leaf disks (N=5) from each genotype were measured daily to convert food fresh weights to dry weights. The difference between the dry weight of the food and the dry weight of the uneaten remains was defined as the amount consumed. Approximate digestibility (AD) was measured as (mg ingested−mg egested)/mg ingested× 100. Efficiency of conversion of digested matter to body mass (ECD) was measured as mg growth/(mg ingested − mg egested)×100. Efficiency of conversion of ingested matter to body mass (ECI) was measured as mg growth/mg ingested× 100 (Waldbauer 1968).

Third-instar *L. dispar* were used to compare larval performance on the intact plants of each genotype. The third instar was chosen to ensure that sufficient leaf material was available to complete this developmental stage. Newly molted larvae were weighed initially and placed at random on separate leaves in fine nylon mesh bags, which were tied

around the petioles. Trees were maintained in the greenhouse (16-hr photoperiod), and pots were rotated daily to provide even exposure of light and heat to all larvae. Five larvae were bagged (one larva per leaf) on four trees per genotype, using leaf LPI 12 through 16. Less than 5% of each leaf was eaten over a 4-d period. Frass was recovered from each bag at the end of the third instar and dried at 70°C. Larvae were frozen upon molting to the fourth instar and then dried to determine their final weights. Larval fresh weights at the beginning of the experiment were converted to dry weights using fresh weight/dry weight ratios from six representative larvae. RER was measured in lieu of RCR.

Statistical Analysis AO activity and ascorbate levels in the four genotypes were compared with one-way analysis of variance (ANOVA; SAS 2003). The normality of residuals was tested with Proc Univariate. Pairwise comparisons between genotypes were made by differences of least squares means (P=0.05 for *a priori* comparisons). To test the hypothesis that the control genotype differed from the three AO genotypes, these two groups were compared with contrasts (weighted as −3, 1, 1, 1) (Proc Mixed). Ascorbate concentrations in midgut fluids of larvae were compared across genotypes and by contrasts, as described above. Ascorbate levels in the foreguts and midguts of *M. sanguinipes* were compared with two-way ANOVA, using plant genotype and gut site as main effects. Ascorbyl radical levels in *L. dispar* fed AO-treated leaf disks were compared across treatment levels with one-way ANOVA. Comparison between levels of semiquinone radicals in *L. dispar* fed control genotype foliage treated with tannins and AO2 foliage treated with tannins were made with the Kruskal–Wallis test (Wilkinson 2000).

RCR, RER, RGR, and nutritional indices (AD, ECD, and ECI) were compared across artificial diets or tree genotypes with analysis of covariance (ANCOVA; Proc Mixed). Main effects used in all models were ascorbate level or tree genotype, respectively. ANCOVA models used to compare insect performance across poplar genotypes were as follows: RGRs were compared with growth rate as the dependent variable and initial dry mass as the covariate. RERs and RCRs were compared across treatments or genotypes with egestion rate or consumption rate as the dependent variable and initial dry mass as the covariate. Developmental times and growth were compared with one-way ANOVA (diet experiment) or ANOVA with contrasts (poplar experiments). ADs were compared by using frass production as the dependent variable and ingested mass as the covariate. ECDs were compared by using growth as the dependent variable and mass digested as the covariate. ECIs were compared with growth as the dependent variable and mass ingested as the covariate. In all cases, models testing for a significant interaction between the dependent variable and covariate were first tested to confirm that the regression slopes were parallel. To test the hypothesis that larvae on the control genotype differed from larvae on the three AO genotypes, these two groups were compared with contrasts, as described above. Where necessary, log or square root transformations were used to normalize residuals, and where indicated, statistical outliers were removed. Data that could not be transformed to meet the assumptions of ANOVA were analyzed with Kruskal–Wallis tests (Wilkinson 2000). Individual insects were used as replicates in all experiments.

Results

Transgenic poplars overexpressing the cucumber AO gene were produced by *Agrobacterium*-mediated transformation, and lines (genotypes) were selected based on transcript accumulation (Fig. 1) and elevated AO activity. AO activities (measured at pH 5.6) were increased by 14- to 37-fold in the transgenic genotypes compared with the control (wild type) genotype (Table 1). Sapling growth rates and leaf morphology showed no obvious differences between AO-overexpressing and untransformed lines when grown to a height of approximately 2 m. However, ascorbate levels in the AO-overexpressing genotypes were significantly lower by 19–54%, compared with the control genotype (contrast P=0.005; Table 1). Thus, AO activity was negatively correlated with ascorbate levels in the poplars (R^2=0.883; Fig. 2). However, when AO activity in AO-overexpressing leaves was measured in pH 10.0 reaction mixtures (representative of many caterpillar midguts), it was reduced to 9% of the activity at pH 5.6 (representative of caterpillar and grasshopper foreguts). Similarly, the activity of purified cucumber AO at pH 10.0 was only 5% of its activity at pH 5.6.

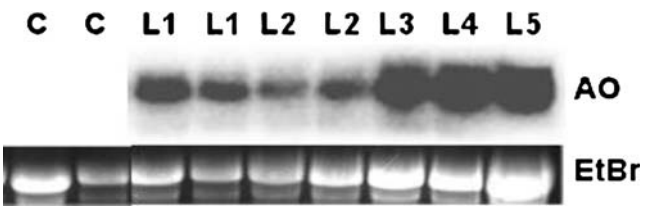

Fig. 1 Northern blot comparing expression of cucumber AO in control (wild type) and transgenic poplar. The blot was probed with a ^{32}P-labeled fragment of the AO-coding sequence. Ethidium bromide-stained ribosomal RNA bands (*EtBr*) are shown as loading controls. *C* refers to untransformed control lines and *L1–L5* represent confirmed AO-overexpressing lines. Lanes with different designations are from independently transformed lines, and lanes sharing the same designation represent separate individuals from the same line. *L1*, *L4*, and *L5* were used in subsequent experiments and were designated genotypes AO1, AO2 and AO3, respectively. No AO RNA bands were visible in the control lines

Table 1 Ascorbate oxidase (AO) activity and ascorbate levels in control (wild type) and transgenic poplar genotypes

Genotype	Ascorbate oxidase (μmol min^{-1} g FW^{-1})	Ascorbate (μmol/g FW)
Control	17.1±3.3a	6.8±0.6c
AO1	276.5±73.3b	5.5±0.5bc
AO2	642.5±67.2c	3.1±0.8a
AO3	250.3±76.8b	4.4±0.8ab
Significance of effects		
Genotype	$P<0.001$	$P=0.008$
Contrast	$P<0.001$	$P=0.005$

Samples of each genotype were analyzed from five dates for ascorbate oxidase and seven dates for ascorbate.

When *L. dispar* larvae consumed leaves from the control or AO-overexpressing genotypes, ascorbate levels did not differ in their midgut contents when contrasted between both types (Fig. 3; $P=0.480$). Thus, AO activity in the control and AO-overexpressing genotypes was not associated with larval ascorbate levels ($R^2=-0.112$). Regardless of the genotype ingested, ascorbate in the midgut contents dropped to only 7.0% (±0.5) of foliar ascorbate levels. Similarly, in *M. sanguinipes*, there were no significant differences between ascorbate levels in the foreguts of adults on control or AO leaf disks (3.0±0.7 and 3.2±0.7 mM, respectively) or in the midguts of adults on control or AO leaf disks (0.16±0.03 and 0.16±0.04 mM, respectively; genotype effect $P=0.818$). Foreguts contained more ascorbate than midguts (gut site effect, $P<0.001$), but there was no significant genotype×gut site interaction ($P=0.830$).

Contrary to expectation, *L. dispar* that fed on AO leaf disks coated with tannins did not have significantly higher levels of oxidative stress than larvae that fed on control genotype leaf disks treated with tannins. Semiquinone

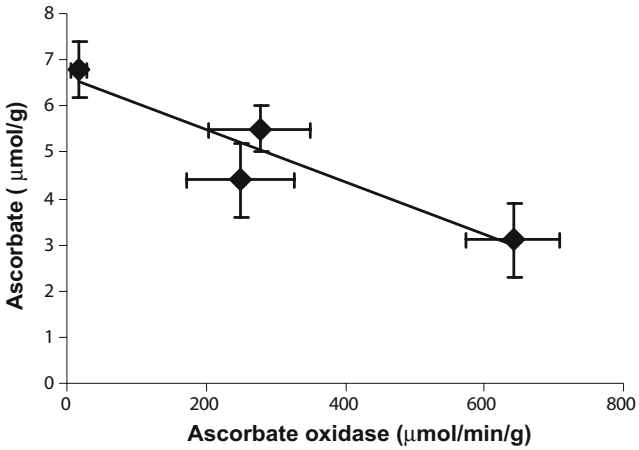

Fig. 2 Negative association between AO activity and ascorbate levels in control and AO-overexpressing poplar leaves ($R^2=0.883$). Standard error bars are presented

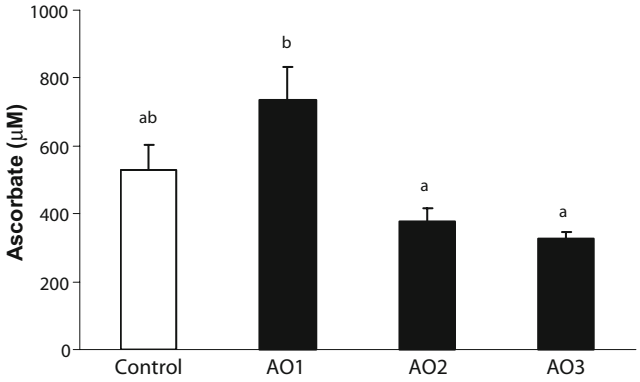

Fig. 3 Ascorbate concentrations in the midgut fluids of fourth-instar *Lymantria dispar* larvae after feeding on control or AO poplar genotype leaf disks. No significant differences were observed between larvae that fed on AO genotypes compared to the control genotype (contrast $P=0.480$)

radical concentrations in the midgut contents were 10.6±1.5 and 8.9±1.7 μM in larvae on the AO transgenic and control genotype foliage, respectively ($P=0.233$). The consumption of AO-coated leaf disks also had no significant effect on oxidative stress in the midgut; similar levels of ascorbyl radicals were present in the midgut across AO treatment levels in the first experiment (229–293 nM; $P=0.644$) and in the second experiment (119–147 nM; $P=0.831$). A similar experiment performed on final-instar *Orgyia leucostigma* larvae also showed a lack of effect from feeding on AO-treated leaf disks ($P=0.570$; unpublished data). No further work was performed on the potential effects of elevated AO levels in transgenic poplar on oxidative stress, since there was no effect of elevated AO levels on ascorbate or phenolic oxidation in *L. dispar* larvae.

When *L. dispar* larvae consumed artificial diet that lacked ascorbate, their performance was significantly decreased: weight gain decreased, developmental time increased, and RGR decreased (Table 2). Relative egestion rate was also decreased in larvae on the ascorbate-free diet, potentially explaining their decrease in performance. However, an analysis of larval growth rate adjusted for frass production (ANCOVA; Fig. 4) demonstrated that the effect of ascorbate was primarily post-ingestive. For any given level of frass production (directly proportional to consumption), larvae on the ascorbate-containing diet had higher growth rates than did larvae on the ascorbate-free diet.

Relative egestion rates did not differ between first-instar caterpillars on the control genotype and the AO-overexpressing genotypes (contrast $P=0.161$; Table 3), suggesting that consumption rates did not differ as a result of AO overexpression. Relative growth rates of first-instar caterpillars also did not differ significantly between larvae on the control genotype and the AO-overexpressing genotypes (contrast $P=0.515$). Similarly, third instars that fed in mesh bags on intact saplings also showed no effect of the high

Table 2 Effects of ascorbic acid on fourth-instar *Lymantria dispar* performance on artificial diets

Diet	Relative egestion rate	Growth (mg)	Development time (days)	Relative growth rate	*N*
+Ascorbic acid	0.881±0.159b	16.1±3.2b	5.1±0.8a	0.349±0.072b	14
−Ascorbic acid	0.548±0.156a	9.6±0.8a	7.0±0.4b	0.143±0.068a	15
Significance of effects					
Diet	$P<0.001$	$P<0.001$	$P=0.004$	$P<0.001$	

Larvae were fed artificial diets. Comparisons of RER and RGR were made using ANCOVA, with initial dry weight as the covariate.
RCR=mg ingested/mg initial body mass/day; RGR=mg growth/mg initial body mass/day

AO activities in the transgenic genotypes on their RER (contrast $P=0.411$) or RGR (contrast $P=0.760$; Table 4). It was assumed that AO activities were unaffected by larval feeding, since no evidence of AO induction by caterpillars has been observed in hybrid poplar (Major and Constabel 2006; Ralph et al. 2006). Finally, the consumption and growth rates and nutritional indices (AD, ECD, and ECI) of fourth instars did not differ when contrasted between the control and AO-overexpressing genotypes (contrast P's$\geq$ 0.690; Table 5). Unlike larvae on an artificial diet without ascorbate, larval growth and developmental times were not significantly altered in *L. dispar* on AO-overexpressing genotypes (data not shown).

Discussion

Contrary to our hypothesis, elevated AO levels in ingested leaf tissues did not compromise the ascorbate-based antioxidant defenses in the midgut lumen of *L. dispar*. As a result, no synergistic interactions were found between AO and tannins on oxidative stress levels in the midguts of *L. dispar*. Neither ingested AO purified from *Cucurbita* or overexpressed in poplar were able to change ascorbate or ascorbyl free radical levels in the midguts of *L. dispar*. Similarly, consumption of AO-overexpressing poplar leaves by the grasshopper *M. sanguinipes* had no impact on levels of ascorbate along the length of its gut. It is noteworthy that ascorbate levels in the guts of *M. sanguinipes* that fed on wheat leaves (Barbehenn 2003) were similar to levels measured in this study on poplar, suggesting that poplar does not have an unusual impact on the post-ingestive fate of ascorbate. The large drop in ascorbate levels observed in the midguts of *L. dispar* on poplar are presumed to be the result of ascorbate oxidation by non-AO reactions, since such decreases in ascorbate concentration can also occur in the midguts of caterpillars feeding on an artificial diet (Barbehenn et al. 2001).

Larval performance was unaffected by high levels of AO, consistent with the inability of ingested AO to oxidize ascorbate in the midgut contents of the tested caterpillars. The results of our artificial diet study suggest that, had AO greatly decreased ascorbate levels available to *L. dispar* larvae, performance would have been decreased as a result of post-ingestive effects on growth rates (Horton and Redak 1993; cf. Lindroth and Weiss 1994). The lack of effect of high levels of AO on midgut ascorbate levels, oxidative stress, or performance calls into question how AO might function more effectively in a wide variety of other plant–insect interactions.

The low levels of oxygen present in the guts of caterpillars and grasshoppers may limit post-ingestive AO

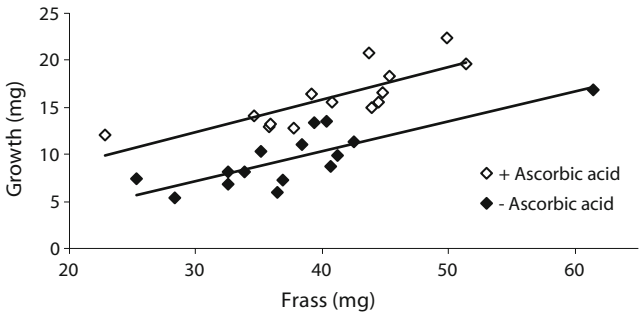

Fig. 4 Effect of ascorbate on growth of fourth-instar *Lymantria dispar* larvae on artificial diets. Previous work showed no effect of ascorbate on the digestibility of the diet, so frass was measured as an estimate of ingestion. In this case, ANCOVA on growth using frass as a covariate provides a comparison of food utilization efficiencies analogous to comparing ECI (efficiency of conversion of ingested matter to body mass)

Table 3 Effects of AO transgenic poplars on the performance of first-instar *Lymantria dispar* on leaf disks

Genotype	Relative egestion rate	Relative growth rate	*N*
Control	1.98±0.09a	0.742±0.043b	14
AO1	2.17±0.08b	0.729±0.036b	14
AO2	1.83±0.07a	0.626±0.032a	13
AO3	2.26±0.05b	0.781±0.026b	14
Significance of effects			
Genotype	$P<0.001$	$P=0.015$	
Contrast[a]	$P=0.161$	$P=0.515$	

Comparisons between genotypes were made using ANCOVA with initial dry weight as the covariate.
RER=mg frass/mg initial body mass/day; RGR=mg growth/mg initial body mass/day
[a] Contrast between control genotype and three AO genotypes

Table 4 Effects of AO transgenic poplars on the performance of third-instar *Lymantria dispar* on intact tree leaves (contrast between control genotype and three AO genotypes)

Genotype	Relative Egestion Rate	Relative Growth Rate	*N*
Control	1.39±0.06a	0.536±0.027a	20
AO1	1.40±0.07a	0.548±0.029a	20
AO2	1.31±0.05a	0.547±0.019a	20
AO3	1.47±0.08a	0.544±0.031a	20
Significance of effects			
Genotype	P=0.307	P=0.975	
Contrast [a]	P=0.411	P=0.760	

Larvae were placed individually in mesh bags on tree leaves. All comparisons were made using ANCOVA.
RER=mg frass/mg initial body mass/day; RGR=mg growth/mg initial body mass/day

activity in these herbivores. The K_m of AO for oxygen is 0.5 mM (Strothkamp and Dawson 1978), while midgut oxygen levels in caterpillars are commonly on the order of 0–0.01 mM, and the foregut and midgut of *M. sanguinipes* have lower oxygen levels than are found in most caterpillars (Johnson and Barbehenn 2000). The large foregut of *L. dispar* has a relatively high oxygen level compared with the foreguts of other caterpillars (approximately 0.06 mM in larvae on artificial diet). However, our results showed that any potential AO activity in the foregut had an inconsequential effect on the levels of ascorbate available to *L. dispar* for absorption or antioxidant defense of the midgut. Other taxa of insect herbivores that have been examined to a more limited extent, including a beetle (Coleoptera) and several grasshoppers, also have nearly anoxic gut contents (Johnson and Barbehenn 2000; Krishnan et al. 2007).

A second factor that also may limit AO activity in the midguts of caterpillars is high pH. At the extremely basic pH typical of caterpillar midguts (e.g., pH 10), rates of ascorbate oxidation by AO from transgenic poplar and *Cucurbita maxima* were reduced to less than 10% of their rates at an acidic pH (Maccarrone et al. 1993; this study). However, the lack of extensive AO activity in the acidic foreguts of either *L. dispar* or *M. sanguinipes* suggests that low oxygen level may be the main factor limiting the oxidation of ascorbate by ingested AO.

If post-ingestive conditions limit AO activity, pre-ingestive AO activity would be a plausible alternative mode of action. Neither oxygen nor high pH would limit AO activity at a herbivore-damaged leaf edge or during ingestion. However, if pre-ingestive AO activity were an important defense mechanism, one might expect greater treatment effects than were observed in this study. No AO effects on the performance of *L. dispar* were observed for neonate and fourth instars on leaf disks or for third instars on intact saplings.

Since high AO levels in the AO-overexpressing genotypes were not able to eliminate ascorbate in the midgut in either of the species tested in this study, our conclusions differ substantially from previous work on the potential role of AO as an inducible plant defense (Felton and Summers 1993). Previous work on induced plants did not isolate the effects of AO from those of other enzymes and non-enzymatic defenses (e.g., Bi et al. 1997). It is possible that effects ascribed to AO previously, such as the loss of ascorbate from the midgut contents of *H. zea* larvae, were produced by other enzymes that can function in the caterpillar midgut, such as peroxidases (unpublished data). It is also unclear to what extent the damage caused to nutrients by AO activity *in vitro* in oxygenated solutions is relevant to the low-oxygen conditions found *in vivo*. Finally, measurement of AO activity in the gut contents of caterpillars presents a quandary, since assays for AO are done at ambient oxygen levels, but AO in the gut is present at nearly anoxic levels. Presumably, the low levels of AO activity measured in the gut lumen and regurgitate of *H. zea* would be even lower or negligible in the low-oxygen conditions found *in vivo*.

Table 5 Effects of AO-overexpressing poplars on fourth-instar *Lymantria dispar* performance

Genotype	RCR	RGR	AD	ECD	ECI	*N*
Control	2.15±0.05a	0.373±0.012b	37.1±0.6a	46.8±0.9b	17.3±0.2b	11
AO1	2.10±0.08a	0.401±0.015c	37.1±0.9a	52.2±1.6c	19.1±0.3c	12
AO2	2.17±0.10a	0.354±0.017b	38.2±0.6a	42.9±1.0a	16.3±0.3a	10
AO3	1.93±0.06a	0.317±0.013a	36.9±0.6a	44.4±1.1ab	16.3±0.3a	12
Significance of effects						
Genotype	P=0.072	P<0.001	P=0.147	P<0.001	P<0.001	
Contrast[a]	P=0.945	P=0.783	P=0.834	P=0.745	P=0.690	

Larvae were fed leaf disks. All comparisons were made using ANCOVA, with the exception of RCR, which was analyzed with ANOVA after log transformation of these data.
RCR=mg ingested/mg initial body mass/day. RGR=mg growth/mg initial body mass/day. AD=(mg ingested−mg egested)/mg ingested×100. ECD=mg growth/(mg ingested−mg egested)×100. ECI=mg growth/mg ingested×100
[a] Contrast between control genotype and three AO genotypes

A variety of ecological functions of induced AO have been examined recently, including responses to stresses such as wounding, pathogens, salinity, and heat (Bi et al. 1997; Sanmartin et al. 2003, 2007; Yamamoto et al. 2005; Fotopoulos et al. 2006; Pignocchi et al. 2006). AO activity in damaged or infected leaf areas would potentially increase the effectiveness of oxidative defenses against microbes that gain entry via insect-feeding sites (e.g., Harris et al. 1980). The formation of ROS is part of the antimicrobial response in plants (Bolwell and Wojtaszek 1997). With decreased ascorbate levels, oxidases such as polyphenol oxidase and peroxidase would have increased net rates of production of quinones and other ROS. Therefore, it may be difficult to distinguish between anti-pathogen and anti-herbivore reactions when plant stress responses such as AO are induced.

In summary, the lack of effect of elevated levels of AO in poplar on ascorbate or ascorbyl radical levels in the midgut contents of *L. dispar* is consistent with the lack of effect of elevated levels of AO on larval performance. The lack of effect of AO on ascorbate levels in the grasshopper *M. sanguinipes* suggests that AO activity is also limited in the guts of insects with acidic pH. As in previous work on polyphenol oxidase-overexpressing poplars (Barbehenn et al. 2007), we conclude that low oxygen levels appear to limit the effectiveness of these oxygen-dependant enzymes as post-ingestive defenses against a variety of leaf-chewing insects.

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References

An, G., Ebert, P. R., Mitra, A., and Ha, S. B. 1988. Binary vectors, pp. 1–19, in S. B. Gelvin, and R. A. Schilperoort (eds.). Plant Molecular Biology Manual. Kluwer, Dordrecht, The Netherlands.

Appel, H. M., and Maines, L. W. 1995. The influence of host plant on gut conditions of gypsy moth (*Lymantria dispar*) caterpillars. *J. Insect Physiol.* 41:241–246.

Barbehenn, R. V. 2003. Antioxidants in grasshoppers: higher levels defend the midgut tissues of a polyphagous species than a graminivorous species. *J. Chem. Ecol.* 29:683–702.

Barbehenn, R. V., Martin, M. M., and Hagerman, A. E. 1996. Reassessment of the roles of the peritrophic envelope and hydrolysis in protecting polyphagous grasshoppers from ingested hydrolyzable tannins. *J. Chem. Ecol.* 22:1901–1919.

Barbehenn, R. V., Bumgarner, S. L., Roosen, E., and Martin, M. M. 2001. Antioxidant defenses in caterpillars: role of the ascorbate recycling system in the midgut lumen. *J. Insect Physiol.* 47:349–357.

Barbehenn, R. V., Poopat, U., and Spencer, B. 2003. Semiquinone and ascorbyl radicals in the gut fluids of caterpillars measured with EPR spectrometry. *Insect Biochem. Mol. Biol.* 33:125–130.

Barbehenn, R. V., Cheek, S., Gasperut, A., Lister, E., and Maben, R. 2005a. Phenolic compounds in red oak and sugar maple leaves have prooxidant activities in the midguts of *Malacosoma disstria* and *Orgyia leucostigma* caterpillars. *J. Chem. Ecol.* 31:969–988.

Barbehenn, R., Dodick, T., Poopat, U., and Spencer, B. 2005b. Fenton-type reactions and iron concentrations in the midgut fluids of tree-feeding caterpillars. *Arch. Insect Biochem. Physiol.* 60:32–43.

Barbehenn, R. V., Jones, C. P., Yip, L., Tran, L., and Constabel, C. P. 2007. Limited impact of elevated levels of polyphenol oxidase on tree-feeding caterpillars: assessing individual plant defenses with transgenic poplar. *Oecologia* 154:129–140.

Bi, J. L., and Felton, G. W. 1995. Foliar oxidative stress and insect herbivory: primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21:1511–1530.

Bi, J. L., Murphy, J. B., and Felton, G. W. 1997. Antinutritive and oxidative components as mechanisms of induced resistance in cotton. *J. Chem. Ecol.* 23:97–117.

Bolwell, G. P., and Wojtaszek, P. 1997. Mechanisms for the generation of reactive oxygen species in plant defence—a broad perspective. *Physiol. Mol. Plant Pathol.* 51:347–366.

Duffey, S. S., and Stout, M. J. 1996. Antinutritive and toxic components of plant defense against insects. *Arch. Insect Biochem. Physiol.* 32:3–37.

Felton, G. W., and Duffey, S. S. 1992. Ascorbate oxidation reduction in *Helicoverpa zea* as a scavenging system against dietary oxidants. *Arch. Insect Biochem. Physiol.* 19:27–37.

Felton, G. W., and Summers, C. B. 1993. Potential role of ascorbate oxidase as a plant defense protein against insect herbivory. *J. Chem. Ecol.* 19:1553–1568.

Fotopoulos, V., Sanmartin, M., and Kanellis, A. K. 2006. Effect of ascorbate oxidase over-expression on ascorbate recycling gene expression in response to agents imposing oxidative stress. *J. Exp. Bot.* 57:3933–3943.

Garcia-Pineda, E., Castro-Mercado, E., and Lozoya-Gloria, E. 2004. Gene expression and enzyme activity of pepper (*Capsicum annuum* L.) ascorbate oxidase during elicitor and wounding stress. *Plant Sci.* 166:237–243.

Gonzalez-Reyes, J. A., Alcain, F. J., Caler, J. A., Serrano, A., Cordoba, F., and Navas, P. 1995. Stimulation of onion root elongation by ascorbate and ascorbate free radical in *Allium cepa* L. *Protoplasma* 184:31–35.

Harris, M. D., Brewer, J. W., and Merril, L. D. 1980. Insect involvement in the transmission of bacterial pathogens, pp. 201–292, in K. F. Harris, and K. Maramorosch (eds.). Vectors of Plant Pathogens. Academic, New York, USA.

Hermsmeier, D., Schittko, U., and Baldwin, I. T. 2001. Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and it natural host *Nicotiana attenuata*. I. Large-scale changes in the accumulation of growth- and defense-related plant mRNAs. *Plant Physiol.* 125:683–700.

Horton, D. R., and Redak, R. A. 1993. Further comments on analysis of covariance in insect dietary studies. *Entomol. Exp. Appl.* 69:263–275.

Johnson, K. S., and Barbehenn, R. V. 2000. Oxygen levels in the gut lumens of herbivorous insects. *J. Insect Physiol.* 46:897–903.

Karban, R., and Baldwin, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, IL, USA.

Kato, N., and Esaka, M. 2000. Expansion of transgenic tobacco protoplasts expressing pumpkin ascorbate oxidase is more rapid than that of wild-type protoplasts. *Planta* 210:1018–1022.

KRISHNAN, N., KODRIK, D., TURANLI, F., and SEHNAL, F. 2007. Stage-specific distribution of oxidative radicals and antioxidant enzymes in the midgut of *Leptinotarsa decemlineata*. *J. Insect Physiol.* 53:67–74.

LARSON, P. R., and ISEBRANDS, J. G. 1971. The plastochron index as applied to developmental studies of cottonwood. *Can. J. For. Res.* 1:1–11.

LEPLÉ, J. C., BRASILEIRO, A. C. M., MICHEL, M. F., DELMOTTE, F., and JOUANIN, L. 1992. Transgenic poplars: expression of chimeric genes using four different constructs. *Plant Cell Rep* 11:137–141.

LINDROTH, R. L., and WEISS, A. P. 1994. Effects of ascorbic acid deficiencies on larvae of *Lymantria dispar* (Lepidoptera: Lymantriidae). *Great Lakes Entomol.* 27:169–174.

MACCARRONE, M., D'ANDREA, G., SALUCCI, M. L., AVIGLIANO, L., and FINAZZI-AGRO, A. 1993. Temperature, pH and UV irradiation effects on ascorbate oxidase. *Phytochemistry* 32: 795–798.

MAJOR, I. T., and CONSTABEL, C. P. 2006. Molecular analysis of poplar defense against herbivory. Comparison of wound- and insect elicitor-induced gene expression. *New Phytol.* 172:617–635.

PIGNOCCHI, C., FLETCHER, J. M., WILKINSON, J. E., BARNES, J. D., and FOYER, C. H. 2003. The function of ascorbate oxidase in tobacco. *Plant Physiol.* 132:1631–1641.

PIGNOCCHI, C., KIDDLE, G., HERNANDEZ, I., FOSTER, S. J., ASENSI, A., TAYBI, T., BARNES, J., and FOYER, C. H. 2006. Ascorbate oxidase-dependent changes in the redox state of the apoplast modulate gene transcript accumulation leading to modified hormone signaling and orchestration of defense processes in tobacco. *Plant Physiol.* 141:423–435.

RALPH, S., ODDY, C., COOPER, D. et al. 2006. Genomics of hybrid poplar (*Populus trichocarpa* × *deltoides*) interacting with forest tent caterpillars (*Malacosoma disstria*): normalized and full-length cDNA libraries, expressed sequence tags, and cDNA microarray for the study of insect-induced defences in poplar. *Mol. Ecol.* 15:1275–1297.

SAMBROOK, J., and RUSSELL, D. W. 2001. Molecular Cloning: A Laboratory Manual. 3rd edn.Cold Spring Harbor Press, Cold Spring Harbor, NY, USA.

SANMARTIN, M., DROGOUDI, P. D., LYONS, T., PATERAKI, I., BARNES, J., and KANELLIS, A. K. 2003. Over-expression of ascorbate oxidase in the apoplast of trangenic tobacco results in altered ascorbate and glutathione redox states and increased sensitivity to ozone. *Planta* 216:918–928.

SANMARTIN, M., PATERAKI, I., CHATZOPOULOU, F., and KANELLIS, A. K. 2007. Differential expression of the ascorbate oxidase multigene family during fruit development and in response to stress. *Planta* 225:873–885.

SAS Institute. 2003. The SAS system for Windows. Version 9.1. SAS Institute, Cary, NC, USA.

SCHULTZ, J. C., and LECHOWICZ, M. J. 1986. Hostplant, larval age, and feeding behavior influence midgut pH in the gypsy moth (*Lymantria dispar*). *Oecologia* 71:133–137.

STROTHKAMP, R. E., and DAWSON, C. R. 1978. A kinetic study of the effects of hydrogen peroxide and pH on ascorbate oxidase. *Biochem. Biophys. Res. Comm.* 85:655–661.

SUMMERS, C. B., and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuiidae): potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Mol. Biol.* 24:943–953.

YAMAMOTO, A., BHUIYAN, M. N. H., WADITEE, R., TANAKA, Y., ESAKA, M. et al. 2005. Suppressed expression of the apoplastic ascorbate oxidase gene increases salt tolerance in tobacco and *Arabidopsis* plants. *J. Exp. Bot.* 56:1785–1796.

YOSHIMURA, K., ISHIKAWA, T., NAKAMURA, Y., TAMOI, M., TAKEDA, T. et al. 1998. Comparative study on recombinant chloroplastic and cytosolic ascorbate peroxidase isozymes of spinach. *Arch. Biochem. Biophys.* 353:55–63.

WALDBAUER, G. P. 1968. The consumption and utilization of food by insects. *Adv. Insect Physiol.* 5:229–289.

WILKINSON, L. 2000. SYSTAT: The system for statistics. SYSTAT, Evanston, IL, USA.

response to root feeders (Gange and Brown 1989; Masters et al. 1993), or the induction of secondary metabolites (Bezemer et al. 2003, 2004). However, these studies focused on tissue feeding root herbivores. In contrast, nematodes such as the gall-forming *Meloidogyne* spp. act as a metabolic sink that directs nutrients to the site of nematode parasitism (Burgeson 1966; McClure 1977).

Secondary plant compounds often have a negative effect on non-adapted herbivores (Karban and Baldwin 1997). Cotton (*Gossypium* spp.) produces a variety of terpenoids that exhibit toxicity to a wide range of herbivores and pathogens (Stipanovic et al. 1990; Bezemer et al. 2004; Wang and Plhak 2004; Xu et al. 2006). Gossypol, a triterpene aldehyde, is found in the roots, foliage, and seed of the cotton plant. Additionally, the terpenoids desoxy-hemigossypol and hemigossypol are present in the roots. Further, so-called gossypol-like terpenoid compounds such as hemigossypolone, heliocide H1, heliocide H2, heliocide H3, and heliocide H4 are found in cotton leaves, flower buds, and seeds. The latter compounds are called heliocides because of their toxicity to *Heliothis* (Nazarova et al. 1981).

Plants may also feature indirect induced defenses that enhance the performance of herbivore natural enemies, rather than acting on herbivores directly. These inducible mechanisms include the production of extrafloral nectar that leads to the recruitment of sugar-seeking carnivores (Wäckers et al. 2001), and the emission of plant volatile chemicals in response to herbivore feeding and egg deposition that serve as signals that allow natural enemies to locate prey (reviews in Röse and Tumlinson 2004; Turlings and Wäckers 2004; Rasmann et al. 2005). In cotton, *Gossypium hirsutum* L. (Malvaceae), much is known about constitutive and induced defenses (Wäckers et al. 2002; Röse and Tumlinson 2004; Anderson and Agrell 2005). Many of the herbivore-induced volatiles involved in cotton defenses have been identified and characterized, including the timing and location of release, and tissue-specific and herbivore-specific release (reviews in Röse and Tumlinson 2004). Recent studies also have shown that nitrogen levels in cotton affect the release of these volatile attractants (Olson et al., unpublished).

Most of the work on below- and aboveground interactions has focused on tissue-feeding herbivores. However, van Dam et al. (2005) found that the migratory endoparasitic root lesion nematode species, *Pratylenchus penetrans* Cobb (Tylenchida: Pratylenchidae), reduced the quality of *Brassica nigra* L. Koch (Brassicaceae) for the larval shoot herbivore species, *Delia radicum* L. (Diptera: Anthomyiidae). This reduction was attributed to enhanced production of phenolics and glucosinolates in shoot tissues following root and shoot feeding. Mateille (1994) reviewed the relatively sparse literature on the mechanisms of plant defense in response to migratory root nematode species and generalized the reactions of hormonal metabolism that produce toxins into those that: (1) are directly toxic to the nematode, (2) regulate defense reactions, and (3) modify tissue development.

In this study, we looked at the sedentary, gall-forming root-knot nematode species, *Meloidogyne incognita* (Kofoid and White) Chitwood. Root-knot nematodes are sedentary endoparasites that retard growth and development of cotton by parasitizing the root system, causing galling, stunting, and other adverse effects (Khoshkhoo et al. 1993). Root-knot nematode parasitism reduces the rate of photosynthesis in plants (Melakeberhan et al. 1987). Although parasitism reduces the rate of nitrogen uptake by roots (Vaast et al. 1998), plants accumulate nitrogen in the parasitized root tissues (Hunter 1958; Shafiee and Jenkins 1963; Burgeson 1966). The effects of root-knot nematode parasitism on the nitrogen content in shoots are not consistent with the parasitism that was reported to increase the percentage nitrogen (Melakeberhan et al. 1985), to have no effect (Hunter 1958; Shafiee and Jenkins 1963), or to decrease the percentage (Mohanty et al. 1990).

In this study, we evaluated whether a root-parasitic nematode (*M. incognita*) and a foliar-feeding insect (*Helicoverpa* (=*Heliothis*) *zea* (Boddie)) interact to affect the direct and indirect defenses of cotton. Our specific objectives were to determine if:

- foliage feeding, root feeding, or their combination affects attraction of the parasitic wasp *Microplitis croceipes* Cresson (Hymenoptera: Braconidae) to cotton plants. The braconid wasp is a solitary and specialized larval parasitoid of *Heliothis* and *Helicoverpa* species;
- terpenoids are induced in cotton plants (whole plant, leaf and root tissue) through foliage feeding, root feeding, or their combination;
- commonly induced volatiles from aboveground herbivore damaged cotton are affected by belowground parasitism by the nematode *M. incognita;*
- the nitrogen level in cotton leaves or petioles is affected by *M. incognita* root parasitism.

Methods and Materials

Insects Corn earworm, *Helicoverpa zea*, were reared at 28° C, 50–70% (RH), and a 16:8 hr L/D photoperiod, as described by Burton (1969). *Microplitis croceipes* were reared on *H. zea* larvae according to the method of Lewis and Burton (1970). *Meloidogyne incognita* inoculum was collected from tomato roots (*Lycopersicon esculentum* Mill. cv. Rutgers) by agitating roots in 0.5% sodium hypochlorite solution for 2 min (Hussey and Barker 1973) approximately 1 hr before inoculation.

Plants Deltapine 5690 cotton plants (*G. hirsutum* L.) were grown in Promix® for 3 wk and transferred to pots (15 cm diameter) with sterilized soil, Tifton sandy loam with 82% sand, 11% clay, 7% silt, and less than 1% organic matter. One gram of ammonium nitrate crystals was added to the soil at transplant and watered with 100 ml of water. Plants were watered as needed over the next 6 wk. For the volatile collections, a single third instar of *H. zea* was placed on each of three mid-level leaves per plant and clipped to the leaf for 2 d with a 23.76-cm^2 cage, with the connecting inner sides of the cage lined with foam to prevent additional leaf damage. The outsides were covered with screen mesh. After the volatile collections, larvae were replaced on the plant for an additional day for the flight tunnel bioassays and the plant tissue analyses. Cages were used to standardize the level of aboveground herbivory: approximately 75% of the leaf area in the cage was consumed by the time of the air sample collection, and approximately 85% of the leaf tissue was consumed at the time of the flight tunnel bioassays and plant tissue collection regardless of treatment. For those treatments that included nematode parasitism, inoculum of 20,000 *M. incognita* race three eggs/pot (approximately 800 eggs/150 cm^3 soil) was distributed into two holes (approximately 2.5 cm deep) and covered with soil when plants were 3 wk old. Pots were watered immediately following inoculation.

When plants were 9-wk-old, they were excised above the point of damage so that four to five leaves remained. Plant tops were placed in a 125-ml glass flask filled with water for the wind tunnel bioassays (below). Prior to the bioassay, a single mature leaf and an immature leaf above the damaged leaves, and a root sample were excised from each plant for the analyses of gossypol and gossypol-like compounds. Nematode damage was classified on a scale of 1 to 10 based on the percentage of the root surface with galls (0=no galling, 1=1–10% of the root surface galled, 2=11–20% of the root surface galled, etc., with 10=91–100% of the root surface galled).

Volatile Collections After 48 hr of herbivore feeding, 9-wk-old plants were placed under a modified 9-l Kimax beaker with the bottom removed and a glass top with a 6.2-mm outlet added. The top 30 cm of each plant was placed within the beaker. This area included the damaged part of the plant. Thus, plant volatiles were collected from the damaged and undamaged parts of the plant after removal of the larvae. At the bottom of the beaker, the plant main stem was guillotined by two Teflon plate halves with a hole in the center, and glass wool was used to fill in space around the main stem. Zero air was introduced into the beaker and adjusted with a panel-mount flow meter (Gilmont GF 6341–1115) to 2.0 l/min. Sample air was pumped out of the beaker from the top with a vacuum pump (Barnant, Model 420-1901) and adjusted to 400 ml/min with a mass flow controller (Aalborg Model AFC2600D). Sampled air was pulled through 1,000 mg of desiccant, followed by 100 mg of SuperQ (Supelco) adsorbent material to trap volatile compounds. Volatiles were trapped on the SuperQ absorbent material for 3 hr for a total of 36 l of air through the trap. Air samples were collected from 12:00 to 3:00 P.M. from a total of 16 plants (four plants per treatment). Collections from plants damaged for 48 hr were made from two treatment plants per day over 8 d. Each treatment was randomly assigned to collection chamber and day of collection. Two additional collections were made without plants as controls for non-plant contamination.

Volatile Extraction and Analyses Adsorbent tubes were eluted with 10 ml of methylene chloride. Eluents were concentrated under a stream of N_2 gas to approximately 0.1 ml. Extracts were then transferred to 0.25-ml glass inserts inside 2-ml autosampler vials. Extract volume was determined by weight. As internal standard, 1 µg methyl palmitate (Sigma-Aldrich, St. Louis, MO, USA) was added to each extract.

GC/MS A ThermoFinnigan DSQII (San Jose, CA, USA) gas chromatograph-quadrupole mass spectrometer was used. The oven was fitted with a 30 × 0.25 mm (i.d.) DB-5 fused silica capillary column (Agilent, Santa Clara, CA, USA). The liquid film thickness of the column was 0.25 µm. Helium carrier gas flow was maintained 1.0 ml min^{-1}. The initial oven temperature (40°C) was held for 1 min. Temperature was then increased to 220°C at 4°C min^{-1} and held for 4 min. Splitless injections were made by using an autosampler with the injection port temperature held at 220°C. Ionization was by electron impact (70 eV) with full scans (m/z^+=33 to 300) made every 0.5 sec. Prior to use, the mass spectrometer was tuned to meet performance criteria for perfluorotributylamine. Authentic standards of a mixture of (α)- and (β)-ocimene (International Flavors and Fragrances, New York, NY, USA), which we refer to as ocimene 1 and ocimene 2, trans-(β)-farnesene (ChromaDex, Irvine, CA, USA), linalool (TCI America, Portland, OR, USA), and indole (Spectrum Chemical MFG CORP., Gardena, CA, USA) were analyzed. Tentative structural assignments for (*E*)-4, 8-dimethyl-1, 3, 7-nonatriene and (*E, E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene were based on comparison to published mass spectra (Maurer et al. 1986). Tentative assignments for (*Z*)-3-hexenyl acetate and (α)-farnesene were made by comparison to the NIST/EPA/NIH Mass Spectral library version 2.0 (NIST, Gaithersburg, MD). Quantification of all compounds was by electronic integration of the base peaks of their mass spectra. The one exception was linalool. In this case, a secondary ion, m/z^+=

93, was used. This was done since linalool was found to co-elute with *n*-undecane, a compound present in all extracts. This ion was prominent in the mass spectrum of linalool (60% of the base peak) as it is in mass spectra of most terpenoids, and it is generally not detected (<0.1% of the total ion current [TIC] of *n*-alkanes such as *n*-undecane). The *n*-undecane contribution to "linalool-undecane" peaks in total ion current (TIC) chromatograms shown in the text was substracted by computing the relative contribution of $m/z^+=93$ to the TIC in the spectrum of our linalool standard and then adjusting the TIC for the peak accordingly.

Nitrogren Analyses A set of eight plants with treatments similar to those used in the bioassays had the leaf and petiole removed from the fifth mature leaf (above the damaged leaves) from the top of the plant. Percent nitrogen was calculated on a per gram weight basis of dried plant material.

Wind Tunnel and General Bioassay Procedure The wind tunnel used in the experiments was described by Drost et al. (1988). All flight responses were tested at 26–28°C, a wind speed of 55 cm/sec, and a light intensity of 2,000 lx. Female *M. croceipes* were mated, fed, and were 3-d-old at the time of the experiments. Each female was allowed to antennate frass of *H. zea* larvae fed on pinto bean diet for 10 sec, given a single sting on a third-instar pinto bean-fed *H. zea* larva, and placed immediately in the wind tunnel 80 cm downwind of the dual choice tests. The dual choices were: 1) control (no damage) vs. *H. zea* damaged plants; 2) control vs. *M. incognita* damaged plants; 3) *H. zea* plus *M. incognita* damaged plants vs. *M. incognita* damaged plants; and 4) *H. zea* plus *M. incognita* damaged plants vs. *H. zea* damaged plants. Each female was used only once, and plants were switched in position after each female tested to minimize potential variance from any side of wind tunnel preferences. Females were given three opportunities to fly upwind and land on a plant. Individuals that failed to do so within 5 min were recorded as no choice. A total of 20 females was tested per dual choice with five females tested per day over a total of 4 d. Fresh plants (9-wk-old) that had 3 d of feeding damage were used each day. Days and/or plants served as replication.

Plant Tissue Analysis All leaf and root material was freeze-dried, ground, and extracted individually for terpenoid analysis. Plant tissues were collected from a total of eight plants per treatment. The methods of sample preparation and HPLC analyses were as described by Bezemer et al. (2004).

Statistics *Chi*-square analyses were used to test the effect of treatment on female parasitoid choice, and analysis of variance (ANOVA) was used to test for effect of treatment on gossypol and gossypol-like compound levels, percentage nitrogen in leaf and petiole samples, and the induced volatiles (i.e., induced terpenes, (*Z*)-3-hexenyl acetate, and indole) (SAS Institute 1998). We log-transformed quantities of volatile terpenes and (*Z*)-3-hexenyl acetate (Levene's test $P>0.117$). No transformation stabilized the variance for indole quantities, but we report the results of the untransformed data.

Results

Microplitis croceipes females chose the corn earworm damaged plants more often than the undamaged control plants (Fig. 1a, $\chi^2=8.89$, $df=1$, $P<0.003$) or the root-knot damaged plants (Fig. 1b, $\chi^2=7.12$, $df=1$, $P<0.008$). There were no significant differences in female choices between

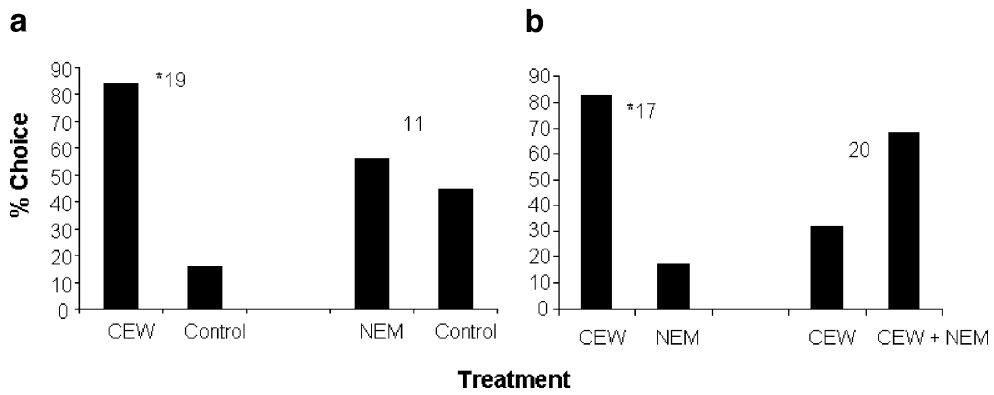

Fig. 1 Percentage flight and landing response to choice of plants damaged by (**a**) corn earworm (*CEW*) and root-knot nematodes (*NEM*) vs. undamaged (*Control*), or plants damaged by (**b**) root-knot nematodes vs. corn earworm, and root-knot nematodes and corn earworm vs. corn earworm only. *Asterisks* indicate statistical significance at $P<0.05$. Numbers *above the bars* are number of females making choices out of a total of 20 females tested

the root-knot nematode damaged and control plants (Fig. 1a; χ^2=0.09, *df*=1, *P*=0.763), nor between plants damaged by corn earworm and those damaged by corn earworm and root-knot nematode (Fig. 1b, χ^2=2.57, *df*=1, *P*=0.108). Root gall ratings of plants damaged by root-knot nematodes varied between 4 and 8 on a 0-to-10 scale (mean ± SD = 6.5±1.2), indicating moderately severe to severe damage.

There were no significant differences in the nitrogen levels from the leaf and petiole samples across treatments (Table 1). Furthermore, root gossypol levels did not differ across treatments (Fig. 2, $F_{3,\ 28}$=0.52, *P*=0.669). Hemigossypolone ($F_{3,\ 28}$=0.02, *P*=0.995), gossypol ($F_{3,\ 28}$=0.27, *P*=0.844), heliocide H1+4 ($F_{3,\ 28}$=0.15, *P*=0.928), heliocide H2 ($F_{3,\ 28}$=0.11, *P*=0.954), and heliocide H3 ($F_{3,\ 28}$=0.23, *P*=0.877) levels in mature leaves and hemigossypolone ($F_{3,\ 28}$=0.44, *P*=0.723), gossypol ($F_{3,\ 27}$=0.44, *P*=0.723), heliocide H1 + 4 ($F_{3,\ 28}$=0.14, *P*=0.142), heliocide H2 ($F_{3,\ 28}$=0.16, *P*=0.922), and heliocide H3 ($F_{3,\ 28}$=0.13, *P*=0.940) levels in immature leaves also did not differ significantly across treatments (Fig. 2).

There was an effect of plant treatment on the release of volatile terpenes ($F_{3,\ 10}$=24.71, *P*<0.001). Plants damaged by corn earworm only and corn earworm plus nematodes had a higher release of volatile terpenes than those plants damaged by nematodes only and undamaged plants (Fig. 3, Table 2). These terpenes were ocimene 1, ocimene 2, linalool, (β)-farnesene, (α)-farnesene, (*E*)-4, 8-dimethyl-1, 3, 7-nonatriene, and (*E, E*)-4, 8, 12-trimethyl-1, 3, 7, 11-tridecatetraene. Plants damaged by nematodes had chemical profiles for volatile terpenes similar to control plants (Fig. 3, Table 2). Chemical analyses of the headspace of "non-plant collections" indicated no contamination within the system.

Plant treatment affected the release of (*Z*)-3-hexenyl acetate ($F_{3,\ 10}$=14.86, *P*<0.001). Plants damaged by corn earworm plus nematodes had significantly higher release rates of (*Z*)-3-hexenyl acetate than those plants damaged by nematodes only, corn earworm (CEW) only, and undamaged plants (Fig. 3, Table 2).

Plants damaged by corn earworm and corn earworm plus nematodes had a higher release of indole than those plants damaged by nematodes only and undamaged plants (Fig. 3, Table 2). Plants damaged by nematodes showed levels of release of indole similar to control plants (Fig. 3, Table 2).

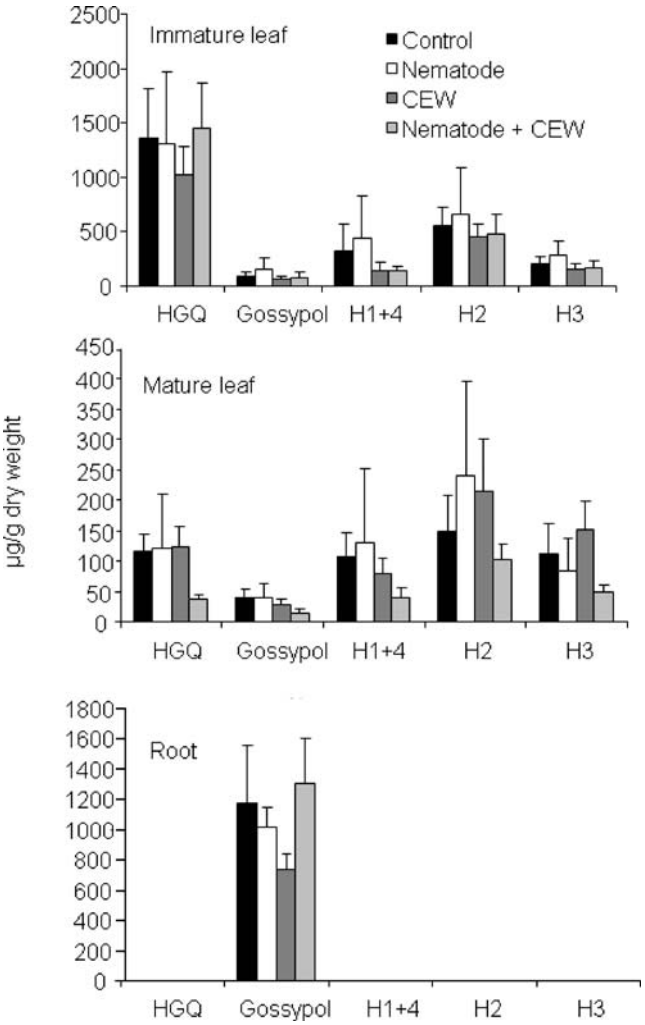

Fig. 2 Mean ± SE µg g^{-1} dry weight hemigossypolone (*HGQ*), gossypol, heliocide 1 plus heliocide 4 (*H1+H4*), heliocide 2 (*H2*), and heliocide 3 (*H3*) in immature leaves, mature leaves and roots of *Gossypium hirsutum* plants not damaged (Control), damaged by root-knot nematodes (Nematode), damaged by corn earworm larvae (CEW), and damaged by root-knot nematodes and CEW larvae (Nematode + CEW). *N*=8

Table 1 Nitrogen (mean percentage of dry weight ± SD) in leaf and petiole samples of the fifth mature leaf from the top of a cotton plant (9 wk after planting)

Treatment	Leaf (% *N*)	Petiole (% *N*)
CEW	3.10±0.11	1.33±0.18
CEW + NEM	2.91±0.20	1.30±0.29
NEM	2.81±0.29	1.21±0.17
CON	2.96±0.24	1.08±0.09

N=8.
CEW corn earworm leaf damage, *CEW + NEM* corn earworm and root-knot nematode damage, *NEM* root-knot nematode damage, *CON* no damage

Discussion

Bezemer et al. (2003) found increased levels of gossypol and gossypol-like compounds in immature leaves of *Gossypium herbaceum* (L.) in response to feeding damage

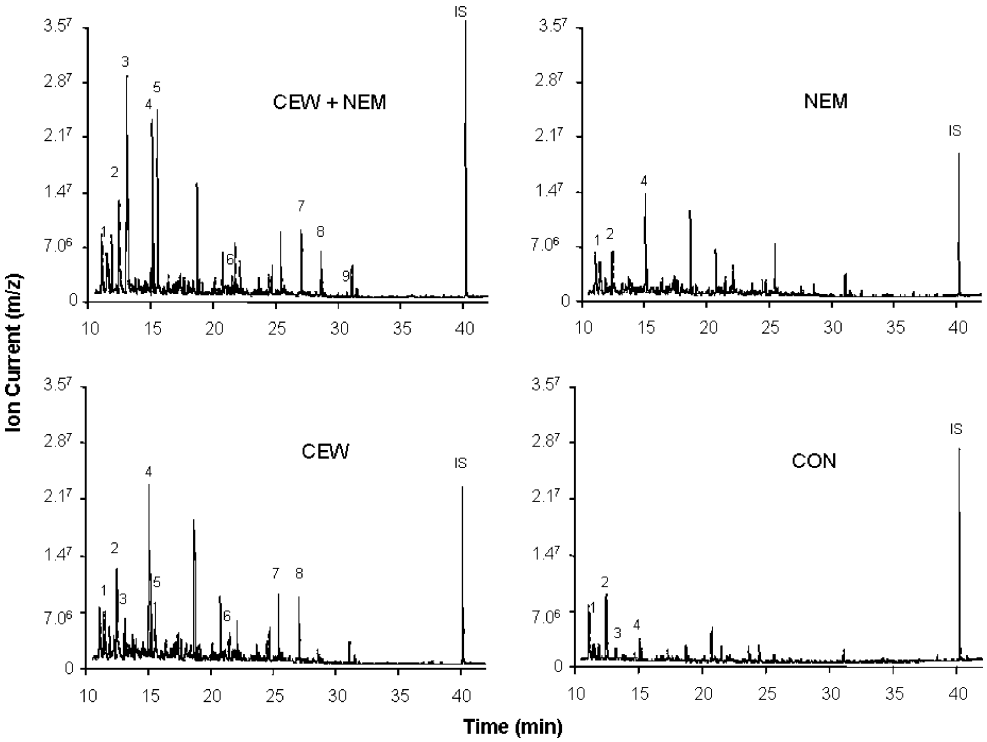

Fig. 3 Total ion current chromatograms of induced volatiles from the upper 30 cm of a cotton (*Gossypium hirsutum*) plant damaged by corn earworm (*Heliocoverpa zea*=CEW), root-knot nematode (*Meloidogyne incognita*=NEM), their combination (= CEW + NEM) and from control (undamaged = CON) plants collected from 12 p.m. to 3 p.m. Compounds: 1) (*Z*)-3-hexenyl acetate 2) ocimene 1, 3) ocimene 2, 4) linalool, 5) (*E*)-4, 8-dimethyl-1,2,7-nonatriene, 6) indole 7) (*β*)-farnesene, 8) (*α*)-farnesene, and 9) (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene. Added internal standard was (IS) methyl palmitate. All unnumbered peaks represent unidentified compounds. *N*=4 plants per treatment

by the foliage feeder, *Spodoptera exigua* (Hübner), as well as in mature and immature leaves in response to the root feeder, *Agriotes lineatus* (L.). In contrast, we found no effect of foliage or root herbivory on induction of hemigossypolone and the heliocides 1–4 in the immature and mature leaves of cotton. Several studies have shown induction of these compounds in the immature leaves of cotton in response to foliage herbivory (McAuslane et al. 1997; McAuslane and Alborn 1998; Bezemer et al. 2004). In these studies, the cotton plants had been damaged by *Spodoptera* spp. for 48 hr, but terpenoid analyses were conducted 1–5 wk later. It is possible that the cotton plants in our study did not have sufficient time (analyses done after 72 hr) to induce the terpenoid aldehydes in the leaves and roots of leaf-damaged plants. There also may be differences in the expression of terpenoids among cotton varieties; one of the above studies did not report the cotton variety used, and the others used varieties different from the one we used. Alternatively, it is possible that damage by corn earworms, a predominately fruit-feeding species, does not have the same effect on levels of gossypol and gossypol-like compounds as feeding by *Spodoptera* spp., which are predominately leaf feeders. The lack of induction of these compounds in response to the nematodes is unlikely to be explained by insufficient induction time or limited infestation, as the plant roots had been exposed to the nematodes for 5 wk, and the level of nematode infestation was high.

Several studies have shown that concentrations of gossypol and gossypol-like compounds in the roots of cotton are enhanced following belowground feeding by root knot nematodes and wireworms, or infection by bacterial or fungal diseases (Mace et al. 1990; Khoshkoo et al. 1993; Zhang et al. 1993; Howell et al. 2000; Bezemer et al. 2003, 2004). Increased gossypol levels were detected in the roots of cotton following *M. incognita* damage that used supple-

Table 2 Mean ± SEM ion current (m/z^+) from volatile terpenes (ocimene 1, ocimene 2, linalool, (*E*)-4,8-dimethyl-1,3,7-nonatriene, (*β*)-farnesene, (*α*)-farnesene, and (*E,E*)-4,8,12-trimethyl-1,3,7,11-tridecatetraene), (*Z*)-3-hexenyl acetate, and indole collected from cotton plants damaged by corn earworms (CEW), nematodes (NEM), corn earworms and nematodes (CEW + NEM), and undamaged control plants (CON)

Compound	CEW + NEM *N*=3	CEW *N*=4	NEM *N*=4	CON *N*=3
Terpenes	$4.76^7 \pm 2.17^7$ a	$7.35^6 \pm 4.13^6$ a	$9.17^4 \pm 2.99^4$ b	$9.02^4 \pm 2.87^4$ b
(Z)-3-hexenyl acetate	$8.07^6 \pm 5.79^6$ a	$7.34^5 \pm 1.89^5$ b	$1.78^5 \pm 5.37^4$ b	$1.31^5 \pm 4.18^4$ b
indole	$5.94^6 \pm 2.66^6$	$1.23^6 \pm 3.15^5$	0	0

The different letters within rows are significantly different at $P<0.050$ using Tukey's HSD

mental applications of bioregulators (Khoshkoo et al. 1993). In the absence of additional bioregulator applications in our study, cotton plants failed to respond to root-knot nematode damage.

Cotton plants damaged at their roots by *M. incognita*, but not at the foliage aboveground, do not emit systemically induced volatiles that are attractive to *M. croceipes*. Plant responses to root-feeding nematodes are more variable and may depend on the type of root-feeding behavior (Matteille 1994; van Dam et al. 2005). As far as we are aware, there are no studies that have looked at comparisons of aboveground plant responses between sedentary gall-forming, migratory nematodes species, and other root herbivores.

Agronomic factors such as plant nutrient availability may also have an effect on both direct and indirect plant resistance mechanisms in cotton plants. Cotton plants with zero or 2 g N applied do not attract the parasitoid species *M. croceipes* as well as those plants with 1 g N, the latter representing the recommended amount of nitrogen (Olson et al., unpublished data). Although *M. incognita* root damage is known to increase nitrogen in the roots of some plants (Hunter 1958; Shafiee and Jenkins 1963; Burgeson 1966), presumably at a cost to the nitrogen levels in shoots, our nitrogen analyses of leaves and petioles indicated that nitrogen in shoots was not affected. Therefore, it is unlikely that the nitrogen was a factor affecting shoot volatile levels.

Microplitis croceipes clearly preferred corn earworm damaged plants over those plants that were undamaged or had been damaged by *M. incognita* alone. They also had a tendency to prefer plants damaged by CEW plus nematodes over those that had been damaged only by CEW. The volatile analyses indicate that *H. zea* feeding on cotton leaf tissue induces release of compounds known to be attractants for parasitoid species, and that adding nematodes to these plants increases the level of these emissions. This suggests that cotton fields that harbor both types of herbivores would not have a detrimental effect on host foraging by this parasitoid species.

Often, loss of either above- or belowground tissue alters the commitment of the plant to the other. Loss of leaf material from herbivores aboveground results in reduced root mass, while root grazing by a variety of nematodes and insect larvae leads to lower leaf mass aboveground (Geiger and Servaites 1991; Mooney et al. 1991). In addition to the quantitative impact on plant biomass, herbivory in one plant compartment usually changes overall plant quality in terms of primary and secondary metabolites, thus affecting herbivore load in the other plant compartment (Seastedt 1985; Seastedt et al. 1988). The magnitude of the response between roots and shoots depends on the plant species, the compounds produced, and the inducing agent (van Dam et al. 2005). The results from this study are unusual as they show no evidence of an interaction between a plant's direct defenses induced by a fruit-feeding aboveground species and a sedentary, gall-forming belowground species. More studies are needed to determine if these results can be generalized for these types of feeding paradigms.

References

ANDERSON, P., and AGRELL, J. 2005. Within-plant variation in induced defense in developing leaves of cotton plants. *Oecologia* 144:427–434.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., and WÄCKERS, F. L. 2003. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101:555–562.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN D, PUTTEN, W. H., and WÄCKERS, F. L. 2004. Above- and belowground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf herbivory. *J. Chem. Ecol* 30:63–67.

BURGESON, G. B. 1966. Mobilization of minerals to the infection site of root-knot nematodes. *Phytopathology* 56:1287–1289.

BURTON, R. L. 1969. Mass rearing the corn earworm in the laboratory. pp. 33–134. US Agr Res Ser/ARS.

DROST, Y. C., LEWIS, W. J., and TUMLINSON, J. H. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals. V. Influence of rearing method, host-plant, and adult experience on host-searching behavior of *Microplitis croceipes* (Cresson), a larval parasitoid of *Heliothis*. *J. Chem. Ecol* 14:1607–1616.

GANGE, A. C., and BROWN, V. K. 1989. Effects of root herbivory by an insect on a foliar-feeding species, mediated through changes in host plant. *Oecologia* 81:38–42.

GANGE, A. C. 2001. Species-specific responses of a root- and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. *New Phytol* 150:611–618.

GEIGER, D. R., and SERVAITES, J. C. 1991. Carbon allocation and response to stress, pp. 103–127, in H. A. Mooney, W. W. Winner, and E. J. Pell (eds.). Response of Plants to Multiple StressesAcademic, New York City.

HOWELL, C. R., HANSON, L. E., STIPANOVIC, R. D., and PUCKHABER, L. S. 2000. Induction of terpenoid synthesis in cotton roots and control of *Rhizoctonia solani* by seed treatment with *Trichoderma virens*. *Phytopathology* 90:248–252.

HUNTER, A. H. 1958. Nutrient absorption and translocation of phosphorus as influenced by the root knot nematode (*Meloidogyne incognita acrita*). *Soil Sci* 86:245–250.

HUSSEY, R. S., and BARKER, K. R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Dis. Rep* 57:1025–1028.

KARBAN, R., and BALDWIN, I. T. 1997. Induced responses to herbivory. University of Chicago, Chicago, IL.

KHOSHKOO, N., HEDIN, P. A., and MCCARTY, J. C. Jr. 1993. Effects of bioregulators on the terpenoid aldehydes in root-knot nematode infected cotton plants. *J. Agric. Food Chem* 41:2442–2446.

LEWIS, W. J., and BURTON, R. L. 1970. Rearing *Microplitis croceipes* in the laboratory with *Heliothis zea* as hosts. *J. Econ. Entomol* 63:202–658.

MACE, M. E., STIPANOVIC, R. D., and BELL, A. A. 1990. Relation between sensitivity to terpenoid phytoalexins and virulence to cotton of *Verticillium dahlia* strains. *Pestic. Biochem. Physiol* 36:79–82.

MASTERS, G. J., and BROWN, V. K. 1992. Plant mediated interactions between two spatially separated insects. *Funct. Ecol* 6:175–179.

MASTERS, G. J., BROWN, V. K., and GANGE, A. C. 1993. Plant mediated interactions between above- and belowground insect herbivores. *Oikos* 66:148–151.

MASTERS, G. J. 1995. The impact of root herbivory on aphid performance: field and laboratory evidence. *Acta Oecol* 16:135–142.

MASTERS, G. J., JONES, T. H., and ROGERS, M. 2001. Plant mediated effects of root herbivory on insect seed predators and their parasitoids. *Oikos* 127:246–250.

MATEILLE, T. 1994. Biology of the plant–nematode relationship: physiological changes and the defense mechanism of plants. *Nematologica* 40:276–311.

MAURER, B., HAUSER, A., and FROLDEVAUX, J. 1986. (E)-4,8-dimethyl-1,3,7-nonatriene and (E,E)-4,8,12-trimethyl-1,3,7,11-tridecatetraene, two unusual hydrocarbons from cardamom oil. *Tetrahedron Lett* 27:2111–2112.

MCAUSLANE, H. J., and ALBORN, H. T. 1998. Systemic induction of allelochemicals in glanded and glandless isogenic cotton by *Spodoptra exigua* feeding. *J. Chem.Ecol* 24:399–416.

MCAUSLANE, H. J., ALBORN, H. T., and TOTH, J. P. 1997. Systemic induction of terpenoid aldehydes in cotton pigment glands by feeding of larval *Spodoptera exigua*. *J. Chem. Ecol* 23:2861–2879.

MCCLURE, M. A. 1977. *Meloidogyne incognita*: a metabolic sink. *J. Nematol* 9:88–90.

MELAKEBERHAN, H., BROOKE, R. C., WEBSTER, J. M., and D'AURIA, J. M. 1985. The influence of *Meloidogyne incognita* on the growth, physiology and nutrient content of *Phaseolus vulgaris*. *Physiol. Plant Pathol* 26:259–268.

MELAKEBERHAN, H., WEBSTER, J. M., BROOKE, R. C., D'AURIA, J. M., and CACKETTE, M. 1987. Effect of *Meloidogyne incognita* on plant nutrient concentration and its influence on the physiology of beans. *J. Nematol* 19:324–330.

MOHANTY, K. C., DAS, S. N., PATNAIK, P. R., and SAHOO, B. 1990. Chemical changes in horse gram plants infected with root knot nematode, *Meloidogyne incognita*. *Comp. Physiol. Ecol* 15:100–102.

MOONEY, H. A., WINNER, W. E., and PELL, E. J. (eds.). 1991. Response of Plants to Multiple Stresses. Academic, New York, p. 422.

MORAN, N. A., and WHITHAM, T. G. 1990. Interspecific competition between root-feeding and leaf-galling aphids mediated by host-plant resistance. *Ecology* 71:050–1058.

NAZAROVA, I. P. 1981. Gossypol-like compounds of the cotton plant. Methods of determining gossypol. *Chem. Nat. Prod* 17:87–102.

RASMANN, S., KÖLLNER, T. G., DEGENHARDT, J., HILTPOLD, I., TOEPFER, S., KUHLMANN, U., GERSHENZON, J., and TURLINGS, T. C. J. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434:732–737.

RÖSE, U. S. R., and TUMLINSON, J. H. 2004. Volatiles released from cotton plants in response to *Heliocoverpa zea* feeding damage on cotton flower buds. *Planta* 218:824–832.

SALT, D. T., FENWICK, P., and WHITTAKER, J. B. 1996. Interspecific herbivore interactions in a high CO_2 environment: root and shoot aphids feeding on *Cardamine*. *Oikos* 77:326–330.

SAS INSTITUTE INC. 1998. SAS/STAT User's, version 6, 4th Edition, Vol. 1–2. SAS, Cary, NC.

SEASTEDT, T. R. 1985. Maximization of primary and secondary productivity by grazers. *Am. Nat.* 126:559–564.

SEASTEDT, T. R., RAMONDO, R. A., and HAYES, D. C. 1988. Maximization of densities of soil animals by foliage herbivory: empirical evidence, graphical and conceptual models. *Oikos* 51:243–248.

SHAFIEE, M. F., and JENKINS, W. R. 1963. Host–parasite relations of *Capsicum frutescens* and *Pratylenchus penetrans*, *Meloidogyne incognita acrita*, and *M. hapla*. *Phytopathology* 53:325–328.

STIPANOVIC, R. D., ELLISALDE, M. H., ALTMAN, D. W., and NORMAN, J. O. 1990. Cell culture bioassay to evaluate allelochemical toxicity to *Heliothis virescens*. *J. Econ. Entomol* 83:737–741.

TURLINGS, T. C. J., and WÄCKERS, F. L. 2004. Recruitment of predators and parasitoids by herbivore-injured plants, pp. 21–75, in R. T. Cardé, and J. Millar (eds.). Advances in Insect Chemical EcologyCambridge University Press, New York.

VAAST, P. H., CASWELL-CHEN, E. P., and ZASOSKI, R. J. 1998. Effects of two endoparasitic nematodes (*Pratylenchus coffeae* and *Meloidogyne konaensis*) on ammonium and nitrate uptake by Arabica coffee (*Coffea Arabica* L.). *Appl. Soil Ecol* 10:171–178.

VAN DAM, N. M., RAAIJMAKERS, C. E., and VAN DER PUTTEN, W. H. 2005. Root herbivory reduces the growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl* 115:161–170.

WÄCKERS, F. L., BONIFAY, C., and LEWIS, W. J. 2002. Conditioning of appetitive behavior in the Hymenopteran parasitoid *Microplitis croceipes*. *Entomol. Exp. Appl* 103:135–138.

WÄCKERS, F. L., ZUBER, D., WUNDERKIN, R., and KELLER, F. 2001. The effect of herbivory on temporal and spatial dynamics of foliar nectar production in cotton and castor. *Ann. Bot* 87:365–370.

WANG, X., and PLHAK, L. C. 2004. Monoclonal antibodies for the analysis of gossypol in cottonseed products. *J. Agric. Food Chem* 52:709–712.

XU, B., CHEN, Y., WU, Y., ZHANG, Q., SU, N., WANG, Y., LIU, X., and YUAN, F. 2006. Effects of CrylAc and secondary metaobolites in Bt transgenic cottonseed on *Lycoriella pleuroti* Yang et Zhang (Diptera: Sciaridae). *Environ. Entomol* 35:807–810.

ZHANG, J. X., MACE, M. E., STIPANOVIC, R. D., and BELL, A. A. 1993. Production and fungitoxicity of the terpenoid phytoalexins in cotton inoculated with *Fusarium oxysporum* f. sp. *vasinfectum*. *J. Phytopathol* 139:247–252.

impact of plant sectoriality on induced metabolic responses to herbivory has only recently been described (Jones et al. 1993; Mutikainen et al. 1996; Orians et al. 2000; Schittko and Baldwin 2003; Viswanathan and Thaler 2004). In these cases, leaves that share the strongest vascular connections (i.e., orthostichous pairs) also tend to transmit the strongest induced responses (Orians 2005; but see Frost et al. 2007 for within-plant volatile signaling).

The consequences of plant vascular architecture for induction have, thus far, only been emphasized in responses that are both elicited and expressed in foliar tissues. In fact, recent studies demonstrate that induced responses span the aboveground and belowground systems (van Dam et al. 2003; Bezemer and van Dam 2005, 2006; Kaplan and Denno 2007). Thus, root herbivory can induce changes in leaf secondary chemistry (Bezemer et al. 2004; Soler et al. 2005; van Dam et al. 2005; van Dam and Raaijmakers 2006; Kaplan et al. 2008), and leaf herbivory can similarly induce changes in root chemistry (Soler et al. 2007). Because resource sharing between leaves and roots is known to be sectored in certain plants (Orians et al. 2004; Bledsoe and Orians 2006; Zanne et al. 2006), vascular architecture may influence aboveground–belowground induction patterns in a manner analogous to orthostichous leaves. Yet, to our knowledge, this possibility has never been tested.

If leaf–root induction is indeed sectored, as is often the case with leaves, this has important ecological implications for understanding the causes of heterogeneity in plant defense traits and their extended effects on consumers. Importantly, leaf and root herbivores are known to be patchily distributed in the aboveground and belowground environments, and thus herbivory on only part of the plant is likely to be ubiquitous under natural growing conditions (Denno and McClure 1983; Ettema and Wardle 2002). When root feeders, for example, predominantly damage one sector of a root system (likely given the poor dispersal capabilities of most belowground consumers), then potential indirect effects on foliar herbivores may be restricted to the same sector of plant aboveground. Similarly, foliar herbivores that damage one or a few leaves may be more likely to affect resource quality for belowground consumers that occur on adjacent root tissue, potentially contributing to the inherent patchiness of soil communities (Ettema and Wardle 2002).

We experimentally manipulated foliar and root herbivory on tobacco (*Nicotiana tabacum*) by using a split-root design and quantified the effects of plant vascular architecture on aboveground–belowground-induced chemical responses.

Methods and Materials

Tobacco plants (var. MD 609) were propagated from seed in a greenhouse (seeds were cultivated in a standard potting mix described below and maintained in a mist room). After 9 week of growth, seedlings were transplanted to pots containing a sterilized growing medium [50% sand, 50% potting mix (SunGro LC1 and professional blend; sphagnum peat moss, bark, perlite, vermiculite, and clay)]. Plants were supplemented with nutrients weekly by applying a soluble fertilizer (20:10:20 NPK) and were maintained at 23–27°C under natural light conditions. A split-root technique was used whereby the roots of all seedlings were divided, and each of the two halves was transplanted into separate, but adjacent, 2-gal pots. Thus, each plant possessed a single root system divided equally between two independent growth environments. Plants were acclimated to these growing conditions for an additional 3 week until use in experiments. At such time, plants remained in the rosette stage of growth and possessed four to five fully expanded leaves and at least five additional developing sink leaves.

Experiment 1—Leaf–Root Vascular Connectivity Vertically aligned leaves and roots are predicted to be more strongly connected via the vascular system than leaves and roots on opposing sides of the plant. To test this hypothesis, we employed a dye tracer technique that used the above described split-root plants (Orians et al. 2000; Viswanathan and Thaler 2004). The first fully expanded leaf on each plant (N=18) was excised with a razor blade, and an Eppendorf tube filled with a 0.025% *w/v* solution of rhodamine-B dye (Sigma, St. Louis, MO, USA) was inserted onto the petiole. After 48 h, the main tap root was dissected by cutting a cross section, and the location of the dye was visually assessed relative to the excised leaf. The intensity of dye staining in the vascular tissue was compared between the root half that occurred closest to the labeled leaf and the remaining half of the root that was opposite from the labeled leaf. We then recorded whether the intensity of vascular dye was greater in the orthostichous root segment, the nonorthostichous root segment, or that there was no difference between the two root segments.

Experiment 2—Aboveground Herbivory Foliar herbivory was manipulated by applying beet armyworm caterpillars, *Spodoptera exigua*, to selected leaves. Beet armyworms are a polyphagous species that are known to feed and induce responses on tobacco (Voelckel and Baldwin 2004). Caterpillars were obtained from a biological supply company (Benzon Research, Inc., Carlisle, PA, USA) and reared on artificial diet until they reached the third instar. Fine-mesh sleeve cages were placed over leaves and fastened to the petiole with a twist tie to ensure that caterpillars only damaged the leaf assigned to the foliar herbivory treatment.

Thirty split-root plants were used; half of these were randomly assigned to caterpillar defoliation (N=15), while

the other half served as undamaged controls. Damage regimes were initiated 21 day after seedlings were transplanted into pots and thus in the rosette stage of growth. The first fully expanded leaf that was orthostichous (i.e., vertically aligned) with one of the two root sections (i.e., pots) was assigned to caterpillar damage. Three third-instar *S. exigua* were placed in sleeve cages and remained for 72 h, during which time they removed 20–50% of the leaf area. Control plants also received sleeve cages, but no caterpillars were added. After 48 h (5 day after the initiation of damage regimes) leaves and roots were harvested for analysis of secondary metabolites. This temporal scale is known to coincide with peak concentrations of nicotine and other induced tobacco secondary compounds following caterpillar herbivory (Keinänen et al. 2001).

Two recently expanded sink leaves (i.e., within 1 week of emergence) were collected from each plant—one leaf that was orthostichous with the caterpillar-damaged leaf and a second leaf that was opposite from the damaged leaf. Rosette-stage tobacco plants follow a 3/8 phyllotaxis whereby each new leaf emerges at a 135° angle from the previous leaf (Allard 1942; Jones et al. 1959; Schittko and Baldwin 2003). Therefore, the leaf that is eight positions higher on the plant from the damaged leaf is vertically aligned. Additionally, root tissue (only fine roots) was harvested from both pots in each plant for chemical analysis.

Tissue samples were frozen immediately in liquid N_2. Secondary chemicals with known antiherbivore properties were extracted and analyzed by high-performance liquid chromatography (HPLC; see Keinänen et al. 2001) on a reverse-phase C18 column (Gemini C18, 150×4.6 mm; Phenomenex). Concentrations of identified alkaloids (nicotine) and phenolics (chlorogenic acid and rutin) were quantified by using calibration curves prepared from commercially available standards. Caffeoyl putrescine is not commercially available and was quantified as chlorogenic acid equivalents as described by Keinänen et al. (2001). A further phenolic compound was putatively identified as 7-methyl esculin by comparison with published retention time and mass spectral data (Vereecke et al. 1997). HPLC–mass spectrometry (MS) was performed under identical HPLC conditions as described above, with the exception of trifluoroacetic acid replacing the phosphoric acid in the mobile phase. MS was performed on a Varian 1200 triple quadrupole instrument (Varian, Palo Alto, CA, USA). Under positive electrospray ionization conditions (needle voltage 4.8 kV; capillary voltage 35 V; drying gas 18.5 psi per 200°C, nebulizer gas 51 psi), the compound produced a $[M+H]^+$ at *m/z* 355. Collision-induced dissociation at a 10-V collision energy yielded a dominant fragment ion at *m/z* 193 corresponding to the 7-methyl esculin fragment or a loss of a hexose (Vereecke et al. 1997). Concentrations of nonnicotine alkaloids and 7-methyl esculin were calculated from peak areas at 254 nm (for alkaloids) and 320 nm (for 7-methyl esculin).

Experiment 3—Belowground Herbivory Root herbivory was manipulated by inoculating tobacco roots with the nematode *Meloidogyne incognita*. This polyphagous species is a sedentary endoparasite that induces a gall at feeding sites (Trudgill and Block 2001). Nematode cultures originated from local agricultural fields and were reared on tobacco prior to use in experiments. Nematode eggs were harvested from the roots of heavily infested plants by using a modified version of the Hussey and Barker (1973) extraction procedure. Galled roots were cut into 2-cm-long segments, placed into a 250-ml flask containing 100 ml of a 0.6% NaOCl dilution, and subsequently poured through nested sieves (250-μm-pore sieve on top of a 25-μm-pore sieve). The resulting eggs were counted and applied to the roots of experimentally inoculated seedlings at transplant. Each seedling assigned to the root herbivory treatment received ~100,000 *M. incognita* eggs. This density falls well within the range documented for *M. incognita*-infested tobacco fields (Barker and Lucas 1984) and also corresponds with inoculum levels used in prior studies on *M. incognita*–tobacco interactions (Hanounik and Osbourne 1975, 1977; Barker and Weeks 1991; Wheeler et al. 1991; Vovlas et al. 2004).

Thirty split-root plants were used—half were assigned to the nematode root herbivory treatment ($N=15$), while the other half acted as control plants. On nematode-treated plants, roots from only one of the two pots were inoculated with nematodes. Thus, in the root herbivory treatment, only half of the root system was galled by nematodes—the other half was not. Twenty-one days after nematode addition, plant leaves and roots were harvested for secondary chemical analysis. This time scale was used because *M. incognita* requires at least 3 week to complete gall development, and this also coincides with prior experimental work on nematode-induced responses in tobacco (Kaplan et al. 2008). Two recently expanded sink leaves were harvested from each plant (nematode root herbivory is known to induce secondary chemical changes in tobacco sink leaves; see Kaplan et al. 2008), one leaf that was orthostichous (i.e., vertically aligned) with the pot containing nematode-inoculated roots and a second leaf that was opposite from the inoculated root section. On control plants, the two leaves were chosen to align with each of the two pots as in the nematode treatment. Secondary plant chemicals were quantified in leaf and root tissue samples as described above (see Exp. 2). Roots in each pot were visually inspected to assess the efficacy of treatment and confirmed that nematode-induced galls only occurred on roots that were inoculated (i.e., there was no migration of juveniles between neighboring pots). Moreover, nematode-inoculated roots were heavily galled

and displayed no evidence of strong resource competition. Roots from each pot were dried (at 60°C for 72 h) and weighed to assess the impact of nematode herbivory on belowground biomass.

Statistical Analyses A *G* test was used to determine whether orthostichy affected patterns of dye accumulation in roots (Sokal and Rohlf 1994). To assess the impact of plant vascular architecture on induced responses to foliar and root herbivory,

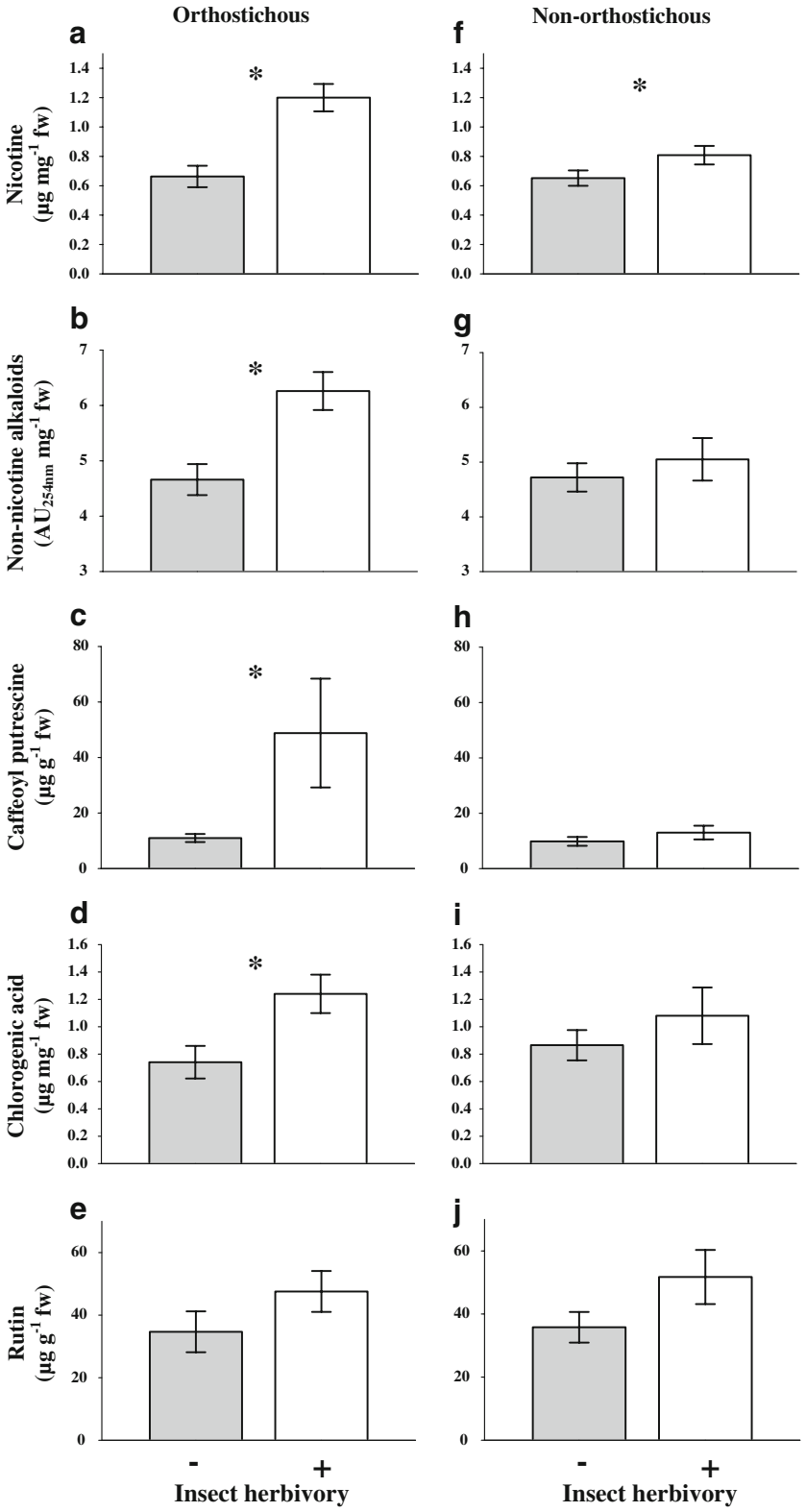

Fig. 1 Effects of aboveground caterpillar herbivory on the expression of secondary plant chemicals in orthostichous (*A–E*) and nonorthostichous (*F–J*) tobacco leaves, including nicotine, nonnicotine alkaloids, caffeoyl putrescine, chlorogenic acid, and rutin (means±SE). *Asterisks* denote significant differences between means ($P<0.05$)

we used multivariate analysis of variance (MANOVA; Proc glm; statistical analyses were performed using SAS, version 9.1; SAS Institute, Inc. 2001), followed by univariate analyses of variance for each secondary chemical measured (Proc mixed). Separate analyses were performed to quantify the impact of aboveground caterpillar herbivory on orthostichous and nonorthostichous leaves and roots, as well as the effect of belowground nematode herbivory on orthostichous and nonorthostichous leaves and roots. Blocks (i.e., spatial groupings of control and herbivory plants) were considered as a random effect in the model. Because the position of the caterpillar-damaged leaf in Experiment 2 varied along the stem (i.e., we were restricted to using the leaf that was growing directly above one of the two pots), we used regression analyses (Proc glm) with leaf number as the predictor variable and root chemistry as the response variable to test for potential relationships between leaf position and belowground induction. *T* tests were used to compare root dry mass on plants with and without nematodes. Data were transformed (square root and log transformations) as needed to meet assumptions of normality and homogeneity of variances.

To compare directly the overall magnitude of secondary chemical induction in orthostichous vs. nonorthostichous plant tissues, effect sizes were calculated (Hedges' *d*) for the impact of caterpillar herbivory on leaf and root chemistry, and the effect of nematode herbivory on leaf chemistry (Rosenberg et al. 2000). Mixed-effects categorical models were then used to compare effect sizes in orthostichous and nonorthostichous plant tissues. For each group, a mean effect size was calculated and reported with 95% bootstrap confidence intervals. Effect sizes greater than zero indicate that herbivory elicited an overall increase in the concentration of secondary plant chemicals. Between-group heterogeneity (Q_B) was tested against a χ^2 distribution to determine whether plant vascular architecture affected the magnitude of aboveground and belowground induction.

Results

The appearance of dye in tobacco roots was affected strongly by leaf position (G=24.95, df=1, P<0.001). In all cases (N=18), greater dye accumulation was found in roots that were vertically aligned with the labeled leaf than in roots that were opposite.

Foliar herbivory elevated the secondary chemistry of tobacco leaves that were both orthostichous (Figs. 1 A–E) and nonorthostichous (Figs. 1 F–J) with the caterpillar-damaged leaf (Table 1). However, the impact of induction was more apparent in leaves sharing strong vascular connections. Four of the five secondary chemicals measured (nicotine, nonnicotine alkaloids, caffeoyl putrescine, and chlorogenic acid) were induced to higher levels in orthostichous leaves (Figs. 1 A–D), compared with only one of five (nicotine) in nonorthostichous leaves (Fig. 1 F).

Similar results were found when comparing the effects of aboveground herbivory on root chemistry in vertically

Table 1 The effects of aboveground and belowground herbivory on the expression of secondary plant chemicals in orthostichous and nonorthostichous leaves and roots of tobacco

Herbivory	Tissue	Compound	Orthostichous			Nonorthostichous		
			df	*F*	*P*	*df*	*F*	*P*
(A) Caterpillar aboveground	Leaf	MANOVA	5, 22	8.98	**<0.001**	5, 24	3.02	**0.030**
		Nicotine	1, 12	19.43	**<0.001**	1, 14	7.83	**0.014**
		Nonnicotine alkaloids	1, 12	13.65	**0.003**	1, 14	0.79	0.389
		Caffeoyl putrescine	1, 12	9.09	**0.011**	1, 14	0.99	0.336
		Chlorogenic acid	1, 12	7.19	**0.020**	1, 14	0.11	0.750
		Rutin	1, 12	2.32	0.154	1, 14	2.49	0.137
(B) Caterpillar aboveground	Root	MANOVA	5, 23	2.33	0.075	5, 18	0.63	0.681
		Nicotine	1, 13	1.88	0.194	1, 10	0.07	0.791
		Nonnicotine alkaloids	1, 13	9.09	**0.010**	1, 10	0.00	0.979
		Caffeoyl putrescine	1, 13	2.64	0.128	1, 10	0.72	0.415
		Chlorogenic acid	1, 13	4.01	0.066	1, 10	0.09	0.765
		7-methyl esculin	1, 13	0.48	0.499	1, 10	4.33	0.064
(C) Nematode belowground	Leaf	MANOVA	5, 23	0.89	0.505	5, 23	0.48	0.784
		Nicotine	1, 13	1.29	0.276	1, 13	0.71	0.416
		Nonnicotine alkaloids	1, 13	0.01	0.935	1, 13	0.09	0.769
		Caffeoyl putrescine	1, 13	0.01	0.910	1, 13	0.00	0.957
		Chlorogenic acid	1, 13	0.05	0.821	1, 13	0.55	0.470
		Rutin	1, 13	1.43	0.254	1, 13	0.27	0.609

Significant effects (P<0.05) are bolded for emphasis

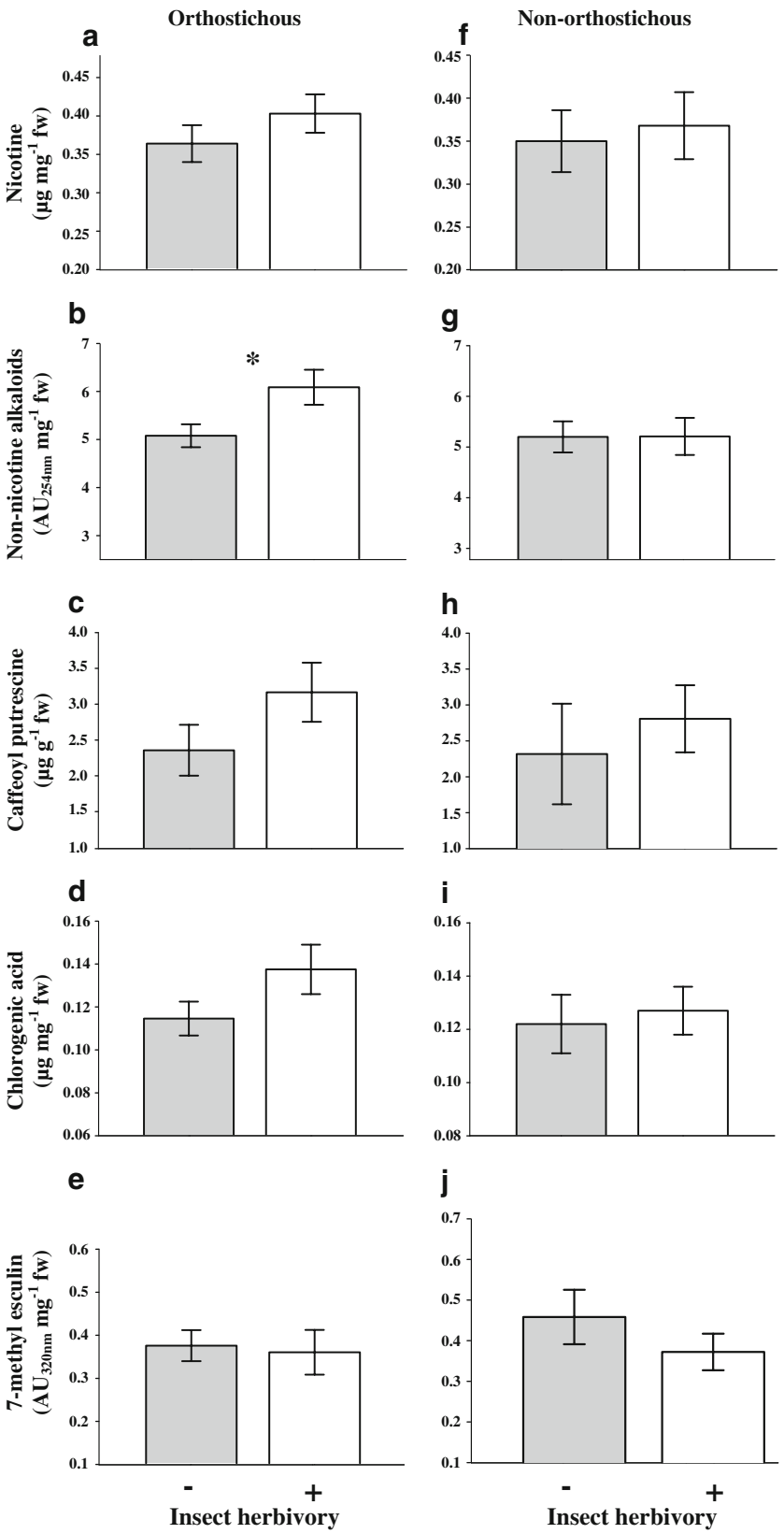

Fig. 2 Effects of aboveground caterpillar herbivory on the expression of secondary plant chemicals in orthostichous (*A–E*) and nonorthostichous (*F–J*) tobacco roots, including nicotine, nonnicotine alkaloids, caffeoyl putrescine, chlorogenic acid, and 7-methyl esculin (means±SE). *Asterisk* denotes significant differences between means ($P<0.05$)

aligned (Figs. 2 A–E) vs. opposing (Figs. 2 F–J) root sections (Table 1). Caterpillar feeding elevated the belowground concentration of certain compounds (e.g., nonnicotine alkaloids; Fig. 2 B), and there was a trend for herbivory to affect overall secondary chemical expression in orthostichous roots (MANOVA: P=0.075). In contrast, aboveground induction did not affect the chemistry of roots that were opposite from the insect-defoliated leaf (Figs. 2

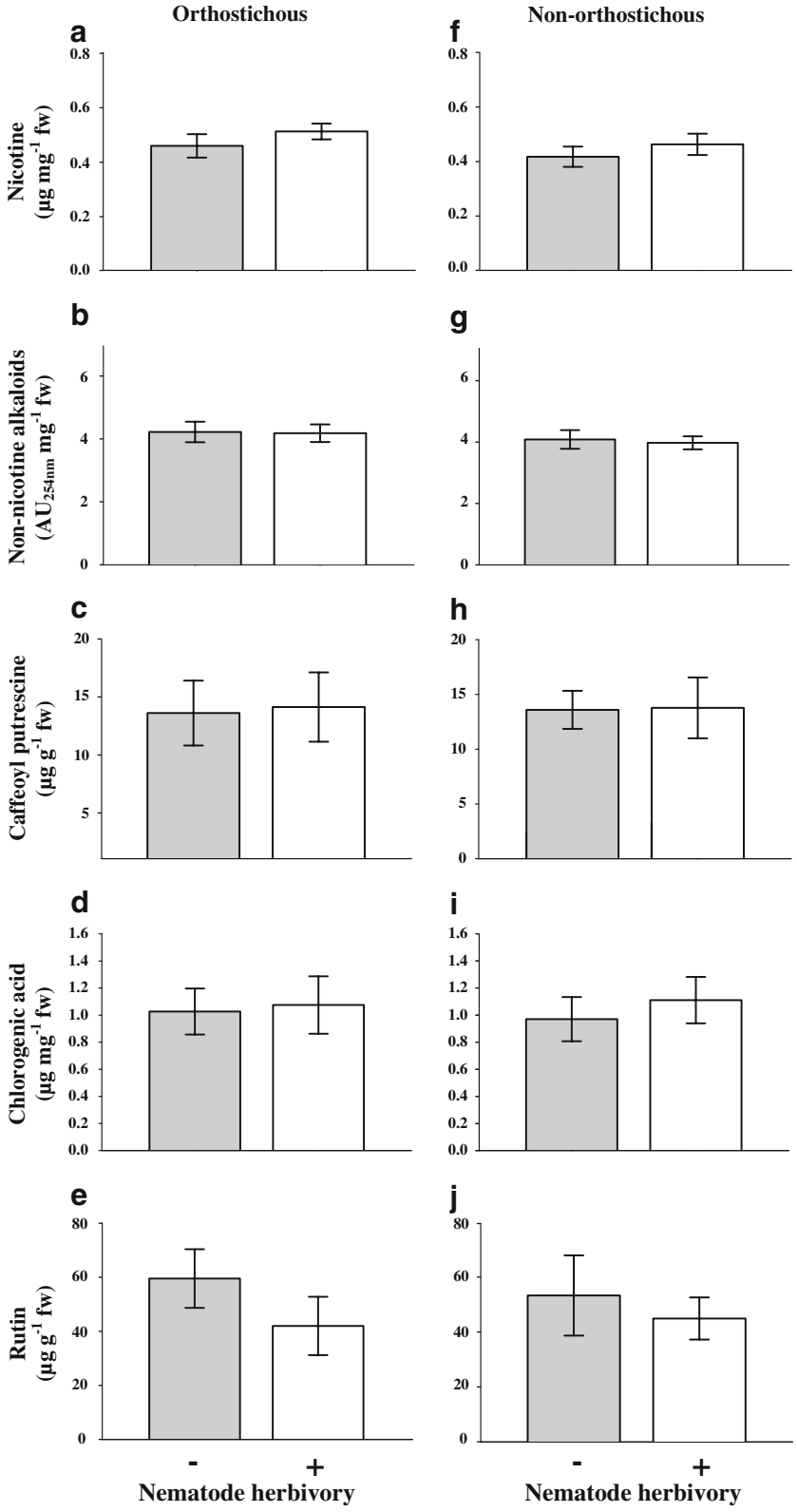

Fig. 3 Effects of belowground nematode herbivory on the expression of secondary plant chemicals in orthostichous (*A–E*) and nonorthostichous (*F–J*) tobacco leaves, including nicotine, nonnicotine alkaloids, caffeoyl putrescine, chlorogenic acid, and rutin (means±SE)

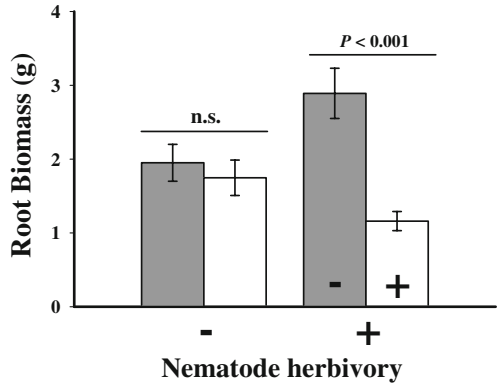

Fig. 4 The impact of nematode root herbivory on belowground plant biomass. The *bars* on the *right* denote plants that were experimentally inoculated with nematodes, whereas the group of *bars* to the *left* represent plants that were not exposed to nematode herbivory. A *positive sign* indicates which of the two split-root sections received nematodes. *n.s.* = nonsignificant

F–J). Moreover, there was no relationship for either root type between the position (age) of the caterpillar-damaged leaf and root chemistry ($P>0.1$ for all secondary metabolites measured).

Nematode root herbivory had no impact on leaf chemistry in either of the two leaf tissue types (Table 1), with nonsignificant effects of nematodes on secondary compounds in both orthostichous (Figs. 3 A–E) and nonorthostichous (Figs. 3 F–J) leaves. However, nematodes induced local changes (i.e., those measured in the roots of pots inoculated with nematodes) in the secondary chemistry of tobacco roots (MANOVA: $F_{5,\ 18}=9.24$, $P<0.001$), including nicotine ($F_{1,\ 22}=19.54$, $P<0.001$), nonnicotine alkaloids ($F_{1,\ 22}=30.93$, $P<0.001$), caffeoyl putrescine ($F_{1,\ 22}=37.14$, $P<0.001$), chlorogenic acid ($F_{1,\ 22}=25.56$, $P<0.001$), and 7-methyl esculin ($F_{1,\ 22}=37.20$, $P<0.001$). In all cases, the concentrations of secondary chemicals were higher in the roots of nematode-damaged plants.

Although nematodes did not affect total root biomass (i.e., pooled belowground dry weights from both split-root sections; $F_{1,\ 28}=0.49$, $P=0.489$), the effects of nematode herbivory on roots were apparent within each split-root pot (Fig. 4). The mass of the two root sections in control pots were not different from one another ($t=0.60$, $P=0.555$), but root mass in nematode-inoculated pots was >50% less than in the opposite pot of the same plant ($t=4.69$, $P<0.001$). This effect was driven partly by nematodes reducing root biomass relative to control plants (average control pot= 1.85±0.18 g, nematode-inoculated pot=1.16±0.13; $F_{1,\ 28}=10.00$, $P=0.004$) and also by plants compensating for nematode herbivory by increasing root biomass in the nematode-free pot of plants assigned to root herbivory (average control pot=1.85±0.18 g, nematode-free pot of root herbivory plants=2.89±0.34 g; $F_{1,\ 28}=7.36$, $P=0.011$).

The overall magnitude of phytochemical induction differed between orthostichous and nonorthostichous plant tissues for one of the three comparisons, i.e., the impact of caterpillar herbivory on leaf chemistry ($Q_B=4.90$, $df=1$, $P=0.027$). In this case, the effect size for responses in the orthostichous leaf was >2× as large as in the nonorthostichous leaf (Fig. 5 A). The difference in root responses to foliar herbivory was nearly significant ($Q_B=2.90$, $df=1$, $P=0.089$), with moderately strong responses in vertically aligned roots (effect size=0.45) and virtually no response in opposing root sections (effect size=0.02; Fig. 5 B). However, aboveground responses to root herbivory were equally weak in both leaf tissue types ($Q_B=0.03$, $df=1$, $P=0.864$; Fig. 5 C).

Discussion

Vascular architecture mediates herbivore-induced plant responses that are both elicited and expressed in leaves, but whether vascular connections similarly shape patterns of induction linking leaves with roots is unknown. In agreement with previously published accounts (Orians et al. 2000; Schittko and Baldwin 2003; Viswanathan and Thaler

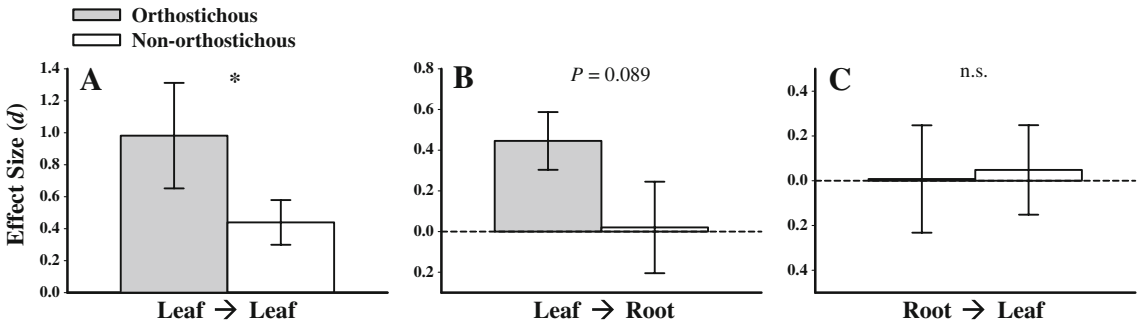

Fig. 5 The magnitude of herbivore-induced responses in orthostichous vs. nonorthostichous plant tissues, including (*A*) leaf responses to foliar herbivory, (*B*) root responses to foliar herbivory, and (*C*) leaf responses to root herbivory (means±95% bootstrap confidence intervals). Effect sizes (Hedges' *d*) were calculated to summarize the cumulative secondary chemical response to herbivory in leaves and roots. *Asterisk* denotes significant differences between means ($P<0.05$). *n.s.* = nonsignificant

2004), we found that the magnitude of phytochemical induction by foliar herbivory was most pronounced in leaves that share strong vascular connections with the caterpillar-damaged leaf (Figs. 1 and 5 A). However, we also document the novel finding that vascular architecture can similarly mediate the effects of foliar herbivory on certain root chemicals (e.g., nonnicotine alkaloids), with stronger induction in roots that are vertically aligned with insect-defoliated leaves (Figs. 2 and 5 B).

Because studies that measure the impact of leaf herbivory on root chemistry do not account for vascular architecture, whereas studies on aboveground induced responses often do, the magnitude of root responses to foliar induction may be underestimated in the literature. For example, in a recently published meta-analysis that merged data from multiple independent studies, evidence was found that foliar induction elicits a relatively weak response in root tissue (Kaplan et al. 2008). This outcome may in part be driven by the lack of control over leaf–root connectivity in previous studies. For this reason, we urge investigators in future studies of aboveground–belowground induction to consider plant sectoriality in the design of experiments. However, leaf responses to foliar herbivory were nevertheless stronger than root responses in the present study, despite controlling for vascular architecture in both cases (leaf–leaf effect size=+0.982, leaf–root effect size=+0.446). Also, the ontogenetic stage of belowground tissues (e.g., fine vs. tap roots) is likely to affect whether or not induced responses are detected in roots (Van Dam and Raaijmakers 2006).

Despite the fact that our experiments support the hypothesis that leaf–root induction follows the vascular architecture of plants, our results also indicate that this phenomenon is more apparent and potentially more important aboveground. Caterpillar damage induced higher concentrations of four of five secondary chemicals measured in orthostichous leaves (compared with only one of five in nonorthostichous leaves), whereas one of five chemicals were induced to higher levels in orthostichous roots (compared with zero of five in nonorthostichous roots). As a result, the overall magnitude of sectorial induction was significant among leaves (P=0.027) but nonsignificant for roots (P=0.089).

Surprisingly, root herbivory did not alter the subset of secondary chemicals that we measured in tobacco leaves (Fig. 3), making it difficult to evaluate the consequences of vascular architecture (Fig. 5C). This outcome was unexpected because in prior experiments strong foliar responses to nematode (*M. incognita*) root herbivory were documented with the same tobacco system and under similar environmental and ecological conditions (Kaplan et al. 2008). More specifically, we previously found that nematodes induced a 40% reduction in leaf nicotine and elevated foliar concentrations of phenolics and diterpene glycosides (two to four times) above control levels. Thus, nematode herbivory in only half of the root system appears to elicit very different aboveground responses when compared with plants experiencing widespread herbivory distributed throughout the entire root system. The quantity of tissue damaged affects the strength of herbivore-induced responses in leaves (see Table 4.5 in Karban and Baldwin 1997). Therefore, lower levels of root herbivory may lead to lower levels of aboveground induction. Because nematodes damage alkaloid biosynthetic sites and interfere with foliar expression (Hanounik and Osborne 1977; Barker and Weeks 1991; Kaplan et al. 2008), another potential explanation is that plants compensate for root damage on one side of the plant by increasing alkaloid production on the other undamaged side (Fig. 4). Moreover, compensatory root growth may come at the expense of foliar chemical responses if plants are limited in their allocation of resources towards growth and defense.

Foliar and root herbivores are often patchily distributed on plant leaves and roots (Denno and McClure 1983; Ettema and Wardle 2002). As a result, their systemic effects on plant defense traits are likely to be heterogeneous. Given the recent emphasis on linkages between aboveground and belowground biota (Wardle et al. 2004; Wardle 2002), our findings provide new mechanistic insight into the ecological circumstances in which foliar and root herbivores affect both plant chemistry and potentially other co-occurring herbivores. Additional studies are needed to assess the relative importance of sectorial induction between leaves and other plant tissues.

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References

ALLARD, H. A. 1942. Some aspects of the phyllotaxy of tobacco. *J. Agric. Res.* 64:49–55.

ARNOLD, T. M., and SCHULTZ, J. C. 2002. Induced sink strength as a prerequisite for induced tannin biosynthesis in developing leaves of *Populus*. *Oecologia* 130:585–593.

ARNOLD, T., APPEL, H., PATEL, V., STOCUM, E., KAVALIER, A., and SCHULTZ, J. C. 2004. Carbohydrate translocation determines the phenolic content of *Populus* foliage: a test of the sink-source model of plant defense. *New Phytol.* 164:157–164.

BARKER, K. R., and LUCAS, G. B. 1984. Nematode parasites of tobacco, pp. 213–242, in W. R. Nickle (ed.). Plant and Insect NematodesMarcel Dekker, New York.

BARKER, K. R., and WEEKS, W. W. 1991. Relationships between soil and levels of *Meloidogyne incognita* and tobacco yield and quality. *J. Nematol.* 23:82–90.

BEZEMER, T. M., and VAN DAM, N. M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *Trends Ecol. Evol.* 20:617–624.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WÄCKERS, F. L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30:53–67.

BLEDSOE, T. M., and ORIANS, C. M. 2006. Vascular pathways constrain ^{13}C accumulation in large root sinks of *Lycopersicon esculentum* (Solanaceae). *Amer. J. Bot.* 93:884–890.

DAVIS, J. M., GORDON, M. P., and SMIT, B. A. 1991. Assimilate movement dictates remote sites of wound-induced gene expression in poplar leaves. *Proc. Natl. Acad. Sci. U. S. A* 88: 2393–2396.

DENNO, R. F., and MCCLURE, M. S. 1983. Variable Plants and Herbivores in Natural and Managed Systems. Academic, New York.

ETTEMA, C. H., and WARDLE, D. A. 2002. Spatial soil ecology. *Trends Ecol. Evol.* 17:177–183.

FROST, C. J., APPEL, H. M., CARLSON, J. E., DE MORAES, C. M., MESCHER, M. C., and SCHULTZ, J. C. 2007. Within-plant signaling via volatiles overcomes vascular constraints on systemic signaling and primes responses against herbivores. *Ecol. Lett.* 10:490–498.

HANOUNIK, S. B., and OSBORNE, W. W. 1975. Influence of *Meloidogyne incognita* on the content of amino acids and nicotine in tobacco grown under gnotobiotic conditions. *J. Nematol.* 7:332–336.

HANOUNIK, S. B., and OSBORNE, W. W. 1977. The relationship between population density of *Meloidogyne incognita* and nicotine content of tobacco. *Nematologica* 23:147–152.

HUNTER, M. D., and PRICE, P. W. 1992. Playing chutes and ladders—heterogeneity and the relative roles of bottom-up and top-down forces in natural communities. *Ecology* 73:724–732.

HUSSEY, R. S., and BARKER, K. R. 1973. A comparison of methods of collecting inocula of *Meloidogyne* spp., including a new technique. *Plant Disease Rep.* 57:1025–1028.

JONES, C. G., HOPPER, R. F., COLEMAN, J. S., and KRISCHIK, V. A. 1993. Control of systemically induced herbivore resistance by plant vascular architecture. *Oecologia* 93:452–456.

JONES, H., MARTIN, R. V., and PORTER, H. K. 1959. Translocation of ^{14}carbon in tobacco following assimilation of ^{14}carbon dioxide by a single leaf. *Ann. Bot. London* 23:493–510.

KAPLAN, I., and DENNO, R. F. 2007. Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. *Ecol. Lett.* 10:977–994.

KAPLAN, I., HALITSCHKE, R., KESSLER, A., SARDANELLI, S., and DENNO, R. F. 2008. Constitutive and induced defenses to herbivory in above- and belowground plant tissues. *Ecology* 89:392–406.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. The University of Chicago Press, Chicago.

KEINÄNEN, M., OLDHAM, N. J., and BALDWIN, I. T. 2001. Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. *J. Agric. Food Chem.* 49:3553–3558.

MUTIKAINEN, P., WALLS, M., and OVASKA, J. 1996. Herbivore-induced resistance in *Betula pendula*: the role of plant vascular architecture. *Oecologia* 108:723–727.

ORIANS, C. 2005. Herbivores, vascular pathways, and systemic induction: facts and artifacts. *J. Chem. Ecol.* 31:2231–2242.

ORIANS, C. M., POMERLEAU, J., and RICCO, R. 2000. Vascular architecture generates fine scale variation in systemic induction of proteinase inhibitors in tomato. *J. Chem. Ecol.* 26:471–485.

ORIANS, C. M., ARDON, M., and MOHAMMAD, B. A. 2002. Vascular architecture and patchy nutrient availability generates within-plant heterogeneity in plant traits important to herbivores. *Am. J. Bot.* 89:270–278.

ORIANS, C. M., VAN VUUREN, M. M. I., HARRIS, N. L., BABST, B. A., and ELLMORE, G. S. 2004. Differential sectoriality in long-distance transport in temperate tree species: evidence from dye flow, ^{15}N transport, and vessel element pitting. *Trees* 18:501–509.

ROSENBERG, M. S., ADAMS, D. C., and GUREVITCH, J. 2000. MetaWin: Statistical Software for Meta-analysis, Version 2.0. Sinauer, Sunderland.

SCHITTKO, U., and BALDWIN, I. T. 2003. Constraints to herbivore-induced systemic responses: bidirectional signaling along orthostichies in *Nicotiana attenuata*. *J. Chem. Ecol.* 29: 763–770.

SHELTON, A. L. 2005. Within-plant variation in glucosinolate concentrations of *Raphanus sativus* across multiple scales. *J. Chem. Ecol.* 31:1711–1732.

SOKAL, R. R., and ROHLF, F. J. 1994. Biometry. Freeman, New York.

SOLER, R., BEZEMER, T. M., VAN DER PUTTEN, W. H., VET, L. E. M., and HARVEY, J. A. 2005. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.* 74:1121–1130.

SOLER, R., BEZEMER, T. M., CORTESERO, A. M., VAN DER PUTTEN, W. H., VET, L. E. M., and HARVEY, J. A. 2007. Impact of foliar herbivory on the development of a root-feeding insect and its parasitoid. *Oecologia* 152:257–264.

SPRUGEL, D. G., HINCKLEY, T. M., and SCHAAP, W. 1991. The theory and practice of branch autonomy. *Annu. Rev. Ecol. Syst.* 22:309–334.

STOUT, M. J., WORKMAN, K. V., and DUFFEY, S. S. 1996. Identity, spatial distribution, and variability of induced chemical responses in tomato plants. *Entomol. Exp. Appl.* 79:255–271.

TRUDGILL, D. L., and BLOK, V. C. 2001. Apomictic, polyphagous root-knot nematodes: exceptionally successful and damaging biotrophic root pathogens. *Annu. Rev. Phytopath.* 39:53–77.

VAN DAM, N. M., and BEZEMER, T. M. 2006. Chemical communication between roots and shoots: towards an integration of aboveground and belowground induced responses in plants, pp. 127–143, in M. Dicke, and W. Takken (eds.). Chemical Ecology: from Gene to EcosystemSpringer, Dordrecht.

VAN DAM, N. M., and RAAIJMAKERS, C. E. 2006. Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology* 16:17–24.

VAN DAM, N. M., HARVEY, J. A., WÄCKERS, F. L., BEZEMER, T. M., VAN DER PUTTEN, W. H., and VET, L. E. M. 2003. Interactions between aboveground and belowground induced responses against phytophages. *Basic Appl. Ecol.* 4:63–77.

VAN DAM, N. M., RAAIJMAKERS, C. E., and VAN DER PUTTEN, W. H. 2005. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.* 115:161–170.

VEREECKE, D., MESSENS, E., KLARSKOV, K., DE BRUYN, A., VAN MONTAGU, M., and GOETHALS, K. 1997. Patterns of phenolic compounds in leafy galls of tobacco. *Planta* 201:342–348.

VISWANATHAN, D. V. and THALER, J. S. 2004. Plant vascular architecture and within-plant spatial patterns in resource quality following herbivory. *J. Chem. Ecol.* 30:531–543.

VOELCKEL, C., and BALDWIN, I. T. 2004. Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. *Ecol. Lett.* 7:770–775.

VOVLAS, N., SIMOES, N. J. O., SASANELLI, N., DOS SANTOS, M. C. V., and ABRANTES, I. M. D. 2004. Host–parasite relationships in tobacco plants infected with a root-knot nematode (*Meloidogyne incognita*) population from the Azores. *Phytoparasitica* 32: 167–173.

VUORISALO, T., and HUTCHINGS, M. J. 1996. On plant sectoriality, or how to combine the benefits of autonomy and integration. *Vegetatio* 127:3–8.

WARDLE, D. A. 2002. Communities and Ecosystems: Linking the Aboveground and Belowground Components. Princeton University Press, Princeton.

WARDLE, D. A., BARDGETT, R. D., KLIRONOMOS, J. N., SETÄLÄ, H. H., VAN DER PUTTEN, W. H., and WALL, D. H. 2004. Ecological linkages between aboveground and belowground biota. *Science* 304:1629–1633.

WATSON, M. A., and CASPER, B. B. 1984. Morphogenetic constraints on patterns of carbon distribution in plants. *Annu. Rev. Ecol. Syst.* 15:233–258.

WHEELER, T. A., BARKER, K. R., and SCHNEIDER, S. M. 1991. Yield–loss models for tobacco infected with *Meloidogyne incognita* as affected by soil moisture. *J. Nematol.* 23: 365–371.

ZANNE, A. E., LOWER, S. S., CARDON, Z. G., and ORIANS, C. M. 2006. ^{15}N partitioning in tomato: vascular constraints versus tissue demand. *Funct. Plant Biol.* 33:457–464.

leading to drought stress symptoms that influence aboveground herbivores (Masters et al. 1993; Poveda et al. 2005). Plant-mediated interactions between root and shoot herbivores likely depend on the plant and herbivore species, as well as on abiotic factors (Wurst and Van der Putten 2007). However, it is unknown if genetic variation in plant defense traits influences belowground-aboveground interactions.

Plants display genetic variation in traits that influence the performance of herbivores. These resistance traits may respond to abiotic and biotic environmental factors that lead to variable defensive phenotypes (Agrawal and Karban 1999). So far, it is largely unknown whether the plant genotype affects the outcome of plant-mediated interactions between root and shoot herbivores. Moran and Whitham (1990) documented that plant resistance to leaf galling abolishes the negative impact of a leaf galling aphid on a root-feeding aphid on *Chenopodium album*. However, it remained unclear whether this effect was mediated by a difference in plant response or by the smaller number of leaf galling aphids on the resistant plants. In general, the way in which a plant responds to herbivores is expected to differ among genotypes and can be considered a trait that is subject to selection. Since root-associated soil organisms may interact with the plant before shoots emerge, root-induced plant responses might have a strong impact on aboveground plant–herbivore interactions and even on higher trophic levels such as predators and parasitoids (Bezemer and Van Dam 2005).

In this study, we investigated the effects of coleopteran root herbivores (*Agriotes* sp. larvae, Elateridae) on secondary plant chemistry of two full-sib families of *Plantago lanceolata* (Plantaginaceae) from lines that were selected for low and high levels of defensive compounds (iridoid glycosides [IG]). IG are known to deter generalist insect herbivores (Bowers and Puttick 1988) and pathogens (Marak et al. 2002a). However, they are used as feeding and oviposition stimulants by specialist herbivores (Bowers 1983). Production of IG can be induced both by herbivores (Darrow and Bowers 1999) and pathogens (Marak et al. 2002b).

Additionally, we investigated whether the production of aboveground volatiles differed between the plant lines and between root herbivore-induced and noninduced plants. While IG may serve as direct defense compounds against herbivores, leaf volatiles may attract carnivorous enemies of herbivores as an indirect defense mechanism (Dicke 1999). Recently, insect root herbivores were shown to change the release of leaf volatiles, which affects aboveground parasitoid behavior (Soler et al. 2007). In a field assay, we monitored leaf damage of root herbivore-induced and noninduced plants of the two lines. The results indicate that plant responses to root herbivory differ between plant lines leading to different defensive phenotypes that aboveground herbivores have to deal with.

Methods and Materials

Experiments were performed with seeds of two full-sib families of *Plantago lanceolata* L. from lines artificially selected for low and high levels of IG (Marak et al. 2000). After four generations, leaf IG concentrations of the "low-IG line" were in average twofold to fourfold lower than of the "high-IG," depending on environmental conditions (Marak et al. 2000, 2003; Biere et al. 2004). The "low-IG" and "high-IG" full-sib families used in the current experiments were the offspring of crosses between two (self-incompatible) parents from the "low-IG line" and between two parents from the "high-IG line", respectively.

On 19 May 2006, seeds of the two *Plantago* lines were surface-sterilized with potassium hypochlorite solution (1%) and sown on wet paper in Petri dishes in the greenhouse (16 h light, 20°C/25°C night/day temperature). Twelve days after sowing, germinated plants were transplanted into seedling trays filled with gamma-sterilized (25 kGy) experimental soil. The experimental soil was a loamy, sandy mineral soil (N=0.13%, C=2.1%, C/N=16.7) that had been sieved through a 0.5-cm mesh.

Experimental Set-Up A total of 48 pots (11.5 cm height, 13 cm diameter) filled with 1,300 g nonsterile experimental soil were placed in a greenhouse with 16 h light and 20°C/25°C night/day temperature. *P. lanceolata* plants with two to three leaves (except the cotyledons) were planted from the seedling trays into the pots on 13 June (day 1 of the experiment). Half the pots were planted with one plant from the "low-IG line," the other half with one plant from the "high-IG line." Two weeks later, two *Agriotes* larvae (fresh biomass added: mean=45.14 mg, SE=1.62) were added to half of the pots of each IG line. *Agriotes* larvae (wireworms) are abundant root herbivores in grasslands, feed on a wide range of plant species, and have a long life cycle with the larval stage taking 4–5 years (Staley et al. 2007). The set-up resulted in a full factorial experimental design with six replicates per treatment and harvest time (two harvests).

The pots were watered with 50 mL demineralized H_2O every second day and redistributed randomly within the greenhouse every second week. In week 8 of the experiment, the stalks were cut and frozen at −80°C to prevent a possible influence of inflorescence odor on the subsequent leaf volatile measurements.

In week 9, the volatile organic compounds (VOC) of 40 experimental plants were measured (ten replicates per

treatment; replicates of harvest 1 and 2 were combined). Plants were placed under 17 L bell-shaped glass cylinders (30 cm height, 24.5 cm diameter) in a controlled climate cabinet (21°C, 72% relative humidity). The glass cylinders were supplied constantly with 300 mL pressurized air (Hoekloos, NL) from the top. The air was cleaned by a Zero Air generator to remove hydrocarbons (Parker Hannifin, Tewksbury, MA, USA). Plant VOC were collected from a potted plant by using a steel trap filled with 150 mg Tenax TA and 150 mg Carbopack B. The trap was connected to a vacuum pump (flow rates 200 mL/min). Traps were removed after 1 h from the pump and kept for approximately 1 week at 5°C until analysis. For control, we measured background VOC profiles from empty glass cylinders (N=2) and VOC profiles from a pot with soil but without a plant inside the cylinder (N=2).

In week 10, half of the plants (N=24) were harvested. Wireworms were collected and weighed during root washing. Since wireworms might have affected colonization of roots by arbuscular mycorrhizal fungi (AMF) with subsequent effects on leaf chemistry and herbivore damage (Gange et al. 1994; Gange 2001; Gange and West 1994), random subsamples of approximately 1.5 g fresh roots were taken, stored in ethanol (50%), stained, and assessed for mycorrhizal fungi colonization (for details see Wurst et al. 2004). Shoots and roots were frozen at −80°C, freeze-dried, weighed, and subsamples were ground for analyses of N and C, glucose, and IG.

The other half of the pots (N=24) was placed in a randomized block design outside on a meadow to expose them to natural herbivory from June until November (field experiment). Fourteen weeks later, these plants were harvested. The leaves of these plants were counted, and their size and extent of herbivore damage (area of holes eaten from the leaf area) were recorded with WinFOLIA (Regent Instruments, Sainte-Foy, Canada). The leaves and roots were frozen at −80°C, freeze-dried, and weighed.

Chemical Analyses Volatiles (VOC) were desorbed from the steel traps by using an automated thermodesorption unit (model Unity, Markes, Pontyclun, UK) at 200°C for 10 min (He flow 30 mL/min) and focused on a cold trap (−10°C). After 1 min of dry purging, trapped volatiles were introduced into the gas chromatograph–mass spectrometer (model Trace, ThermoFinnigan, Austin, TX, USA) by heating the cold trap for 3 min to 270°C. Split rate was set to 1:4, and the column used was a 30-m×0.32-mm ID RTX-5 Silms, film thickness 0.33 μm. Temperature raised from 40°C to 95°C at 3°C/min, then to 165°C at 2°C/min, and finally to 250°C at 15°C/min. The VOC were detected by the mass spectrometer operating at 70 eV in EI mode. Mass spectra were acquired in full scan mode (33–300 AMU, three scans per second). Compounds were identified (a) by their mass spectra by using deconvolution software (AMDIS) and comparison with mass spectra from NIST 98 and Wiley 7th edition spectral libraries, (b) by their linear retention indices and comparison with values reported in the literature (C.A. Hordijk, personal database), and (c) by analyzing reference substances commonly reported to be detected in plant volatile profiles (octanal, nonanal, decanal, limonene, benzylcyanide, *cis*-3-hexenyl acetate, *cis*-3-hexen-1-ol, dimethyl trisulfide [Sigma-Aldrich, St Louis, MO, USA], methyl salicylate [Merck, Darmstadt, Germany], *cis*-3-hexenyl isobutyrate [Oxford Chemicals, Harlow, Essex, UK], and 4,8-dimethyl 1,3,7-nonatriene [kindly provided by Dr. TA Van Beek, Wageningen University, The Netherlands]) and comparing their mass spectra with those of the compounds detected. Peak areas of identified compounds were integrated by the Xcalibur software (Version 1.3, Finnigan). To exclude potential interference by coeluting compounds, specific quantifier ions were carefully selected for each individual compound of interest. In general, these quantifier ions were similar to the most intense model ions extracted from the raw mass spectrum by AMDIS. The integrated absolute signal of the quantifier ion(s) were used for comparison between the treatments. Peak areas in each sample were divided by the total volume in milliliters (calculated as total volume= $(\text{flow}_{\text{begin}}+\text{flow}_{\text{end}})/2\times$sampling time in minutes) that was sampled over the trap. This procedure corrects for differences in flow rates over individual traps that arose during the sampling procedure and minor difference in sampling times between replicate sets.

IG and glucose from 25 mg freeze-dried and ground root samples were extracted overnight with 70% methanol. The concentrations of the IG aucubin and catalpol were analyzed using a Dionex (Sunnyvale, CA, USA) BioLC equipped with a GP50 gradient pump, a Carbopac PA1 anion-exchange guard (2×50 mm) and analytical column (2×250 mm), and an ED50 electrochemical detector for pulsed amperometric detection. Compounds were eluted with NaOH (1 M) and ultrapure water (10%:90%). Retention times were 3.5 and 5.0 min for aucubin and catalpol, respectively. Concentrations were analyzed with Chromeleon Software Release 6.60 (Dionex). Contents of total N and C in leaves and roots were measured by using a C/N analyzer (Flashea Series1112, Interscience, Breda, NL, USA).

Statistical Analyses Data were analyzed by factorial analyses of variance (ANOVA) in a general linear model (GLM, Statistica 6.0, Statsoft) with the class factors "*Plantago* line" and "wireworms." The data were tested for normality (Kolmogorov–Smirnov one-sample test) and homogeneity of variances (Levene test) and log-transformed or arcsine transformed (for percentage data) if necessary. Volatiles

were analyzed by a redundancy analysis (RDA) followed by a Monte Carlo 999 permutation test in CANOCO for Windows 4.5. The linear method RDA was justified by the length of the gradients being shorter than 3.0 in a detrended canonical correspondence analysis (Lepš and Šmilauer 2003). Only volatiles that were not detected in the background controls (i.e., empty glass cylinders) and were detected in at least 70% of the ten replicates of one treatment were used for statistical analysis (Table 1).

Results

Harvest 1 (Greenhouse Experiment) Leaf biomass was greater in plants from the "high-IG line" ($F_{(1,\ 20)}$=21.74, P<0.001) (Fig. 1), while plants from the "low-IG line" produced more inflorescences ("low-IG line": mean=2.50, SE=0.26; "high-IG line": mean=1.00, SE=0.17; $F_{(1,\ 44)}$= 23.53, P<0.001) and higher reproductive biomass ("low-IG line": mean=0.41 g dry weight, SE=0.03; "high-IG line": mean=0.15 g dry weight, SE=0.02; $F_{(1,\ 37)}$=33.74, P< 0.001). Note, however, that the inflorescences were cut in week 8 (prior to VOC analyses), while the leaves and roots were harvested in week 10 (after VOC analyses). Overall, the total aboveground biomass did not differ between the lines. Plants from the "high-IG line" had a greater root biomass ($F_{(1,\ 20)}$=8.00, P<0.05), resulting in a greater total biomass than plants from the "low-IG line" ($F_{(1,\ 20)}$=7.18, P<0.05). Wireworms did not affect the biomass of roots and inflorescences, but enhanced the leaf biomass ($F_{(1,\ 20)}$= 7.46, P<0.05) and the total shoot biomass ($F_{(1,\ 20)}$=6.64, P<0.05) (Fig. 1). The effects of wireworm feeding on plant biomasses did not depend on the *Plantago* line.

Carbon concentration (percent dry weight) in leaves was slightly but significantly higher in plants from the "high-IG line" (mean=41.62, SE=0.23) than in plants from the "low-IG line" (mean=40.73, SE=0.34; $F_{(1,\ 20)}$=4.79, P<0.05). Nitrogen concentrations in leaves (mean=1.15, SE=0.11) and roots (mean=0.89, SE=0.16) and carbon concentrations in roots (mean=37.65, SE=0.98) were not affected by the plant line or by wireworms. Glucose concentration in shoots (mean=0.55%, SE=0.07) was unaffected by the treatments, while glucose concentration in roots increased when wireworms fed upon the "high-IG line," but decreased in the "low-IG line" (interaction between *Plantago* line and wireworms: $F_{(1,\ 20)}$=6.86, P<0.05; Fig. 2).

Catalpol concentration (percent dry weight) in leaves was higher in the "high-IG line," but only in the absence of wireworms. When wireworms were present, plants from the "high-IG line" reduced leaf catalpol concentrations, while plants from the "low-IG line" showed enhanced concentration of this IG in leaves (interaction between *Plantago* line and wireworms: $F_{(1,\ 20)}$=9.21, P<0.01; Fig. 3). The same pattern was observed for the concentration of total IG (aucubin+catalpol) in leaves (interaction between *Plantago* line and wireworms: $F_{(1,\ 20)}$=6.36, P<0.05). The leaf aucubin concentration did not differ significantly between treatments (mean=2.86%, SE=0.34), but tended to show the same pattern (interaction between *Plantago* line and wireworms: $F_{(1,\ 20)}$=3.62, P=0.07). In roots of both lines, the catalpol concentration was enhanced in the presence of wireworms ($F_{(1,\ 20)}$=4.85, P<0.05; Fig. 3). The concentrations of aucubin (mean=2.52%, SE=0.18) and total IG (mean=2.89%, SE=0.19) in roots were not significantly affected by wireworms or plant line.

Colonization of roots by AMF (mean=41.17%, SE= 2.24) was not affected by plant line or wireworms. Recovery rates of wireworms at harvest 1 were as follows: 37.5% as larvae, 12.5% as pupae, and 12.5% as adult beetles. In 83.33% of the wireworm-treated pots, at least one *Agriotes* individual was recovered. Since the *Agriotes* specimens recovered were of different life stages, no potential effect of the plant line on wireworm weight could be measured.

Table 1 Volatiles of *Plantago lanceolata* that were detected in ≥70% of the ten replicates of at least one treatment and not present in the background controls

Terpenes (RI)	Green leaf volatiles (RI)	Others (RI)
cis-β-ocimene (1048)	*cis*-3-hexen-1-yl-acetate (1010)	2-Methyl furan (0604)
(3-*Trans*)-4,8-dimethyl-1,3, 7-nonatriene (1117)	1-Hexyl-acetate (1015)	Heptanoic acid (1081)
		2-Nonanone (1091)
β-elemene (1385)		Octanoic acid (1184)
cis-caryophyllene (1408)		Nonanoic acid (1274)
Trans-α-bergamotene (1429)		
Unknown sesquiterpene (1434)		
Unknown sesquiterpene (1439)		
Trans-β-farnesene (1454)		
(δ or γ)-Cadinene (1511)		
Germacrene A (1494)		

RI retention index

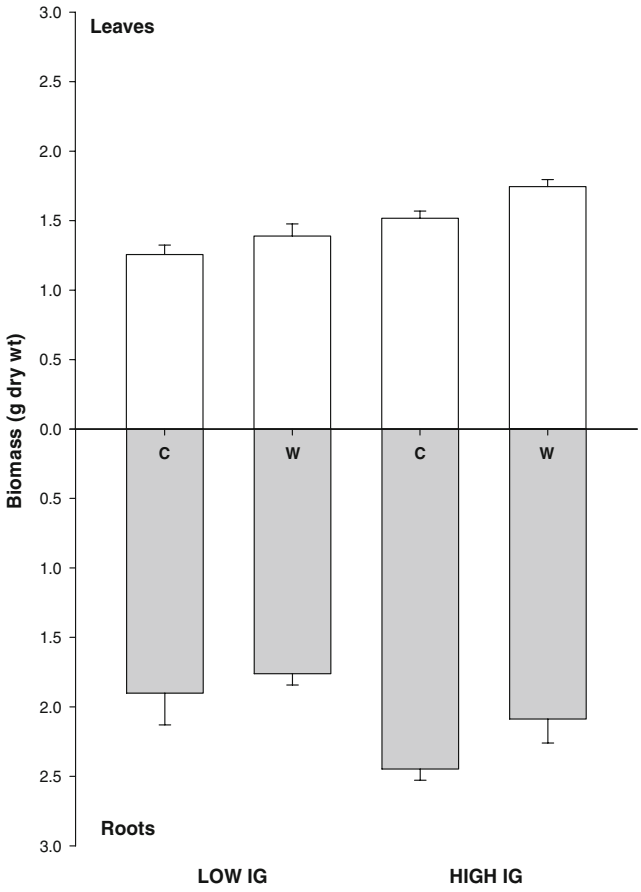

Fig. 1 Effects of plant line (low IG and high IG) and wireworms (*C* without wireworms, *W* with wireworms) on the biomass (mean+SE) of roots (*gray*) and leaves (*white*) of *Plantago lanceolata*. For the significant main and interaction effects of ANOVA, see text

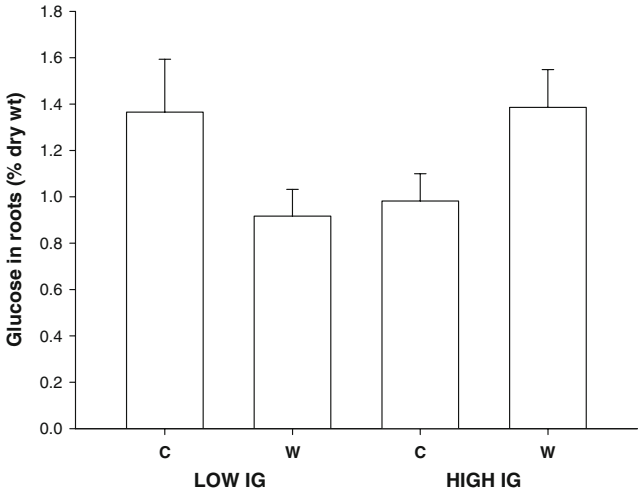

Fig. 2 Effects of plant line (low IG and high IG) and wireworms (*C* without wireworms, *W* with wireworms) on the glucose concentration (mean+SE) in roots of *Plantago lanceolata*. Significant interaction between *Plantago* line and wireworms ($F_{(1,\ 20)}$=6.86, P<0.05), ANOVA

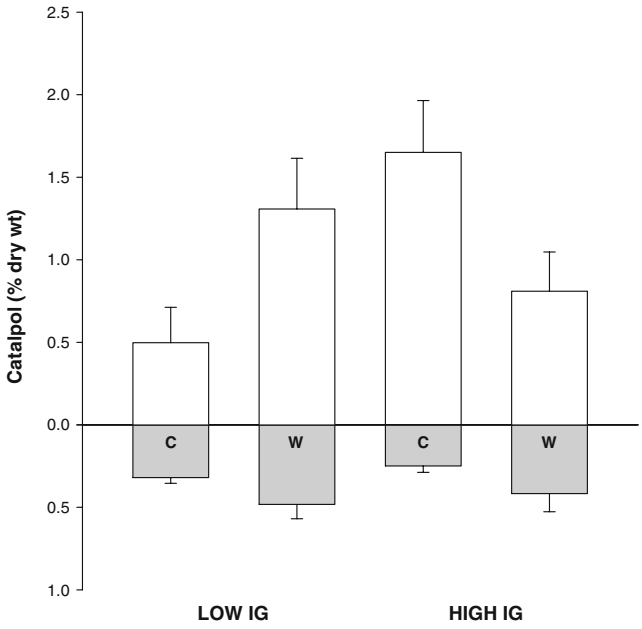

Fig. 3 Effects of plant line (low IG and high IG) and wireworms (*C* without wireworms, *W* with wireworms) on the concentration of catalpol (mean+SE) in shoots (*white*) and roots (*gray*) of *Plantago lanceolata*. For the significant main and interaction effects of ANOVA, see text

Volatiles The RDA showed that the environmental variable "*Plantago* line" explained 14.5% of the total variability in volatile emission (999 Monte Carlo permutations test: F= 6.42, P<0.01; Fig. 4). No effects of wireworms or an interaction between wireworms and *Plantago* line on the volatile blends were detected. Germacrene A and β-elemene were only detected in "high-IG" plants. Cadinene (either the gamma- or the delta-isomer), an unknown sesquiterpene with retention index 1434, and *cis*-3-hexen-1-yl-acetate were omitted in higher quantities from "high-IG" plants. When corrected for leaf biomass, the emission of *cis*-3-hexen-1-yl-acetate and cadinene did not differ between the plant lines, while the unknown sesquiterpene was still emitted in higher quantities from "high-IG" plants.

Harvest 2 (Field Experiment) After more than 3 months in the field, the "low-IG line" and the "high-IG line" did not differ in root (mean=2.12 g, SE=0.07), remaining leaf (mean=1.07 g, SE=0.05), and total biomass (mean=3.19 g, SE=0.10) and were no longer affected by the wireworms. While the number of leaves (mean=24.96, SE=2.03) did not differ between lines, the rosette and average leaf area of plants from the "high-IG line" were 32% and 40% larger than rosette and average leaf area of plants from the "low-IG line," respectively (rosette area: $F_{(1,\ 20)}$=4.44, P<0.05; leaf area: $F_{(1,\ 20)}$=7.38, P<0.05). Total leaf damage (measured as the hole area within the leaf area) of the plants was enhanced in wireworm-induced "low-IG" plants,

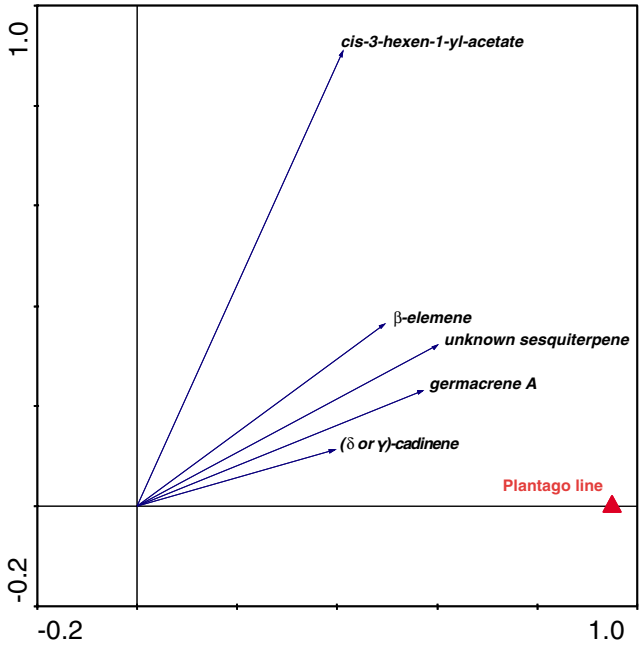

Fig. 4 Species–environment biplot from RDA summarizing differences in the leaf volatile compounds between the *Plantago* lines studied. The substrates shown are well-fitted by the sample scores on the first (*horizontal*) ordination axis, i.e., at least 14.5% of their variability is explained by the *Plantago* line. The second (*vertical*) ordination axis represents the residual variance

but tended to be reduced in "high-IG" plants affected by wireworms (interaction between *Plantago* line and wireworms: $F_{(1,\ 20)}=5.35$, $P<0.05$; Fig. 5). From the added wireworms, 12.5% were recovered as larvae and 4.2% as imagines.

Discussion

Both *Plantago* lines responded to wireworms by increasing the levels of catalpol in roots, thus indicating a defense response upon root herbivory. Induction of secondary metabolites in roots due to root feeding by insect larvae has been reported for a number of plant and herbivore combinations (Birch et al. 1992; Bezemer et al. 2003, 2004; Borowicz et al. 2003; Van Dam and Raaijmakers 2006) and might be a general plant response similar to aboveground induction due to shoot herbivores (Karban and Baldwin 1997). Irrespective of plant line, wireworms had no significant effect on root biomass, but led to an increase in leaf biomass, pointing to compensatory shoot growth (Wurst and Van der Putten 2007). However, the indirect effect of wireworms on leaf chemistry differed between plant lines. While the "low-IG line" showed an increase of catalpol concentration in leaves in response to wireworms, the "high-IG lines" responded with a reduction. By contrast, wireworm feeding led to a reduction of glucose concentration in roots of the "low-IG line," but to an increase of glucose concentration in the roots of the "high-IG line." Whether the contrasting patterns observed for catalpol and glucose are related to a trade-off response in the plants is unclear. The carbon and nitrogen concentrations and the AMF colonization of roots were not affected by wireworms. Consistently, another insect root herbivore (*Otiorhynchus sulcatus*—Coleoptera: Curculionidae) did not affect AMF colonization of roots (Gange et al. 1994; Gange 2001)

The leaf volatile profiles differed between the plant lines, but were not affected by the wireworms. In contrast, Soler et al. (2007) reported changes in leaf volatile profiles of *Brassica nigra* due to root herbivory by *Delia radicum*. The "high-IG line" emitted higher amounts of cadinene and higher amounts of an unknown terpene and the green leaf volatile *cis*-3-hexen-1-yl-actetate. Germacrene A and β-elemene were only detected in the volatile blend of plants from the "high-IG line." The higher emission of the green leaf volatile *cis*-3-hexen-1-yl-acetate and the terpene cadinene could be explained by the greater leaf biomass of the "high-IG line." The other terpenes were emitted only or to a greater extent by the "high-IG" plants, irrespective of the leaf biomass. This might be considered a characteristic trait of the "high-IG line," possibly leading to enhanced indirect defense by attraction of parasitoid or predators. Thus, we found no evidence for a trade-off between direct and indirect defenses in *P. lanceolata*.

Plants from the two lines differed in a number of traits. As expected, noninduced plants of the "high-IG line" contained twofold higher levels of total IG and threefold higher levels of catalpol in the leaves, but levels of these

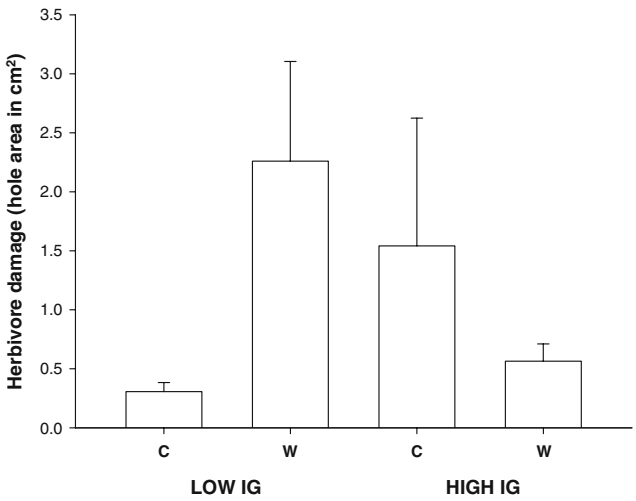

Fig. 5 Effects of plant line (low IG and high IG) and wireworms (*C* without wireworms, *W* with wireworms) on leaf damage by herbivores (mean+SE; measured as total hole area in square centimeter) of *Plantago lanceolata* in the field. Significant interaction between *Plantago* line and wireworms ($F_{(1,\ 20)}=5.35$, $P<0.05$), ANOVA

compounds in roots were similar between lines. Leaf carbon concentration was generally higher in the "high-IG line," while the carbon concentration in roots and the nitrogen concentration in roots and shoots were not affected by the plant line. As in previous studies (Marak et al. 2003), plants from the "high-IG line" produced fewer flower stalks, but produced more root and leaf biomass than plants from the "low-IG line." Former studies also did not detect costs in terms of vegetative biomass for plants producing high levels of IG (Bowers and Stamp 1992; Adler et al. 1995; Marak et al. 2003).

After placing half of the experimental plants for more than 3 months in the field, the root and remaining leaf biomass of the plant lines did not differ and were not affected by the wireworm treatment anymore. However, the rosette area and average leaf area of plants from the "high-IG line" was still greater compared to the "low-IG line." Leaf damage by naturally occurring herbivores was enhanced in wireworm-induced plants from the "low-IG line." Interestingly, aboveground herbivore damage was reduced in wireworm-induced plants from the "high-IG line." During the field experiment, shoot herbivores such as flea beetles, caterpillars, cicadas, and snails were observed on the plants. Since the "low-IG line" increased leaf catalpol concentrations, while the "high-IG line" decreased them after root herbivory, one of the possible explanations for the observed pattern in leaf damage could be the attraction of specialist herbivores to plants containing higher levels of catalpol (Bowers 1983; Bowers and Puttick 1988). For example, flea beetles (*Longitarsus*) are specialist herbivores of plants containing IG (Willinger and Dobler 2001) and might use these compounds as feeding stimulants.

In summary, the two *Plantago* lines examined both showed compensatory leaf growth and enhanced catalpol concentrations in roots in response to wireworm feeding. However, other physiological plant responses to wireworms differed between the "low-IG line" and the "high-IG line." An interaction between *Plantago* line and root herbivory was found for the catalpol concentration in leaves and the glucose concentration in roots. The different responses of the plant lines to root herbivory led to different aboveground defensive phenotypes which herbivores had to deal with. In the field, greater leaf damage was observed on plants with higher leaf catalpol concentration, possibly caused by some specialist herbivores. The present study documents for the first time that intraspecific variation in plant defense can affect the outcome of plant-mediated interactions between root and shoot herbivores.

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References

ADLER, L. S., SCHMITT, J., and BOWERS, M. D. 1995. Genetic variation in defensive chemistry in *Plantago lanceolata* (Plantaginaceae) and its effect on the specialist herbivore *Junonia coenia* (Nymphalidae). *Oecologia* 101:75–85.

AGRAWAL, A. A., and KARBAN, R. 1999. Why induced defenses may be favored over constitutive strategies in plants, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible DefensesPrinceton University Press, Princeton.

BEZEMER, T. M., and VAN DAM, N. M. 2005. Linking aboveground and belowground interactions via induced plant defenses. *TREE* 20:617–624.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., and WÄCKERS, F. L. 2003. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101:555–562.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WÄCKERS, F. L. 2004. Above- and belowground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30:53–67.

BIERE, A., MARAK, H. B., and VAN DAMME, J. M. M. 2004. Plant chemical defense against herbivores and pathogens: generalized defense or trade-offs? *Oecologia* 140:3430–441.

BIRCH, A. N. E., GRIFFITHS, D. W., HOPKINS, R. J., SMITH, W. H. M., and MCKINLAY, R. G. 1992. Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia floralis*) larvae. *J. Sci. Food Agric.* 60:1–9.

BOROWICZ, V. A., ALBRECHT, U., and MAYER, R. T. 2003. Effects of nutrient supply on citrus resistance to root herbivory by *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae). *Environ. Entomol.* 32:1242–1250.

BOWERS, M. D. 1983. The role of iridoid glycosides in host-plant specificity of checkerspot butterflies. *J. Chem. Ecol.* 9:475–493.

BOWERS, M. D., and PUTTICK, G. M. 1988. Response of generalist and specialist insects to qualitative allelochemical variation. *J. Chem. Ecol.* 14:319–334.

BOWERS, M. D., and STAMP, N. E. 1992. Chemical variation within and between individuals of *Plantago lanceolata* (Plantaginaceae). *J. Chem. Ecol.* 18:985–995.

DARROW, K., and BOWERS, M. D. 1999. Effects of herbivore damage and nutrient level on induction of iridoid glycosides in *Plantago lanceolata*. *J. Chem. Ecol.* 25:61427–1440.

DICKE, M. 1999. Evolution of induced indirect defense of plants, in R. Tollrian, and C. D. Harvell (eds.). The Ecology and Evolution of Inducible DefensesPrinceton University Press, Princeton.

GANGE, A. C. 2001. Specie-specific responses of a root- and shoot-feeding insect to arbuscular mycorrhizal colonization of its host plant. *New Phytol.* 150:611–618.

GANGE, A. C., and WEST, H. M. 1994. Interactions between arbuscular mycorrhizal fungi and foliar-feeding insects in *Plantago lanceolata* L. *New Phytol.* 128:79–87.

GANGE, A. C., BROWN, V. K., and SINCLAIR, G. S. 1994. Reduction of black vine weevil larval growth by vesicular–arbuscular mycorrhizal infection. *Entomol. Exp. Appl.* 70:115–119.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. The University of Chicago Press, Chicago.

LEPŠ, J., and ŠMILAUER, P. 2003. Multivariate analysis of ecological data using CANOCO. Cambridge University Press, Cambridge.

MARAK, H. B., BIERE, A., and VAN DAMME, J. M. M. 2000. Direct and correlated responses to selection on iridoid glycosides in *Plantago lanceolata* L. *J. Evol. Biol.* 13:985–996.

MARAK, H. B., BIERE, A., and VAN DAMME, J. M. M. 2002a. Two herbivore-deterrent iridoid glycosides reduce the in-vitro growth of a specialist but not of a generalist pathogenic fungus of *Plantago lanceolata* L. *Chemoecology* 12:185–192.

MARAK, H. B., BIERE, A., and VAN DAMME, J. M. M. 2002b. Systemic, genotype-specific induction of two herbivore-deterrent iridoid glycosides in *Plantago lanceolata* L. in response to fungal infection by *Diaporthe adunca* (Rob.) Niessel. *J. Chem. Ecol.* 28:2429–2448.

MARAK, H. B., BIERE, A., and VAN DAMME, J. M. M. 2003. Fitness costs of chemical defense in *Plantago lanceolata* L.: effects of nutrient and competition stress. *Evolution* 57:2519–2530.

MASTERS, G. J., BROWN, V. K., and GANGE, A. C. 1993. Plant mediated interactions between above- and below-ground insect herbivores. *Oikos* 66:148–151.

MORAN, N. A., and WHITHAM, T. G. 1990. Interspecific competition between root-feeding and leaf-galling aphids mediated by host-plant resistance. *Ecology* 71:1050–1058.

POVEDA, K., STEFFAN-DEWENTER, I., SCHEU, S., and TSCHARNTKE, T. 2005. Effects of decomposers and herbivores on plant performance and aboveground plant–insect interactions. *Oikos* 108:503–510.

SOLER, R., BEZEMER, T. M., VAN DER PUTTEN, W. H., VET, L. E. M., and HARVEY, J. A. 2005. Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. *J. Anim. Ecol.* 74:1121–1130.

SOLER, R., HARVEY, J. A., KAMP, A. F. D., VET, L. E. M., VAN DER PUTTEN, W. H., VAN DAM, N. M., STUEFER, J. F., GOLS, R., HORDIJK, C. A., and BEZEMER, T. M. 2007. Root herbivores influence the behavior of an aboveground parasitoid through changes in plant-volatile signals. *Oikos* 116:367–376.

STALEY, J. T., HODGSON, C. J., MORTIMER, S. R., MORECROFT, M. D., MASTERS, G. J., BROWN, V. K., and TAYLOR, M. E. 2007. Effects of summer rainfall manipulations on the abundance and vertical distribution of herbivorous soil macro-invertebrates. *Eur. J. Soil Biol.* 43:189–198.

VAN DAM, N. M., and RAAIJMAKERS, C. E. 2006. Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology* 16:17–24.

VAN DAM, N. M., HARVEY, J. A., WÄCKERS, F. L., BEZEMER, T. M., VAN DER PUTTEN, W. H., and VET, L. E. M. 2003. Interactions between aboveground and belowground induced responses against phytophages. *Basic Appl. Ecol.* 4:63–77.

VAN DAM, N. M., RAAIJMAKERS, C. E., and VAN DER PUTTEN, W. H. 2005. Root herbivory reduces growth and survival of the shoot feeding specialist *Pieris rapae* on *Brassica nigra*. *Entomol. Exp. Appl.* 115:161–170.

VAN DER PUTTEN, W. H., VET, L. E. M., HARVEY, J. A., and WÄCKERS, F. L. 2001. Linking above- and belowground multitrophic interactions of plants, herbivores, pathogens, and their antagonists. *TREE* 16:547–554.

WILLINGER, G., and DOBLER, S. 2001. Selective sequestration of iridoid glycosides from their host plants in *Longitarsus* flea beetles. *Biochem. Syst. Ecol.* 29:335–346.

WURST, S., and VAN DER PUTTEN, W. H. 2007. Root herbivore identity matters in plant-mediated interactions between root and shoot herbivores. *Basic Appl. Ecol.* 8:491–499.

WURST, S., DUGASSA-GOBENA, D., LANGEL, R., BONKOWSKI, M., and SCHEU, S. 2004. Combined effects of earthworms and vesicular–arbuscular mycorrhizas on plant and aphid performance. *New Phytol.* 163:169–176.

largely responsible for the characteristic flavors of crucifers. The glucosinolate-myrosinase system is regarded as an important defense strategy for the Brassicaceae as it may protect plants from some generalist pests and pathogens (Giamoustaris and Mithen 1995). However, glucosinolates and their breakdown products also act as stimulants and attractants for insects specialized on brassicaeous hosts (Roessingh et al. 1992; van Loon et al. 1992; Renwick et al. 2006). Many studies to date have concentrated solely on the effect of herbivory on glucosinolate induction in plants. An increase in total glucosinolates, especially those containing an indole-based side chain, has often been observed in plant tissues after herbivore damage (Koritsas et al. 1991; Bartlet et al. 1999; van Dam and Raaijmakers 2006).

In addition to the stress caused by herbivory, other factors also affect glucosinolate concentrations in plants including competition for light, nutrients, and water (Yosuf and Collins 1998; Radovich et al. 2005). While the effects of herbivory have received much attention, little work has been done to establish the effect of plant–plant interactions on the concentrations of secondary compounds in brassicaceous plants; even less effort has been made to investigate the combined effect of herbivory and plant competition. When subjected to herbivory, plants growing in competitive environments may not have access to, or allocate, the same amount of resources towards the synthesis of secondary compounds. Theory predicts that the biological costs of plant defense should increase in environments that expose plants to intense competition (Siemens et al. 2002). The growth-differentiation balance (GDB) hypothesis predicts how plants balance the processes that allocate resources between growth and differentiation depending on environmental conditions (Herms and Mattson 1992). One consequence of this may be that brassicaceous plants grown in highly competitive environments allocate a lower proportion of their resources to the production of defensive compounds, in this case, glucosinolates. Because of reduced attacks from pest insects (Risch 1983; Theunissen 1997) and infestations of specialized insects on brassicaceous plants (Theunissen et al. 1995; Finch and Kienegger 1997; Björkman et al. 2007), intercropping has received increased attention. The main mechanism for pest reduction in intercropped systems is thought to be a disruption of host-plant searching behavior of the herbivore (Finch and Collier 2000) and possibly an enhanced effect of natural enemies suppressing the negative effects of the pest insect (Root 1973). In addition, intercropping often creates an environment where crops have to compete with companion plants for mutual resources. The changes, brought about in the crop plants by the competitive environment, are also likely to affect herbivores in intercropping systems.

It is of interest to investigate how the glucosinolate content of plants is impacted by herbivory in a competitive environment, such as intercropping, due to the role of these chemicals in plant defense. The present study examined the combined effect of intercropping with red clover and larval feeding by the turnip root fly, *Delia floralis* Fall. (Diptera: Anthomyiidae), on the glucosinolate content of cabbage (*Brassica oleracea* var. *capitata*). The aims were to investigate how the concentration of total and individual glucosinolates in both above and below ground cabbage tissues were affected by (1) competition from different densities of clover, (2) the damage of different densities of *D. floralis*, and (3) whether the co-occurrence of simultaneous stress from clover competition and *D. floralis* herbivory had any additional affects on glucosinolate concentrations.

Methods and Materials

Plant Material Plant material was grown in a greenhouse, under natural light supplemented with 400-W sodium lamps to give an 18:6 h L/D regime. Cabbage (*B. oleracea* var. *capitata* genotype Castello) and red clover (*Trifolium pratense* genotype Betty) were planted in small pots (50×50×50 mm) containing potting soil (Hasselfors Garden AB, Hasselfors, Sweden). Seven-week-old clover plants and 5-week-old cabbage plants were transplanted into plastic boxes (L×W×D=360×255×215 mm) filled with 15 L of a sand-potting soil mixture (one third washed sand − grain size 0.5 to 5 mm and two thirds potting soil).

Insect Material A culture of the turnip root fly, *D. floralis* Fall. (Diptera: Anthomyiidae), was established at the Department of Ecology (Swedish University of Agricultural Sciences) from field-collected pupae [collected in 2002–2003 from an experimental site growing cabbage at Röbäcksdalen, Umeå (63°45′ N; 20°15′ E)]. Adult flies were kept at 20°C, 70% RH, 16:8 h L/D regime and fed with a standardized diet of milk, yeast-powder, and honey (based on Finch and Coaker 1969). A Petri dish containing a piece of fresh swede (*Brassica napus* ssp. *rapifera*) was placed on top of moist sand (grain size 1.0–1.6 mm) as an oviposition site. Eggs were floated from the sand with water and sieved through a black cloth. Eggs were taken from the cloth and inoculated onto the plants with a fine paintbrush.

Experimental Procedure Cabbage plants were transplanted into the boxes alone (M) or with one of the two clover densities (IC1 or IC2). Clover plants were transplanted into the boxes at either one row (IC1) of four plants on each side (10 cm from) of the cabbage, or two rows (IC2) of four plants (a total of eight plants) on each side of the cabbage. The boxes were left for 2 weeks in the greenhouse to allow cabbage and clover plants to establish.

The experiment consisted of ten blocks with one block set up each day for ten successive days. Within each block, there were four cabbage plants grown with each clover density (M, IC1, and IC2). Throughout the experiment, the greenhouse was kept at 17°C during the day and 12°C during the night. Light conditions were natural light, supplemented with 400-W sodium lamps when natural light was below 100 W/m^2, between 6 AM and 9 PM. After 2 weeks of plant establishment *D. floralis* eggs (<24-h old; either 0, 10, 20, or 40) were inoculated on each of the four cabbage plants within the three growing conditions, leading to a total of 12 different treatments within each block. As there were ten blocks, each treatment was replicated ten times. Eggs where inoculated onto the stem base of cabbage plants with a fine brush according to the methods of Birch et al. (1992). At the time of egg inoculation, cabbage plants were at the 6–7 true leaf stage.

Throughout the experiment, only pure water was added to the boxes to create a competitive environment in terms of space, light, and nutrients in the intercropped treatments. Plants were watered according to their requirements, i.e., boxes with clover were given more water so that drought did not influence survival of the turnip root fly larvae. Water was added in moderate doses two times a day. For the first week after egg inoculation, a spray bottle was used to keep the soil near the stem base moist to avoid dehydration of unhatched eggs and to avoid eggs from being washed away from the stem base by ordinary watering. There was no significant difference in the percentage pupation of *D. floralis* depending on the density of eggs applied or the density of clover (Björkman, unpublished data). Consequently, the number of eggs applied is equivalent to herbivore density.

Prior testing of *D. floralis* developmental time under similar conditions revealed that the development time was approximately 6 weeks. Therefore, 6 weeks after egg inoculation, the experiment was terminated at a rate of one block per day. Green cabbage parts were cut to ground level and the cabbage roots were freed from soil. A 6–6.4-g foliage sample and a 1.3–1.7-g root sample were cut from each individual plant and immediately frozen in liquid nitrogen, prior to being lyophilized and milled for glucosinolate analysis. Foliage samples were taken from the youngest leaves, while root samples were taken from the main root. Freeze-dried samples from blocks 1–5 and blocks 6–10 were bulked and analyzed together, with the consequence that each treatment was twice replicated in the chemical analyses.

Glucosinolate Identification Glucosinolate analysis was performed by hplc according to Nilsson et al. (2006). Glucosinolates were identified by their retention times (Rt) and comparison with authentic standards. A rapeseed (*B. napus*) standard with added sinigrin was used to validate the Rt for all glucosinolates except for glucoraphanin, for which a radish (*Raphanus sativus*) seed sample was used.

Statistical Analyses The overall analysis of glucosinolate content was performed with separate multivariate analyses of variance (MANOVAs) for foliage and root samples. Total glucosinolate concentration, amount of aliphatic or indole glucosinolates, and individual analyses of glucosinolates were carried out with general linear model (GLM). Intercrop and egg densities were used as variables in the model as well as the squared egg density. The latter density was to reveal nonlinear responses to an increase in damage or plant competition. Data for root gluconapin were analyzed by using a log-link to achieve a normal distribution. GLMs were simplified by removing variables that did not improve the explanatory power. This was done by comparing the simplified model with the original model by using an F test (Crawley 2003). Variables with a P values <0.1 were retained in the model (and are presented in the tables). Nonsignificant variables were excluded as long as they were not included in a significant interaction. Excluded variables are marked with a hatched line in tables, and nonsignificant variables that were retained in the model are marked with N.S. As the number of analyses per root and shoot sample was as many as nine for root and foliage glucosinolates, respectively, a Bonferroni correction would give a corrected P value of 0.006 for a 5% risk of making a Type I error. All analyses were made using S-PLUS statistical software (Insightful Corp. Seattle, USA. S-PLUS® 6.0).

Results

Five aliphatic and four indole glucosinolates were found in the plant samples—all nine were present in both foliage and roots. The aliphathic glucosinolates were sinigrin (2-propenyl), gluconapin (3-butenyl), glucoiberin (3-[methylsulphinyl] propyl), progoitrin (2[*R*]-2-hydroxy-3-butenyl), and glucoraphanin (4-[methylsulphinyl] butyl). The indole glucosinolates were glucobrassicin (indol-3-ylmethyl), neoglucobrassicin (1-methoxyindol-3-ylmethyl), 4-hydroxyglucobrassicin (4-hydroxyindol-3-ylmethyl), and 4-methoxyglucobrassicin (4-methoxyindol-3-ylmethyl).

Total glucosinolate concentration of in cabbage foliage did not changed in response to treatment, although there was a tendency toward a decrease in glucosinolate concentration both due to egg and clover densities (Table 1). Total root glucosinolate concentration was decreased by clover density, particularly at high egg densities (Table 2). In monocultured control plants, the concentration of foliage glucosinolates was 26±6 μmol g^{-1} freeze-dried matter (FDM) and in roots

Table 1 Changes in glucosinolate concentrations in cabbage foliage due to *D. floralis* egg density and clover density

	Trivial name	Eggs	Clover	Egg × clover	Egg^2
Aliphatic	Glucoiberin	NS	- - -	- - -	- - -
	Progoitrin	0.03 (−)	0.03 (−)	- - -	- - -
	Glucoraphanin	NS	- - -	- - -	0.066 (+, −)
	Sinigrin	0.053 (−)	- - -	- - -	- - -
	Gluconapin	0.056 (−)	<0.001 (−)	- - -	- - -
	Total Aliphatic	0.059 (−)	- - -	- - -	- - -
Indole	Hydroxyglucobrassicin	- - -	<0.001 (−)	- - -	- - -
	Methoxyglucobrassicin	0.063 (−)	- - -	- - -	- - -
	Glucobrassicin	- - -	<0.01 (−)	- - -	- - -
	Neoglucobrassicin	0.04 (−)	- - -	- - -	- - -
	Total Indole	- - -	0.018 (−)	- - -	- - -
	Total glucosinolates	0.058 (−)	0.053 (−)	- - -	- - -

Table presents *P* values, followed by (+) or (−) to describe the changes in concentrations. When nonlinearity was observed (Egg^2), + and – are used to describe the appearance of the curve. A *dashed line* represents variables excluded from the analysis when they did not improve the explanatory power of the GLM.

10±0.6 μmol g^{-1} FDM. The concentrations of individual glucosinolates were generally higher in foliage than in roots, although this was not the case for hydroxyglucobrassicin or for neoglucobrassicin at high egg densities. MANOVA showed that there was an effect of intercropping density on individual glucosinolates for both foliage (approximately F=2.70; $d.f.$=2; P=0.017) and roots (approximately F=4.60; $d.f.$=2; P<0.001). Root glucosinolates were also affected by egg density (approximately F=5.63; $d.f.$=1; P=0.008). GLM analyses suggested that individual glucosinolates responded variously to treatment, even within the aliphatic and indole groups.

Of the foliage glucosinolates, glucobrassicin and aliphatic sinigrin were the dominant compounds in the foliage of monocultured control plants with concentrations of 12±2 and 10±3 μmol g^{-1} FDM, respectively. Glucobrassicin levels decreased in response to intercropping but were not significantly altered by herbivory. The concentrations were almost identical for cabbage plants grown with high and low densities of clover and intercropping at the different egg densities and resulted in a decrease in glucobrassicin by nearly half. No statistically significant responses were found for sinigrin, although there was a tendency toward lower concentrations in infested plants. Of the remaining aliphatic glucosinolates, gluconapin concentration decreased sharply in intercropped plants (Table 1). This compound was found in small amounts in monocultured control plants (0.69± 0.15 μmol g^{-1} FDM) and had a mean reduction of 54% in intercropped plants for all egg densities (mean of IC1 and IC2). Progoitrin (2.1±0.35 μmol g^{-1} FDM in monocultured control plants) was the second most common aliphatic glucosinolate, and the only glucosinolate in the foliage that responded to both egg and clover density—in both cases with a reduction in concentration (Table 1). Glucoiberin and glucoraphanin were not changed by either turnip root fly damage or intercropping, as was the total concentration of

Table 2 Changes in glucosinolate concentrations in cabbage roots in response to *D. floralis* egg density and clover density

	Trivial name	Eggs	Clover	Egg × clover	Egg^2
Aliphatic	Glucoiberin	0.011	<0.001	- - -	- - -
	Progoitrin	0.043	<0.001	- - -	- - -
	Glucoraphanin	0.007	0.08	- - -	- - -
	Sinigrin	<0.001	0.006	0.006	0.041
	Gluconapin	<0.001	0.003	<0.001	<0.001
	Total Aliphatic	<0.001	0.001	0.008	0.018
Indole	Hydroxyglucobrassicin	NS	<0.001	0.004	- - -
	Glucobrassicin	- - -	0.003	- - -	- - -
	Methoxyglucobrassicin	<0.001	<0.001	- - -	0.042
	Neoglucobrassicin	<0.001	- - -	- - -	0.001
	Total Indole	<0.001	- - -	- - -	0.057
	Total glucosinolates	NS	0.004	0.021	- - -

Table presents *P* values. A *dashed line* represents variables excluded from the analysis when they did not improve the explanatory power of the GLM.

aliphatic glucosinolates (Table 1). The latter was probably influenced by the lack of response by the dominant sinigrin. The concentration of hydroxyglucobrassicin also decreased significantly in intercropped plants. Hydroxyglucobrassicin was a trace glucosinolate component (0.18±0.06 μmol g^{-1} FDM) in monocultured control plants, and decreased by a mean of 53% in intercropped plants at all egg densities (mean of IC1 and IC2). Neoglucobrassicin, the second most common indole glucosinolate (0.89±0.23 μmol g^{-1} FDM in monocultured control plants), decreased in concentration in response to increasing egg densities while the concentration of methoxyglucobrassicin remained unchanged (0.41± 0.15 μmol g^{-1} FDM in monocultured control plants). Similar to glucobrassicin, the dominant glucosinolate in foliage, the total concentration of foliage indole glucosinolates responded to treatment with a decrease with clover density (Table 1).

Root glucosinolates responded differently and in a more complex manner than foliar glucosinolates. Sinigrin was the dominant glucosinolate (6.8±0.3 μmol g^{-1} FDM in monocultured control plants) in roots. Both monocultured and intercropped plant roots contained similar sinigrin concentrations. However, the concentration of sinigrin decreased with egg density in intercropped plants. In monocultured roots, sinigrin concentration was similar to the levels in control plants at all egg densities except for the ten egg treatments where the concentration was somewhat lower. This mixed response caused both an interaction between eggs and clover and a tendency toward a nonlinear response (Table 2, Fig. 1). Progoitrin, another aliphatic glucosinolate, occurred in relatively large amounts in monocultured control plants (1.1±0.2 μmol g^{-1} FDM) and decreased in concentration with increasing clover density (Table 2, Fig. 1). Glucoiberin decreased in concentration in response to both egg and clover densities, while glucoraphanin tended to increase as a result of damage by *D. floralis* (Table 2, Fig. 1). Gluconapin showed a strong nonlinear response as it decreased rapidly with egg inoculation at low

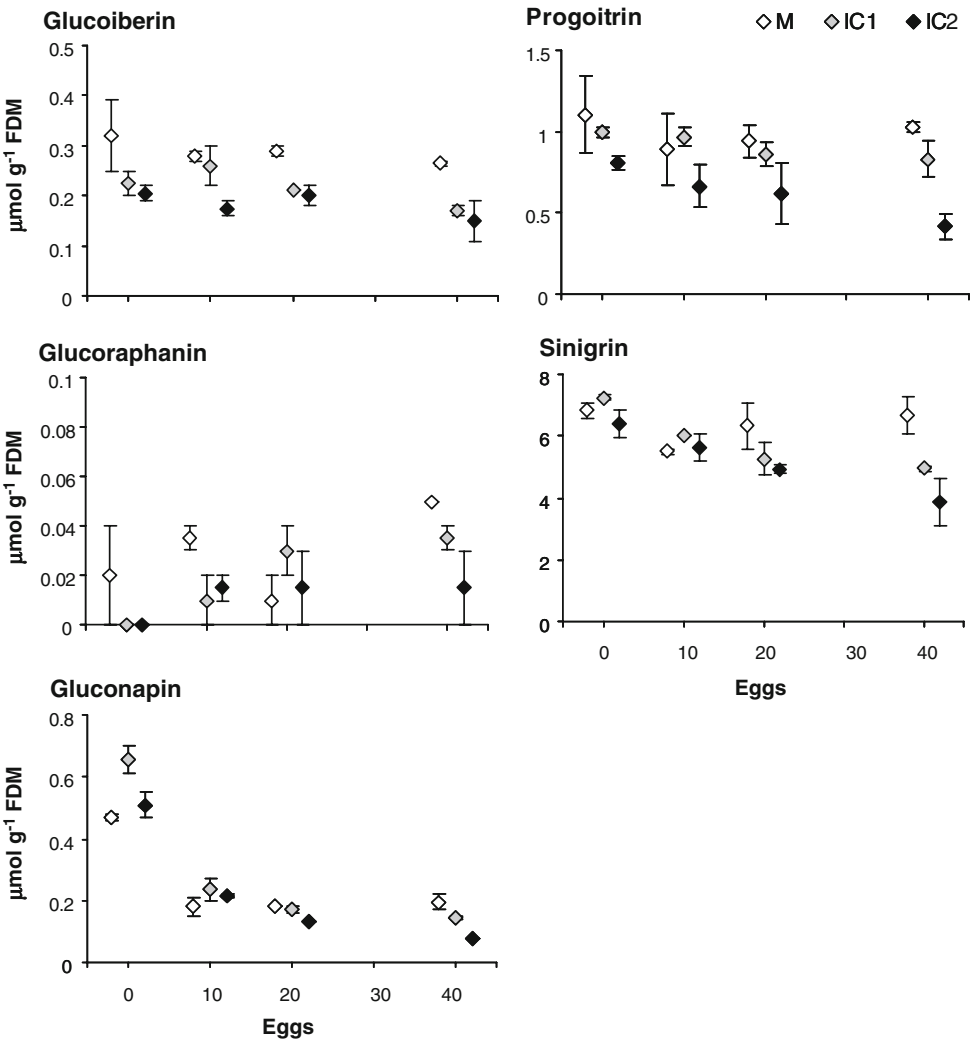

Fig. 1 Changes in concentration of individual aliphatic glucosinolates (μmol g^{-1} FDM) in cabbage roots caused by an increase in clover density (*IC1* and *IC2*) compared with monoculture (*M*), and increasing *D. floralis* egg density (0, 10, 20, and 40 eggs; *N*=2)

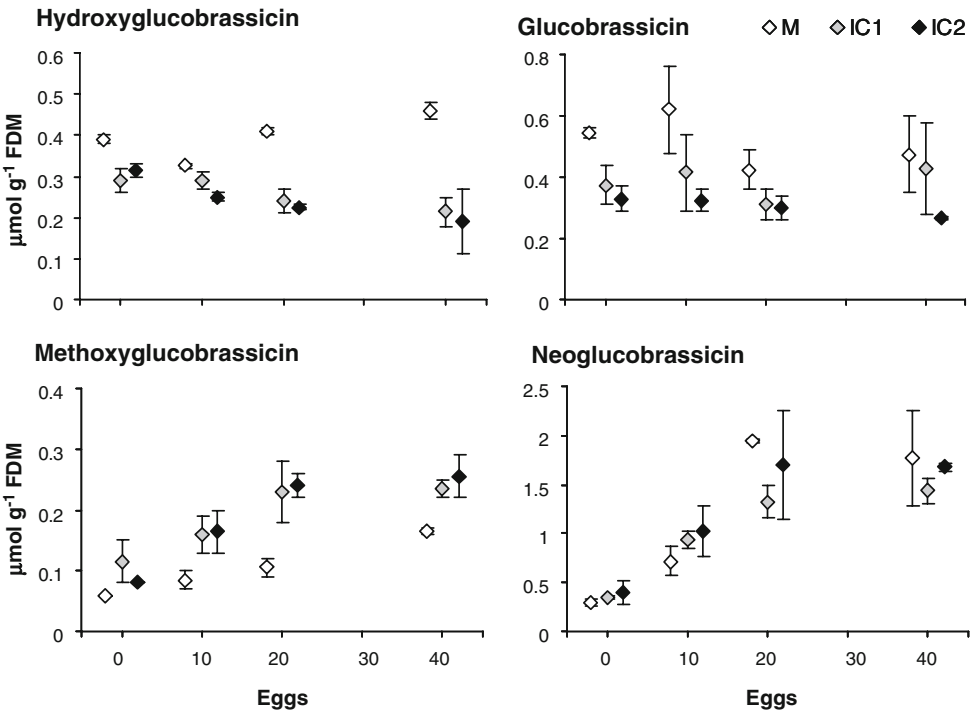

Fig. 2 Changes in concentration of individual indole glucosinolates (μmol g^{-1} FDM) in cabbage roots caused by an increase in clover density (*IC1* and *IC2*) compared with monoculture (*M*), and increasing *D. floralis* egg density (0, 10, 20, and 40 eggs; *N*=2)

egg densities, while additional eggs did not result in much further decreases (Table 2, Fig. 1). The higher concentrations in intercropped control plants and the lower concentrations in intercropped plants with higher egg densities caused an interaction effect between egg and clover (Table 2, Fig. 1). As the aliphatic glucosinolates were dominated by sinigrin, the response of the group as a whole was almost identical to that of sinigrin (Table 2, Figs. 1 and 3).

Of the indole glucosinolates found in roots, neoglucobrassicin responded strongly to egg density and concentrations increased from 0.39±0.01 μmol g^{-1} FDM in monocultured control plants to 1.95±0.03 μmol g^{-1} FDM in monocultured plants at a density of 20 eggs.

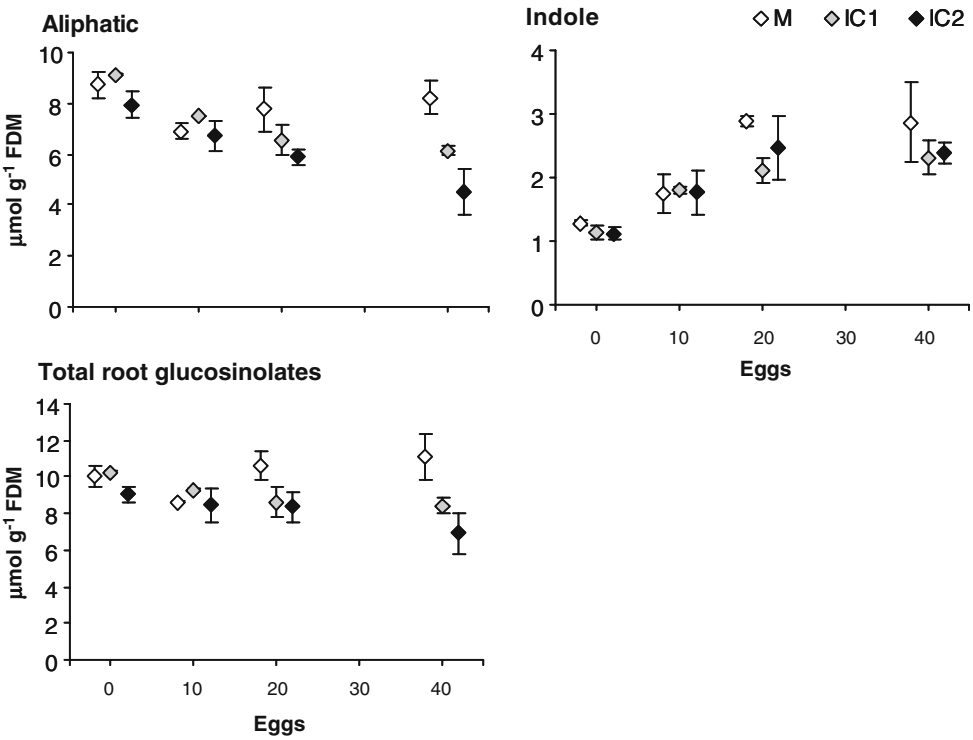

Fig. 3 Changes in concentration of aliphatic, indole, and total glucosinolates (μmol g^{-1} FDM) in cabbage roots caused by an increase in clover density (*IC1* and *IC2*) compared with monoculture (*M*), and increasing *D. floralis* egg density (0, 10, 20, and 40 eggs; *N*=2)

Additional increases did not occur in plants inoculated with 40 eggs, thus, resulting in a significant nonlinear response in monocultured plants (Table 2, Fig. 2). The concentration of hydroxyglucobrassicin had contrasting patterns in cabbage grown in monoculture vs. intercropping. As hydroxyglucobrassicin concentration decreased with egg density at both clover densities, it increased somewhat in the monoculture (Table 2, Fig. 2). The concentration of glucobrassicin was consistently higher when grown in monoculture (Table 2, Fig. 2). Methoxyglucobrassicin increased with egg density. In addition, the concentrations of this glucosinolate were higher in intercropped plants than in monocultured plants (Table 2, Fig. 2). Neoglucobrassicin and methoxyglucobrassicin were the only glucosinolates in roots to respond to egg inoculation with increased concentrations. This means that neoglucobrassicin had opposite reactions in shoots and roots as this glucosinolate tended to decrease with egg density in the foliage. The total concentration of indole glucosinolates increased in response to egg density similar to the neoglucobrassicin, the dominate glucosinolate (Table 2, Figs. 2 and 3).

Discussion

Earlier studies that examined glucosinolate levels in brassicaceous host plants following herbivory by root flies considered larval damage as the sole stress factor. The responses to larval feeding found previously correspond to herbivore-induced changes that occurred in the present study. Indole glucosinolate levels in roots, particularly neoglucobrassicin and glucobrassicin, tend to increase in response to root herbivory in most studies (Birch et al. 1992, 1996; Griffiths et al. 1994; Hopkins et al. 1998a; van Dam and Raaijmakers 2006). Whether or not these studies showed a net increase in the total amount of root glucosinolates seemed to depend on the relative size of the increase in indole glucosinolates vs. the decrease in aliphatic glucosinolates (Birch et al. 1992; Hopkins et al. 1998a). In our study, damage-induced increases in indole glucosinolates and the corresponding decrease in aliphatic glucosinolates did not balance each other out in intercropped roots (Table 2, Fig. 3).

The lack of response to root damage by total leaf glucosinolate content has previously been shown by Birch et al. (1992), although they reported decreases in foliar indole glucosinolates in several brassica cultivars, as also shown for cabbage in this study. It is clear that glucosinolate responses to root fly larval feeding in roots and leaves vary among plant species and genotype (Birch et al. 1992, 1996), as well as on a temporal scale (Birch et al. 1996). Our glucosinolate sampling was performed 6 weeks after egg inoculation at the time of pupation, when larval damage was completed. It is likely that the glucosinolate profile of both roots and leaves may have been different at earlier, or later, sampling dates (Bodnaryk 1992; Griffiths et al. 1994; Birch et al. 1996; van Dam and Raaijmakers 2006).

Besides herbivory, an additional stress factor was added in the present student, namely competition with red clover. The competitive environment within the intercropping is likely to have had consequences for the cabbage plants' ability to defend themselves against *D. floralis* larval feeding. The GDB hypothesis suggests that a plant can either grow or allocate resources to defense, depending on resource availability (Stamp et al. 2004). This should lead to reduced concentrations of defense compounds in competition-stressed plants. The ability of a plant to acquire resources for growth or defense is affected not only by competition but also by root damage, since such damage impairs the plant's ability to acquire resources. Decreased concentrations of several glucosinolates in aerial cabbage parts and the strong tendency towards an overall reduction in glucosinolates (Table 1) suggest that competing plants, as well as plants with root herbivory, do not allocate the same resources as unchallenged plants towards sustaining levels of foliar defense compounds. Total root glucosinolate levels were reduced in intercropped treatments but only at higher egg densities, while glucosinolate levels in monoculture treatments were fairly constant. This suggests that plants subjected to high *D. floralis* infestations are not able to allocate resources to maintain glucosinolate root levels if they are also competing with the clover for light and nutrients. Our findings on secondary compounds in relation to plant competition confirm the results of other studies. For example Stamp et al. (2004) found that foliar concentrations of chlorogenic acid, rutin, and tomatine in tomato (*Lycopersicon esculentum*) decreased with increasing competition. Furthermore, Cipollini and Bergelson (2001) reported decreasing nutrient availability through plant competition-mediated, density-dependent reductions in trypsin inhibitor levels of in *B. napus* seedlings. They later showed that this decrease in defense led to higher levels of leaf herbivory in a field situation (Cipollini and Bergelson 2002). From the results of this study, it is clear that within the usual division of glucosinolates into aliphatic, aromatic, and indole-based, that certain glucosinolates may not respond uniformly to herbivory or plant competition. Even if relevant to describe changes in different glucosinolate groups classified according to structure, this is likely to conceal the fact that individual glucosinolates within each group may respond in different manners. For cabbage roots, this was the case for methoxyglucobrassicin, which, in contrast to other indole glucosinolates, increased in intercropped treatments (see Fig. 2). It was also evident that the changes that occur within a glucosinolate group may be strongly influenced by changes in a single compound

(Griffiths et al. 1994; Hopkins et al. 1998a; van Dam and Raaijmakers 2006). In cabbage foliage, only indole glucosinolates responded to treatment with increasing clover density by decreasing the dominating glucobrassicin. The responses of aliphatic and indole glucosinolate groups in roots were dominated by sinigrin and neoglucobrassicin, respectively Our results confirm the importance of considering nonlinear responses, as they were observed to occur in several instances. Root gluconapin and neoglucobrassicin concentrations had the strongest nonlinear responses. In swede roots (Hopkins et al. 1998a), neoglucobrassicin and gluconapin had nearly identical nonlinear responses to increased egg load as found with cabbage in this study. A nonlinear response of glucobrassicin has also been shown for *B. napus* cotyledons wounded mechanically through an increasing number of needle punctures (Bodnaryk 1992). The additional stress from competition in this study also affected nonlinear responses to herbivory. The interaction between intercropping and egg density for gluconapin in roots could be explained by the nonlinear response to an increased herbivore load being more marked in intercropped roots than for monocultured cabbage.

Both root and leaf herbivories could have negative implications for the subsequent development of specialist leaf herbivores through systemic induced changes in the glucosinolate-myrosinase system in undamaged leaves (van Dam et al. 2005; Martin and Müller 2007). An indication of systemic induction of glucosinolate concentrations in response to larval damage in our study was the minor, but significant, decrease in foliar progoitrin and neoglucobrassicin. The effect of intercropping, which decreased concentrations of gluconapin, hydroxyglucobrassicin, and glucobrassicin in intercropped cabbage foliage, is likely to be a result of both above- and below-ground competition, as intercropped cabbage plants at the end of the experiment were almost overgrown by the clover. Glucobrassicin and gluconapin have been identified as oviposition stimulants for *D. radicum* and *D. floralis* (Roessingh et al. 1992; Simmonds et al. 1994; Gouinguené and Städler 2006), as well as other specialists, such as *Pieris brassicae* (van Loon et al. 1992). A decrease in the concentration of these compounds in aerial parts resulting from plant competition could lead to less attractive cabbage plants for specialized pest insects in intercropped systems. In contrast, lower leaf glucosinolate levels may have implications on the generalist herbivore community, as infestation levels may increase as defense levels decrease (Cipollini and Bergelson 2002). Changes in the glucosinolate profile of roots by competition and herbivory are most likely important for subsequent *D. floralis* and *D. radicum* females approaching plants for the purpose of oviposition. *D. radicum* females prefer to lay eggs on plants with moderate damage compared with undamaged or heavily infested plants (Baur et al. 1996a), and cues perceived from the roots of these plants are likely to be of importance for host selection by females (Baur et al. 1996b).

To evaluate the importance of the changes in this and other studies for host seeking and preference of brassica-specialized insects, one needs to investigate how the actual changes are perceived by herbivores. Evaluations of single factors in complex systems are far from easy, as other plant characteristics change at the same time as the concentrations of secondary compounds change, e.g., plant biomass, nutrient and fiber content (Björkman, unpublished data). Adding competition to the study of herbivore-induced glucosinolate responses has revealed complex relationships between responses and causal agents. This shows the importance of investigating the impact of multiple challenges on plant composition and the possible implications for herbivore host plant search and acceptance.

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References

BARTLET, E., KIDDLE, G., WILLIAMS, I., and WALLSGROVE, R. 1999. Wound-induced increases in the glucosinolate content of oilseed and forage rape and their effect on subsequent herbivory by a crucifer specialist. *Ent. Exp. Appl.* 91:163–167.

BAUR, R., KOSTAL, V., and STÄDLER, E. 1996a. Root damage by conspecific larvae induces oviposition in cabbage root flies. *Ent. Exp. Appl.* 80:224–227.

BAUR, R., KOSTAL, V., PATRIAN, B., and STÄDLER, E. 1996b. Preference for plants damaged by conspecific larvae in ovipositing cabbage root flies: Influence of stimuli from leaf surface and roots. *Ent. Exp. Appl.* 81:353–364.

BIRCH, A. N. E., GRIFFITHS, D. W., HOPKINS, R. J., MACFARLANE SMITH, W. H., and MCKINLAY, R. G. 1992. Glucosinolate responses of swede, kale, forage and oilseed rape to root damage by turnip root fly (*Delia floralis*) larvae. *J. Sci. Food. Agric.* 60:1–9.

BIRCH, A. N. E., GRIFFITHS, D. W., HOPKINS, R. J., and MACFARLANE SMITH, W. H. 1996. A time-course study of chemical and physiological responses in Brassicas induced by turnip root fly (*Delia floralis*) larval feeding. *Ent. Exp. Appl.* 80:221–223.

BJÖRKMAN, M., HAMBÄCK, P. A., and RÄMERT, B. 2007. Neighboring monocultures enhance the effect on the turnip root fly (*Delia floralis*) in intercropping systems. *Ent. Exp. Appl.* 124:319–326.

BODNARYK, R. P. 1992. Effects of wounding on glucosinolates in the cotyledons of oilseed rape and mustard. *Phytochemistry* 31:2671–2677.

CIPOLLINI, D. F., and BERGELSON, J. 2001. Plant density and nutrient availability constrain constitutive and wound-induced expression of trypsin inhibitors in *Brassica napus*. *J. Chem. Ecol.* 27:593–609.

CIPOLLINI, D. F., and BERGELSON, J. 2002. Interspecific competition affects growth and herbivore damage of *Brassica napus* in the field. *Plant. Ecol.* 162:227–231.

CLOSSAIS-BESNARD, N., and LARHER, F. 1991. Physiological role of glucosinolates in *Brassica napus*. Concentration and distribution pattern of glucosinolates among plant organs during a complete life cycle. *J. Sci. Food. Agric.* 56:25–38.

CRAWLEY, M. J. 2003. Statistical Computing. An Introduction to Data Analysis Using S-Plus. John Wiley and Sons Ltd, Chichester.

FINCH, S., and COAKER, T. H. 1969. A method for the continuous rearing of the cabbage root fly *Erioischia brassicae* (Bouché) and some observations on its biology. *Bull. Ent. Res.* 58:619–627.

FINCH, S., and COLLIER, R. H. 2000. Host-plant selection by insects - a theory based on 'appropriate/inappropriate landings' by pest insects of cruciferous plants. *Ent. Exp. Appl.* 96:91–102.

FINCH, S., and KIENEGGER, M. 1997. A behavioural study to help clarify how undersowing with clover affects host plant selection by pest insects of brassica crops. *Ent. Exp. Appl.* 84:165–172.

GIAMOUSTARIS, A., and MITHEN, R. 1995. The effect of modifying the glucosinolate content of leaves of oilseed rape on its interaction with specialist and generalist pests. *Ann. Appl. Biol.* 126:347–363.

GOUINGUENÉ, S. P. D., and STÄDLER, E. 2006. Comparison of the egg-laying behaviour and electrophysiological responses of *Delia radicum* and *Delia floralis* to cabbage leaf compounds. *Phys. Ent.* 31:382–389.

GRIFFITHS, D. W., BIRCH, A. N. E., and MACFARLANE SMITH, W. H. 1994. Induced changes in the indole glucosinolate content of oilseed and forage rape (*Brassica napus*) plants in response to either turnip root fly (*Delia floralis*) larval feeding or artificial root damage. *J. Sci. Food. Agric.* 65:171–178.

HALKIER, B. A., and GERSHENZON, J. 2006. Biology and biochemistry of glucosinolates. *Annu. Rev. Plant. Biol.* 57:303–33.

HERMS, D. A., and MATTSON, W. J. 1992. The dilemma of plants – to grow or defend. *Q. Rev. Biol.* 67:283–335.

HOPKINS, R. J., GRIFFITHS, D. W., BIRCH, A. N. E., and MCKINLAY, R. G. 1998a. Influence of increasing herbivore pressure on modification of glucosinolate content of swedes (*Brassica napus* spp. *rapifera*). *J. Chem. Ecol.* 24:2003–2019.

HOPKINS, R. J., EKBOM, B. E., and HENKOW, L. 1998b. Glucosinolate content and susceptibility for insect attack of three genotypes of *Sinapis alba* L. *J. Chem. Ecol.* 24:1203–1216.

KORITSAS, V. M., LEWIS, J. A., and FENWICK, G. R. 1991. Glucosinolate responses of oilseed rape, mustard and kale to mechanical wounding and infestation by cabbage stem flea beetle (*Psylliodes chrysocephala*). *Ann. Appl. Biol.* 118:209–221.

LAMBDON, P. W., HASSALL, M., BOAR, R. R., and MITHEN, R. 2003. 2003 Asynchrony in the nitrogen and glucosinolate leaf-age profiles of *Brassica*: Is this a defensive strategy against generalist herbivores? *Agr. Ecosyst. Environ.* 97:205–214.

MARTIN, N., and MÜLLER, C. 2007. Induction of plant responses by a sequestering insect: Relationship of glucosinolate concentration and myrosinase activity. *Basic. Appl. Ecol.* 8:13–25.

NILSSON, J., OLSSON, K., ENQVIST, G., EKVALL, J., OLSSON, M., NYMAN, M., and ÅKESSON, B. 2006. Variation in the content of glucosinolates, hydroxycinnamic acids, carotenoids, total antioxidant capacity and low-molecular-weight carbohydrates in *Brassica* vegetables. *J. Sci. Food. Agric.* 86:528–538.

RADOVICH, T. J. K., KLEINHENZ, M. D., and STREETER, J. G. 2005. Irrigation timing relative to head development influences yield components, sugar levels, and glucosinolate concentrations in cabbage. *J. Am. Soc. Hortic. Sci.* 130:943–949.

RENWICK, J. A. A., HARIBAL, M., GOUINGUENÉ, S. P. D., and STÄDLER, E. 2006. Isothiocyanates stimulating oviposition by the diamondback moth, *Plutella xylostella*. *J. Chem. Ecol.* 32:755–766.

RISCH, S. J. 1983. Intercropping as cultural pest control: Prospects and limitations. *Environ. Manage.* 7:9–14.

ROESSINGH, P., STÄDLER, E., FENWICK, G. R., LEWIS, J. A., NIELSEN, J. K., HURTER, J., and RAMP, T. 1992. Oviposition and tarsal chemoreceptors of the cabbage root fly are stimulated by glucosinolates and host plant extracts. *Ent. Exp. Appl.* 65: 267–282.

ROOT, R. 1973. Organisation of a plant-arthropod association in simple and diverse habitats. The fauna of collards (*Brassica oleracea*). *Ecol. Monogr.* 43:95–124.

SIEMENS, D. H., GARNER, S. H., MITCHELL-OLDS, T., and CALLAWAY, R. M. 2002. Cost of defence in the context of plant competition: *Brassica rapa* may grow and defend. *Ecology* 83:505–517.

SIMMONDS, M. S. J., BLANEY, W. M., MITHEN, R., BIRCH, A. N. E., and LEWIS, J. 1994. Behavioural and chemosensory responses of the turnip root fly (*Delia floralis*) to glucosinolates. *Ent. Exp. Appl.* 71:41–57.

STAMP, N., BRADFIELD, M., LI, S., and ALEXANDER, B. 2004. Effect on competition on plant allometry and defence. *Am. Mid. Nat.* 151:50–64.

THEUNISSEN, J. 1997. Intercropping in field vegetables as an approach to sustainable Horticulture. *Outlook. Agr.* 26:95–99.

THEUNISSEN, J., BOOIJ, C. J. H., and LOTZ, L. A. P. 1995. Effects of intercropping white cabbage with clovers on pest infestation and yield. *Ent. Exp. Appl.* 74:7–16.

ULMER, B. J., and DOSDALL, L. M. 2006. Glucosinolate profile and oviposition behaviour in relation to the susceptibilities of Brassicaceae to the cabbage seedpod weevil. *Ent. Exp. Appl.* 121:203–213.

VAN DAM, N. M., and RAAIJMAKERS, C. E. 2006. Local and systemic induced responses to cabbage root fly larvae (*Delia radicum*) in *Brassica nigra* and *B. oleracea*. *Chemoecology* 16:17–24.

VAN DAM, N. M., RAAIJMAKERS, C. E., and DER PUTTEN, W. H. 2005. Root herbivory reduces growth and survival of the shoot feeding *Pieris rapae* on *Brassica nigra*. *Ent. Exp. Appl.* 115: 161–170.

VAN LOON, J. J. A., BLAAKMEER, A., GRIEPINK, F. C., BEEK, T. A., SCHONOOVEN, L. M., and GROOT, A. 1992. Leaf surface compound from *Brassica oleracea* (Cruciferae) induces oviposition by *Pieris brassicae* (Lepidoptera: Pieridae). *Chemoecology* 3:39–44.

YOSUF, S. W., and COLLINS, G. G. 1998. Effect of soil sulphur levels on feeding preference of *Brevicoryne brassicae* on Brussels sprouts. *J. Chem. Ecol.* 24:417–424.

illustrated by R-(+)-pulegone's ability to depolarize root membrane potentials (Maffei et al. 2001) and inhibit bacterial growth in low concentrations (<5 mg dm^{-3}; Oumzil et al. 2002). R-(+)-pulegone, known as a major constituent of pennyroyal, is hepatotoxic and pulmotoxic in rats, mice, and humans (Sullivan et al. 1979; Gordon et al. 1982; Thorup et al. 1983; Madsen et al. 1986; Anderson et al. 1996). Pennyroyal tea has been used by people against various ailments and as an abortifacient, sometimes leading to death because of the toxicity of R-(+)-pulegone (EMEA 2005).

Domestic goats and sheep have browsed and grazed the Mediterranean and Middle East for thousands of years. These animals have decimated many palatable plant species. The resulting "antipastoral vegetation" is dominated by plants with spines, thorns, leathery, or woolly leaves and stalks, and strongly aromatic or bitter compounds thought to act as chemical defenses against herbivores. Aromatic compounds, essential oils comprising mixtures of mono- and sesquiterpenoids, may deter herbivores either by immediate sensory experiences (i.e., taste or smell) or by causing negative postingestive consequences resulting in conditioned food aversions (Duncan et al. 2005). We studied monoterpene levels in thyme basil (*A. suaveolens*) growing in Greece under two environmental conditions: on rangeland heavily used by goats and sheep and on inaccessible rock pillars in the same area.

Methods and Materials

Study Site A unique group of rock pillars in Greece (Fig. 1) carries vegetation and is surrounded by heavily pastured livestock range with many plants of the same species as on the pillar. This permits a comparison of plant defenses in individuals of the same plant species that are inaccessible to goats and sheep (exclosure effect) to those that are exposed to browsing. The approximately 100 Metéora rock pillars consist of conglomerate and are located in central Greece's Plains of Thessaly in the valley of the Pinios River (approximately 21°30′ E and 39°30′ N), bounded by the Pindus Mountains in the West and the Khasia Range to the East. The Ambaria, Dupiani, and Aghia Apostoli (more commonly known as Grosse Heilige) pillars range between 150 and 300 m high (Stutte and Hasse 1986). In this study, DMS observed that local grazing sheep and goats ignore thyme basil, *A. suaveolens*, as they moved through stands of this plant.

Field Collection Terpenoid-based defenses show large between-individual variability (Sjödin et al. 2000; Latta et al. 2000, 2003; Langenheim 2003; Thoss et al. 2007) that makes detecting an environmental influence on overall production or composition in the field more challenging. In this study, we addressed inherent variability by taking nine samples per site and using six different sites, three of which were located on rock pillars and three in the rangeland surrounding these pillars.

The pillars are accessible only by technical rock climbing. On the summits of these pillars and on the livestock range below, DMS harvested at each location about ten nonflowering shoots from each of three–five plants during late April. Back at base, three shoots were randomly selected and five leaves plucked from each on the day of collection. The leaves were cut into thirds: leaf base, middle, and tip. Leaf bases of the five leaves were put

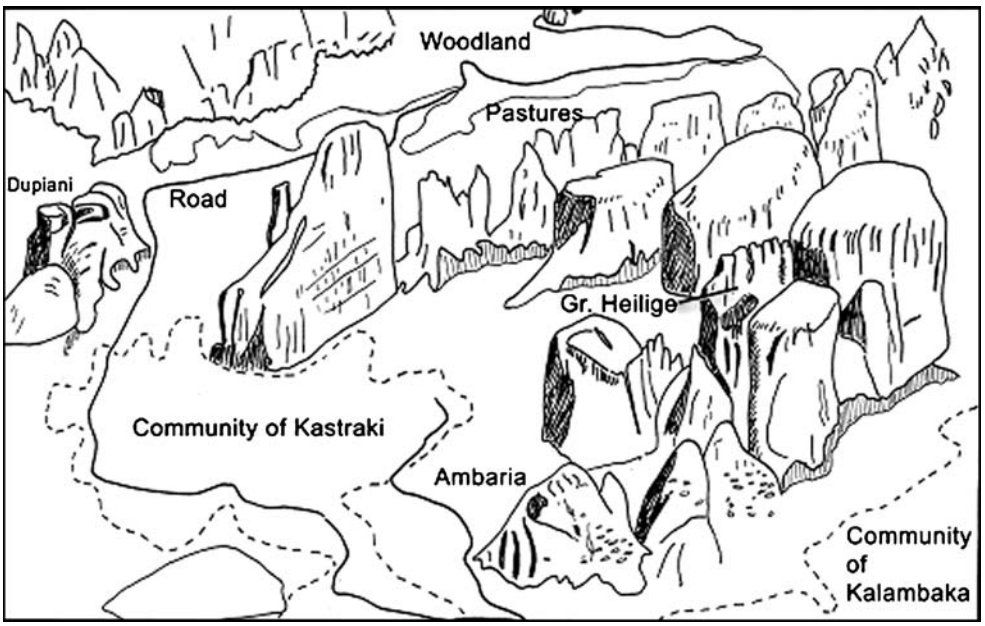

Fig. 1 Rock pillars at Meteora. The locations of the sampling sites Dupiani, Grosse Heilige (Aghia Apostoli), and Ambaria are shown. Approximate distance from *left* to *right* is 1.5 km. Redrawn with permission after Rohweder 1996

together in one screw cap vial (5 ml) with hexane (1 ml) and menthone (10 mg dm^{-3}) as procedural standard. The middle sections of five leaves were placed into another vial and the tips in a third vial. Upon completion of the collection, samples were air-shipped from Athens to Aberdeen.

Chemical Analysis The solvent extract was removed from the leaf sections and fenchone was added as internal standard to a final concentration of 20 mg dm^{-3}. The concentration of menthone (after subtraction of added procedural standard) and R-(+)-pulegone were determined on a Trace GC 2000 version 1.3 with a Trace DSQ version 1.3.0 SP4 mass spectrometer. The column was Sol Gel 10–25 (30 m length× 0.25 mm i.d. from SGE, UK). Injection volume was 1 µl with a 1:80 splitting ratio. The temperature program started at 70°C (held for 2 min), followed by a ramp of 10°C up to 130°C, followed by a 50°C ramp up to 200°C. The software for data capture and analysis was Xcalibur™ version 1.3. Identification and quantification was based on commercial R-(+)-pulegone (technical grade, 85% purity) and menthone (>99% purity) standards (from Sigma). The leaf material was dried at 50°C to obtain dry matter (DM).

Statistical Analyses Minitab Release 14 was used for statistical treatment of the data. At each location, for example "Range at Dupiani", 27 samples were taken, giving 162 samples in total. These samples related to nine different sets of plants, which had been split into top, middle, and bottom leaf sections. Since significant differences were not found between top, middle, and bottom sections (one-way analysis of variance (ANOVA)), the means for each sample derived from top, middle, and bottom sections were used for further calculation. The data was transformed by taking the square root of the concentration to achieve normality (Kolmogorov–Smirnov test). A two-way ANOVA was conducted for R-(+)-pulegone and for menthone, respectively. Factors used were "range or pillar" and "location" referring to the three different areas sampled.

Results

The average concentrations in *A. suaveolens* determined in this study were 8.4±1.4 mg R-(+)-pulegone, 3.7±0.14 mg menthone, and 12.1±1.4 mg total monoterpenoids (all per g DM). R-(+)-pulegone levels were higher ($P<0.001$) in plants growing on the range at the foot of the rock pillars where sheep and goats roamed freely than on the pillars, while the concentrations of menthone were similar (Table 1; Fig. 3). The results suggest that herbivory may be associated with higher absolute and relative concentrations of R-(+)-pulegone. This is true for all three pillars sampled (Fig. 2). Levels of R-(+)-pulegone and menthone did not differ significantly among tips, middle sections, and leaf bases, although there was a nonsignificant tendency for lower levels at the tips of the leaves than at their base or middle.

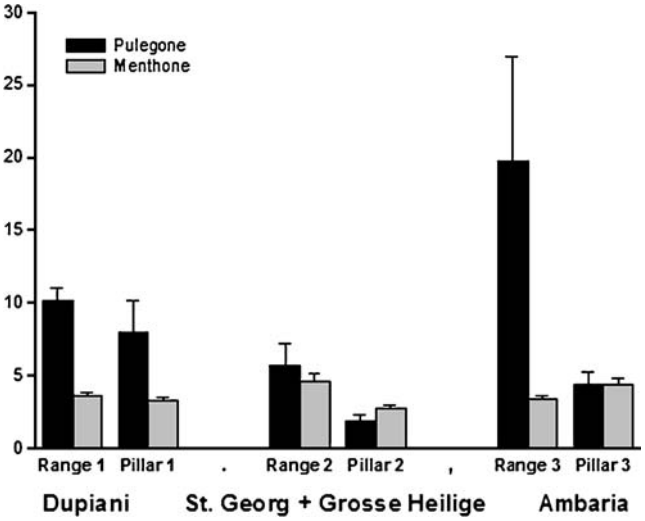

Fig. 2 The mean concentrations of R-(+)-pulegone and menthone in *A. suaveolens* leaves collected from three different pillar and range locations. *Error bars* are ±1 SE (N=9)

Discussion

The biosynthesis of menthone from R-(+)-pulegone via (+)-pulegone reductase in peppermint has been documented (Gershenzon et al. 2000; McConkey et al. 2000; Turner and Croteau 2004). Surprisingly, the genetic modification of peppermint to reduce menthofuran synthesis by down-regulating cytochrome P450 menthofuran synthases resulted in reduced R-(+)-pulegone content (Mahmoud and Croteau 2003). Despite elucidation of the genetic basis

Table 1 R-(+)-pulegone and menthone concentrations in thyme basil (*A. suaveolens*) from range vs. pillars (means±standard error (SE); *F*- and *P*-values from two-way ANOVA on square root transformed data)

	Range (N=27)	Pillar (N=27)	*P*-value	*F*-value
R-(+)-pulegone (mg (g DM)$^{-1}$)	11.72±2.38	4.73±0.92	<0.001	16.84
Menthone (mg (g DM)$^{-1}$)	3.89±0.19	3.45±0.22	0.076	3.29
(mg (g DM)$^{-1}$)	15.61±2.38	8.18±1.00	0.001	12.66
R-(+)-pulegone (%)	66.3±3.5	47.5±4.0	<0.001	12.52

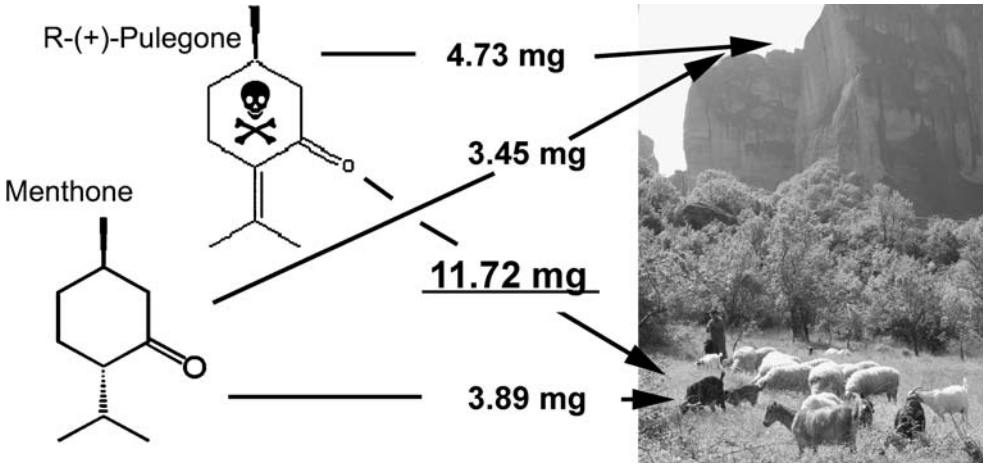

Fig. 3 Visual summary of the main results: concentrations of menthone and R-(+)-pulegone on *summits* and range *below summits*

for monoterpenoid production in peppermint, the actual content and composition seem to depend to a large extent on environmental conditions (Burbot and Loomis 1967; Voirin et al. 1990). Thyme basil produces two monoterpenoids and, therefore, its biosynthetic complexity is reduced compared to peppermint. In addition, the plasticity of (+)-pulegone reductase seems to support our findings of a significantly higher proportion and absolute concentration of R-(+)-pulegone in thyme basil plants accessible to herbivores. That environmental factors influence gene expression is further supported by significant differences among the three locations at Dupiani, Grosse Heilige, and Ambaria (P=0.001 and F=8.00; Fig. 2).

One difference between R-(+)-pulegone and menthone is the reported toxicity of either compound. For menthone, the no-observed-effect level orally for rats was <200 mg/kg body weight per day (Madsen et al. 1986), which is a magnitude higher than the 20 mg per kg body weigh per day for R-(+)-pulegone (Thorup et al. 1983). In addition, the mode of action of R-(+)-pulegone toxicity has been shown to involve covalent binding of the compound to liver proteins (McClanahan et al. 1989) through destruction of microsomal cytochrome P-450 (Moorthy et al. 1991). Our results show that, depending on the location and selective pressures it is exposed to, thyme basil responds by a selective increase in the more biologically active compound, requiring less energy to be expended for a similar level of protection.

Goats consume at least 3% of their body weight in dry plant matter every day. Assuming an adult goat weighing 50 kg, its daily consumption of dry plant matter is at least 1.5 kg. In the Metéora with its antipastoral vegetation, the intake is likely to be higher. In humans, at a concentration range between 90 to 150 mg per kg body weight, R-(+)-pulegone causes moderate to severe toxicity, while an estimated intake of about 130–215 mg R-(+)-pulegone per kg body weight in the form of pennyroyal essential oil is lethal due to centrilobular necrosis of the liver, pulmonary edema, and internal hemorrhage (EMEA/HMPWP/52/04). With the mean concentration of R-(+)-pulegone in the range plants studied, the consumption of 500 g DM of *A. suaveolens* is likely to be hepatotoxic for an adult goat. As goats are capable of associating negative postingestive consequences with a specific blend of volatile components (Duncan et al. 2005), their avoidance of *A. suaveolens* as observed in the field might be caused by previous experiences of R-(+)-pulegone toxicity.

This study adds further supportive evidence to the influence of mammalian browsing on plant chemical defense. Since plants on the pillars still face insect predation, they are expected to maintain certain levels of chemical defense. The studied system permits the separation of these two different herbivore pressures on plants. Absence of mammalian herbivores can affect plants in complex ways. For example, exclusion of large herbivores such as elephants and giraffes from stands of whistling thorn acacia trees (*Acacia drepanolobium*) leads to reduced secretion of "nectar" from leaf base glands. This makes the tree less attractive to a certain species of ant that defends the tree against mammals. Less defended by ants, the trees now succumb to other insects such as longhorn beetles (Palmer et al. 2008). In the case of *Acinos* at Metéora, other environmental factors such as water stress on the pillars with their thin soil cover cannot be ruled out at this point.

Of particular interest is the selective increase in the 'more toxic' R-(+)-pulegone in plants available to goats. Whether the measured concentrations and composition reflect a genetic difference among the plants, whether it occurred as induced response to browsing, or a combination of both, requires further study. Genetic isolation is unlikely, as seeds and pollen can travel between the pillars via wind, birds, and/or insects. Given individual variation between plants, livestock browsing may select for better defended specimens. Alternatively, browsing may phenotypically boost monoterpenoid levels in the same plant.

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References

ANDERSON, I. B., MULLEN, W. H., MEEKER, J. E., KHOJASTEDH-BAKHT, S. C., and BLANC, P. D. 1996. Pennyroyal toxicity: measurement of toxic metabolite levels in two cases and review of the literature. *Ann. Intern. Med.* 124:726–734.

BURBOT, A. J., and LOOMIS, W. D. 1967. Effects of light and temperature on the monoterpenes of peppermint. *Plant Physiol.* 42:20–28.

COULADIS, M., TZAKOU, O., DEMETZOS, C., and PERDETZOGLOU, D. 2002. Chemical composition and antibacterial activity of the oil of *Acinos suaveolens* (Sibth. et Sm.) G. Don f. from Greece. *J. Essent. Oil Res.* 14:139–140.

DUNCAN, A. J., REID, S. A., THOSS, V., and ELSTON, D. A. 2005. Browse selection in response to simulated seasonal changes in diet quality through postingestive effects. *J. Chem. Ecol.* 31:729–744.

EMEA 2005. Final public statement on the use of herbal medicinal products containing pulegone and menthofuran. European Agency for the Evaluation of Medicinal Products. Reference EMEA/HMPC/138386/2005.

GERSHENZON, J., and DUDAREVA, N. 2007. The function of terpene natural products in the natural world. *Nat. Chem. Biol.* 3:408–414.

GERSHENZON, J., MCCONKEY, M. E., and CROTEAU, R. B. 2000. Regulation of monoterpene accummulation in leaves of peppermint. *Plant Physiol*. 122:205–214.

GORDON, W. P., FORTE, A. J., MCMURTRY, R. J., GAL, J., and NELSON, S. D. 1982. Hepatotoxicity and pulmonary toxicity of pennyroyal oil and its constituent terpenes in the mouse. *Toxicol. Appl. Pharmacol*. 65:413–424.

LANGENHEIM, J. H. 2003. Plant Resins: Chemistry, Evolution, Ecology and Ethnobotany. Timber, Portland, Cambridge.

LATTA, R. G., LINHART, Y. B., LUNDQUIST, L., and SNYDER, M. A. 2000. Patterns of monoterpene variation within individual trees in ponderosa pine. *J. Chem. Ecol*. 26:1341–1357.

LATTA, R. G., LINHART, Y. B., SNYDER, M. A., and LUNDQUIST, L. 2003. Patterns of variation and correlations in the monoterpene composition of xylem oleoresin within populations of ponderosa pine. *Biochem. Syst. Ecol.* 31:451–465.

LINCOLN, D. E., and LANGENHEIM, J. H. 1976. Geographic patterns of monoterpenoid composition in *Satureja douglasii*. *Biochem. Syst. Ecol.* 4:237–248.

LINCOLN, D. E., and LANGENHEIM, J. H. 1979. Variation of *Satureja douglasii* monoterpenoids in relation to light intensity and herbivory. *Biochem. Syst. Ecol.* 7:289–298.

LINCOLN, D. E., and LANGENHEIM, J. H. 1981. A genetic approach to monoterpenoid compositional variation in *Satureja douglasii*. *Biochem. Syst. Ecol*. 9:153–160.

MADSEN, C., WURTZEN, G., and CARSTENSEN, J. 1986. Short-term toxicity study in rats dosed with menthone. *Toxicol. Lett.* 32:147–152.

MAHMOUD, S. S., and CROTEAU, R. B. 2003. Menthofuran regulates essential oil biosynthesis in peppermint by controlling a downstream monoterpene reductase. *Proc. Natl. Acad. Sci. U.S. A.* 100:14481–14486.

MAFFEI, M., CAMUSSO, W., and SACCO, S. 2001. Effect of *Mentha x piperita* essential oil and monoterpenes on cucumber root membrane potential. *Phytochemistry* 58:703–707.

MCCLANAHAN, R. H., THOMASSE, N. D., SLATTERY, J. T., and NELSON, S. D. 1989. Metabolic activation of (R)-(+)-pulegone to a reactive enonal that covalently binds to mouse liver proteins. *Chem. Res. Toxicol.* 2:349–355.

MCCONKEY, M. E., GERSHENZON, J., and CROTEAU, R. B. 2000. Developmental regulation of monoterpene biosynthesis in the glandular trichomes of peppermint. *Plant Physiol*. 122:215–224.

MILEWSKI, A. V., YOUNG, T. P., and MADDEN, D. 1991. Thorns as induced defenses: experimental evidence. *Oecologia* 86:70–75.

MOORTHY, B., MADYASTHA, P., and MADYASTHA, K. M. 1991. Destruction of rat liver microsomal cytochrome P-450 *in vitro* by a monoterpene ketone, pulegone: A hepatotoxin. *Indian J. Chem., Sect. B* 30:138–146.

OUMZIL, H., GHOULAMI, S., RHAJAOUI, M., ILIDRISSI, A., FKIH-TETOUANI, S., FAID, M., and BENJOUAD, A. 2002. Antibacterial and antifungal activity of essential oils of *Mentha suaveolens*. *Phytother. Res.* 16:727–731.

PALMER, T. M., STANTON, M. L., YOUNG, T. P., GOHEEN, J. R., PRINGLE, R. M., and KARBAN, R. 2008. Breakdown of an ant-plant mutualism follows the loss of large herbivores from an African savanna. *Science* 319:192.

PULLIN, A. S., and GILBERT, J. E. 1989. The stinging nettle, *Urtica dioica*, increases trichome density after herbivore and mechanical damage. *Oikos* 54:275–280.

RICE, R. L., LINCOLN, D. E., and LANGENHEIM, J. H. 1978. Palatability of monoterpenoid compositional types of *Satureja douglasii* to a generalist molluscan herbivore, *Ariolimax dolichophallus*. *Biochem. Syst. Ecol.* 6:45–53.

ROHWEDER, A. 1996. Meteora Panoramic Map. Karto Atelier, Forch, Switzerland. ISBN 3-906593-06-1.

SJÖDIN, K., OERSSON, M., FÄLDT, J., EKBERG, I., and BORG-KARLSON, A.-K. 2000. Occurrence and correlations of monoterpenes hydrocarbon enantiomers in *Pinus sylvestris* and *Picea abies*. *J. Chem. Ecol.* 26:1701–1720.

STUTTE, H. L., and HASSE, D. 1986. Meteora, Greece: Climbing and Hiking. 2nd edn.Stutte, Wolfratshausen, Germany.

SULLIVAN, J. B., RUMACK, B. H., THOMAS, H., PETERSON, R. G., and BRYSON, P. 1979. Pennyroyal oil poisoning and hepatotoxicity. *J. Am. Med. Assoc.* 242:2873–2874.

THORUP, I., WURTZEN, G., CARSTENSEN, J., and OLSEN, P. 1983. Short term toxicity study in rats dosed with pulegone and menthol. *Toxicol. Lett.* 19:207–210.

TURNER, G. W., and CROTEAU, R. 2004. Organization of monoterpene biosynthesis in menthe. Immunocytochemical localization of geranyl diphosphate synthase, limonene-6-hydrozylase, isopiperitenol dehydrogenase, and pulegone reductase. *Plant Physiol.* 136:4215–4227.

THOSS, V., O'REILLY-WAPSTRA, J., and IASON, G. R. 2007. Intra-specific and phenological variability in monoterpenes of Scots pine (*Pinus sylvestris)* foliage: implications for ecological research. *J. Chem. Ecol.* 33:477–491.

VOIRIN, B., BRUN, N., and BAYET, C. 1990. Effects of daylength on the monoterpene composition of leaves of *Mentha x piperita*. *Phytochemistry* 29:749–755.

of large-scale patterns of phenolic plant compounds. Factors that regulate carbon allocation to secondary compounds in plants are usually investigated by small-scale experiments, where one or two ecological factors are manipulated. It is poorly known how concentrations of phenolics vary with geographic or landscape changes. Earlier chemotaxonomic studies in conifers indicated that the concentrations of some flavonoids and monoterpenes vary in different latitudes (Hiltunen 1975; Laracine-Pittet and Lebreton 1988; Nerg et al. 1994). Latitudinal and regional variations in the concentrations of phenolics in naturally occurring white birch (*Betula pubescens* EHRH) populations have so far not been investigated. In birches, phenolics constitute a large fraction of foliar biomass. Concentrations vary with several different factors, such as soil nutrient availability (Ruohomäki et al. 1996; Mutikainen et al. 2000; Keski-Saari and Julkunen-Tiitto 2003; Stark et al. 2007), UV radiation (Tegelberg et al. 2001; Keski-Saari et al. 2005), and herbivory (Tuomi et al. 1990; Ruohomäki et al. 1996; Kaitaniemi et al. 1999; Mutikainen et al. 2000; Henriksson et al. 2003; Riipi et al. 2005).

In order to test whether variation in phenolic concentrations at the landscape level agree with the experimental evidence for the ecological factors that regulate phenolic concentrations in white birch, we conducted two separate investigations. First, we conducted a climatic transect of birch populations in boreal forests from south to north (from 60° to 70° north) in Finland and analyzed the concentrations of phenolics in birch populations along the transect. Second, we investigated the correlation between foliar phenolics and site characteristics, such as soil element concentrations, exposure on the sunny or shady slope in the landscape, temperature sum, and foliar biomass on a regional scale. Flavonoids in plants have beneficial properties for human health and, therefore, the potential for their use for antioxidative and anti-inflammatory purposes has received increasing attention (Rauha et al. 2000; Wojdylo et al. 2007). Our objective was also to examine whether the landscape variation in the concentration of phenolics could be used as a tool to select sites for gathering birch leaves with high concentrations of flavonoids.

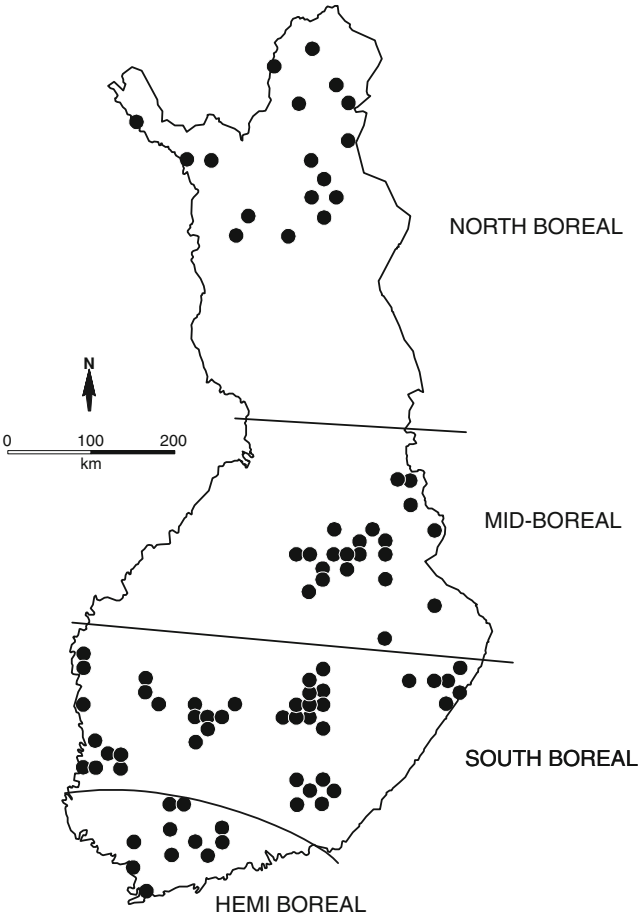

Fig. 1 Map of the study sites used in the north–south transect for investigating latitudinal variation and the effect of climatic zone on the concentration of flavonoids and condensed tannins in the white birch foliage (91 study sites)

Methods and Materials

The South–North Climatic Transect We collected leaf samples from *B. pubescens* in 91 study sites that belong to the National Forest Inventory network in Finland (Fig. 1). Following approximately the division of vegetation zones in Finland (Kalela 1958), we classified the study sites into the following four climatic zones: hemiboreal, southern boreal, midboreal, and northern boreal. Genetic variation among birch trees can sometimes override the effects of nutrient availability, simulated herbivory, or enhanced or excluded UVB radiation (Laitinen et al. 2000, 2004; Mutikainen et al. 2000; Keski-Saari et al. 2005). Our aim, however, was to identify differences between large areas and to cover the variation among the birch clones; we collected leaf samples randomly from at least ten different birch trees at each site. In the north, *B. pubescens* may hybridize with *Betula nana* and form the subspecies *B. pubescens* ssp. *czerepanovii*, the mountain birch. We did not distinguish between pure white birch and the mountain birch but collected random samples of trees. Our data thus represent the large-scale patterns of the concentration of phenolic compounds in birch that may result from either genotypic differences between birch populations, or phenotypic responses to environmental conditions.

Site Characteristics that Correlate with the Concentration of Flavonoids and Condensed Tannins In addition to the general investigation on the north–south gradient, we investigated factors that correlate with the concentrations

of flavonoids and condensed tannins on a regional scale. For this purpose, 38 sites were selected randomly in a study area in northern Finland (Fig. 2; latitude 65–68° north). These sites do not overlap with the sites used in the climatic gradient but constitute a selection of young forest sites. Therefore, data in this study represent a situation in which variation caused by the development stage of the forest is excluded. The following criteria were used in the selection of sites: (1) the sites represent young successional development stages and (2) the proportion of birch coverage is at least 50%. From each site, leaf samples were collected from ten trees. A forest inventory was conducted for birch density, height, and leaf biomass during June and early July, 2006. Mean birch leaf biomass was estimated by selecting a median tree, and the foliar biomass was removed and weighed. Leaf biomass (dry weight) then was used for scaling foliar analysis from a concentration basis into amounts per tree and per hectare.

Forest data for the analysis were provided by Metsähallitus (the government agency that manages state-owned forests in Finland). Geochemical data from the Geological Survey of Finland, which gave concentrations of 37 different elements, then was added to the data files. The following parameters were added to the data: (1) long-term *annual temperature sum*, obtained by a model (Ojansuu and Henttonen 1983); (2) *slope*, calculated by the differences in altitude; (3) *hill shade*, describing the sun exposure in each pixel in the digital elevation model and calculated by the angle of the sun, the direction of the slope, and the angulation of the slope; and (4) *topographic wetness index*, which combines local upslope contributing area and slope.

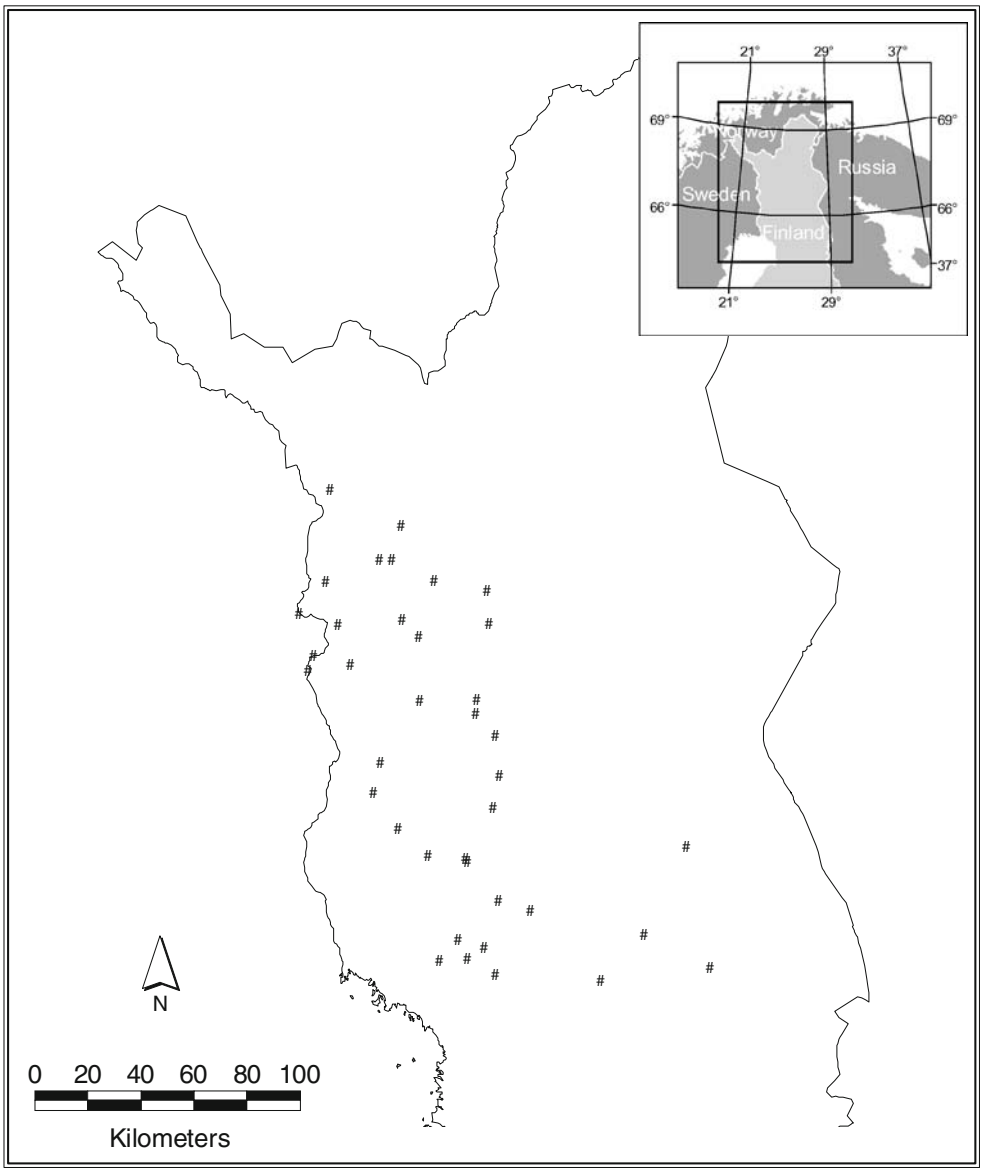

Fig. 2 Map of the study sites used to investigate factors that correlate with the concentration of flavonoids and condensed tannins on a regional basis (38 study sites)

Chemical Analysis of Birch Leaves Leaf samples were oven-dried at +48°C for 48 h and then milled to a fine powder, which was extracted with methanol for the high performance liquid chromatography (HPLC) analysis of phenolics (Julkunen-Tiitto 1985). Briefly, 8 mg of dried material were extracted three times in 400 µl of methanol. Methanol from the combined extracts (1.2 ml) was evaporated under a flow of nitrogen gas. Dried samples were redissolved in 200 µl of methanol and 200 µl of water and analyzed by HPLC with an RP C18 column and an injection volume of 10-µl HPLC conditions. Identification of the compounds was based on retention times, UV spectra, and HPLC–mass chromatography detection as described (Keski-Saari and Julkunen-Tiitto 2003). A wavelength of 320 nm was used for quantification of flavonoids. Concentrations were calculated with the following standards: kaempferol 3-glucoside for kaempferol derivatives, apigenin 7-glucoside for apigenin derivatives, quercetin 3-galactoside for quercetin derivatives, and myricetin 3-rhamnoside for myricetin derivatives. Condensed tannins were determined directly from dried and milled material by an acid butanol assay (Porter et al. 1986), which gives the total amount of condensed tannins (both soluble and cell-bound condensed tannins) in the leaves. Purified condensed tannin from *B. nana* leaves was used as the standard. All results were calculated as concentrations per unit leaf dry mass (mg g^{-1}). Results for the regional study were also calculated as amount per tree and per hectare.

Data Analysis Concentrations of birch flavonoids change with the age of leaves and are higher in young leaves compared to mature ones (Salminen et al. 2001; Laitinen et al. 2002; Riipi et al. 2002). Similarly, concentrations of condensed tannins are lower in young leaves compared to mature leaves (Riipi et al. 2002; Stark et al. 2007). For investigating latitudinal differences, it was crucial to collect leaf samples in exactly the same stage of development in different climatic zones. After conducting the analysis, phenological observations conducted regularly by the Finnish Forest Research Institute (http://www.metla.fi/metinfo/fenologia/index-en.htm) were used to standardize the leaf development stage in samples taken from the different climatic zones. The observations distinguish three stages of development: emergence of leaves, onset of leafing, and full growth of leaves. In each climatic zone, we used only samples collected at the onset of leafing for the statistical analysis. This reduced the total number of replicates from 91 to 54. Samples from the following dates were used: 29th May–4th June in the hemiboreal zone, 23rd May–4th June in the southern boreal zone, 5th June–15th June in the midboreal zone, and 8th June–19th June in northern boreal zone.

For testing the role of the climatic zone in concentrations of flavonoids, data were tested with one-way analysis of variance (ANOVA) with Tukey's multiple comparisons as a post hoc test. The effect of climatic zone on the concentrations of flavonoids was analyzed both for individual substances and substances pooled into the flavonoid groups (quercetin, myricetin, kaempferol, apigenin, and naringenin derivatives), and the pooled concentrations were used for data analysis. The sum of all flavonoids was calculated, and correlation between the phenolics and latitude was examined by using linear regression. Correlations with the site characteristics and concentrations of flavonoids on a regional basis were examined with stepwise linear regression. All statistical analyses were performed with SPSS 12.0 statistical software.

Results

Concentrations of Flavonoids and Condensed Tannins in a North–South Transect A total of 16 flavonoid substances were identified in the birch leaf samples, and the concentration of nine of these was influenced significantly by the climatic zone (Table 1; one-way ANOVA). When individual flavonoids were grouped into compound groups, there was a significant effect of climatic zone on the concentration of quercetin, apigenin, and naringenin derivatives and condensed tannins (Table 1; one-way ANOVA). The concentrations of quercetin derivatives in the northern boreal zone were significantly higher compared to other zones, whereas there were no differences between the hemiboreal, south boreal, and midboreal zones (Tukey's test; Fig. 3). By contrast, the concentrations of both apigenin and naringenin derivatives were significantly higher in the south boreal zone compared to the other zones. Concentrations of condensed tannins in the midboreal zone were significantly higher than in the other zones. There was no difference among the climatic zones in the concentration of kaempferol and myricetin derivatives, although some individual kaempferol derivatives differed by climate zone (Table 1). There was a significant influence of the climatic zone on the total sum of flavonoids (calculated as the sum of all identified substances), which was lower in the midboreal zone than in the other zones (Tukey's test, values not shown).

Analysis by linear regression showed that latitude correlated positively with concentrations of quercetin derivatives and negatively with the concentrations of apigenin and naringenin derivatives (Fig. 4). There was no correlation between latitude and concentrations of total flavonoids and condensed tannins. There was significant negative correlation between the concentration of total flavonoids and condensed tannins.

Correlations of Flavonoid and Condensed Tannin Concentrations with Site Characteristics—the Regional Study The

Table 1 ANOVA table for the effects of climatic zone on the concentrations of different phenolic compounds and compound groups in wild white birch (*B. pubescens*) populations (between groups *df* 3, within groups *df* 50)

	F	*P*
Quercetins	6.9	0.001
Quercetin-3-galactoside	4.1	0.011
Quercetin-3-glucoside+glucuronide	5.1	0.004
Quercetin-3-arabinoside	2.4	0.075
Quercetin-glycoside 1	1.0	0.423
Quercetin-glycoside 2	0.8	0.511
Quercetin-der. 1	4.5	0.007
Apigenins	3.3	0.028
Apigenin 1	7.1	<0.001
Apigenin der-2	2.8	0.051
Naringenins	4.0	0.013
Naringenin-der. 1	4.0	0.013
Naringenin-der. 2	2.4	0.075
Naringenin-der. 3	3.4	0.024
Kaempferols	1.8	0.164
Kaempferol-der. 1	3.1	0.034
Kaempferol-der. 2	0.3	0.773
Kaempferol-der. 3	5.1	0.004
Kaempferol-der. 4	1.2	0.333
Myricetins	1.3	0.290
Myricetin-3-glucoside+glucuronide		
Sum of all flavonoids	4.4	0.008
Condensed tannins	3.4	0.025

concentration of foliar flavonoids in the white birch populations was best explained by the temperature sum and the concentration of P in the geochemical mapping data, which together accounted for 53.1% of the variation in flavonoid concentrations (stepwise linear regression, $F=19.2$, $P<0.001$). The correlation coefficients in the analysis showed that the flavonoid concentration was correlated positively with the temperature sum, which explained a large proportion of the variation. However, flavonoid concentration correlated negatively with the P concentration (Fig. 5). When different compound groups were tested separately, the concentration of kaempferol derivatives ($F=12.4$, $P=0.001$, $R^2=25.7$), apigenin derivatives ($F=13.0$, $P=0.001$, $R^2=26.5$), and naringenin derivatives ($F=19.6$, $P<0.001$, $R^2=35.3$) were correlated positively with the temperature sum, whereas no correlation was observed for quercetin or myricetin derivatives (values not shown). Of the separate compound groups, only the concentration of apigenin derivatives was correlated negatively with soil P concentrations ($F=6.0$, $P=0.019$, $R^2=14.4$). The concentration of condensed tannins in birch leaves correlated negatively with slope, which explained 24.9% of the variation in the condensed tannin concentrations, but not with any other parameters used in the analysis (stepwise linear regression, $F=11.6$, $P=0.002$). There was no correlation between concentrations of flavonoids and condensed tannins.

When calculated as the amount of flavonoids per tree, flavonoids were best explained by leaf biomass and tree diameter together with temperature sum and slope ($F=76.8$, $P<0.001$, $R^2=0.903$, stepwise linear regression). The amount of flavonoids calculated per hectare was not correlated with any of the site characteristics other than the number of trees per hectare and the leaf biomass ($F=50.9$, $P<0.001$, $R^2=0.744$). Similarly, the amount of condensed tannins per tree was correlated with the leaf biomass and the slope ($F=172.1$, $P<0.001$, $R^2=0.902$), but the amount of condensed tannins per hectare was correlated only with the number of trees per hectare and the leaf biomass ($F=62.6$, $P<0.001$, $R^2=0.782$).

Discussion

In our investigation along a climatic transect from southern to northern Finland, we found that the concentrations of quercetin derivatives in the white birch foliage increased with latitude, while the concentrations of apigenin and naringenin derivatives decreased with latitude. The compound-specific latitudinal gradients compensated each other, resulting in no changes in the total concentration of flavonoids with latitude. Thus, our results demonstrate a qualitative, but not quantitative latitude-associated gradient in the foliar flavonoids of white birch. As the randomly selected study sites differed greatly in their properties, such as the successional state of the forest, the age of trees, and site fertility, the flavonoid concentrations varied to a great extent also within the climatic zones. In addition to the variation mediated by site characteristics, in our data of 53 study sites across Finland, the latitude explained 10–17% of the variation of the flavonoid groups.

The increasing concentrations of quercetin derivatives towards the north indicate higher need for protection against photooxidative stress there than in the south. White birch responds to enhanced UVB radiation with enhanced synthesis of quercetin derivatives (Keski-Saari et al. 2005). Excess light in relation to the photosynthetic capacity in plants causes formation of reactive chemicals that may cause oxidative damage in plant tissue (e.g., Asada 1994). Due to their chemical features, such as two hydroxyls in the B ring in an optimal location and 2,3-double bond with the 4-oxo function in the C ring, quercetin derivatives exert strong antioxidant activity (Larson 1988; Rice-Evans et al. 1996), and their antioxidant activity is many times higher (4.7 mM) than that of e.g., apigenin and naringenin (1.5 mM; Rice-Evans et al. 1997). Although the concentration of total flavonoids does not change with latitude, the change in the

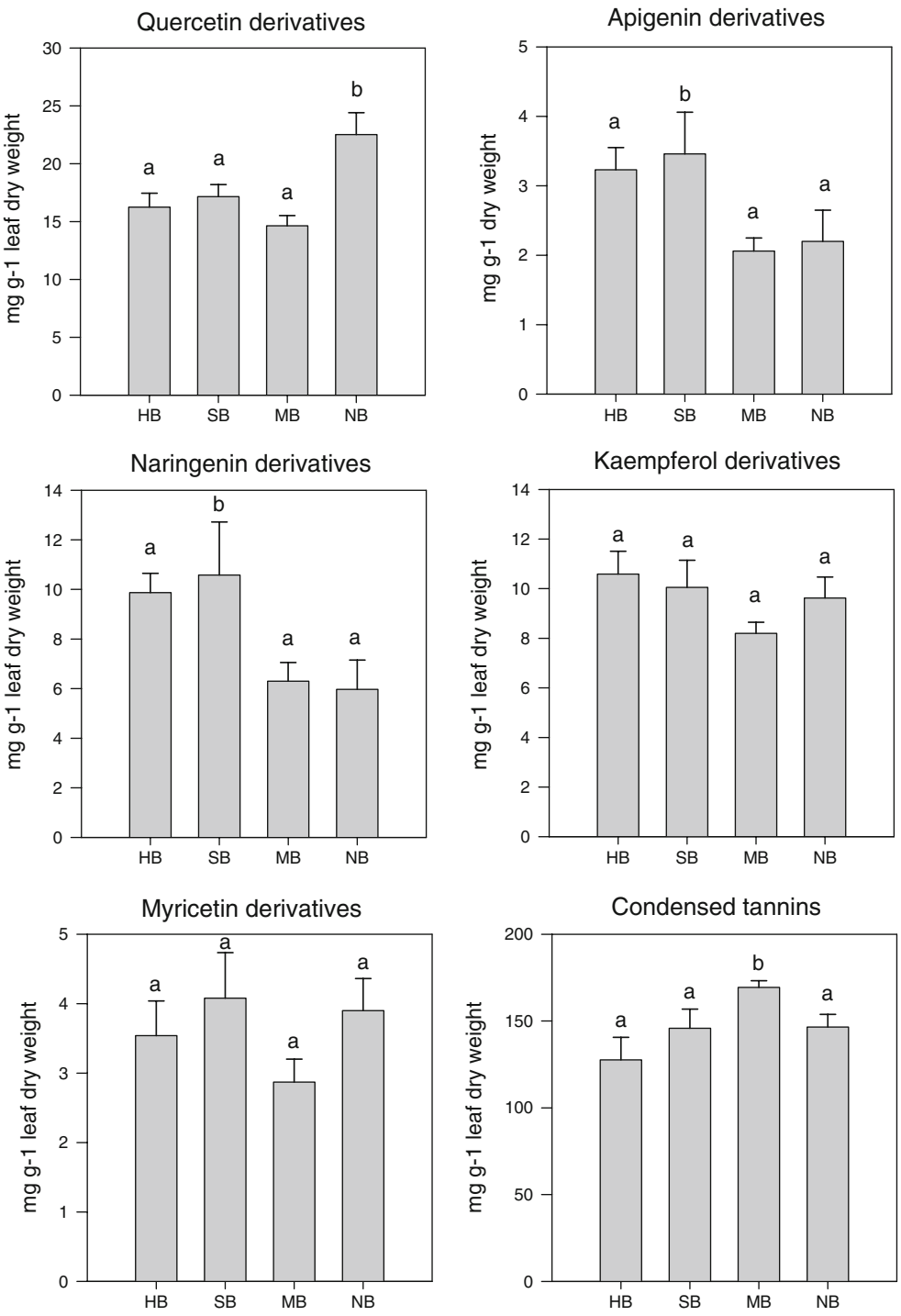

Fig. 3 The concentration of flavonoids in different compound groups in the different climatic zones. Values are mean + standard error, $N=54$ study sites. Significant differences ($P<0.05$) determined by Tukey's test are indicated by *different letters*. The climatic zones are: *HB* hemiboreal ($N=6$ study sites), *SB* south boreal ($N=16$ study sites), *MB* middle boreal ($N=17$ study sites), *NB* north boreal ($N=15$ study sites)

proportions of quercetin in relation to other flavonoids causes the antioxidant capacity in the leaves to be higher in the north than in the south. Low temperatures generally enhance photooxidative stress in relation to light intensity by limiting the capacity of photosynthetic enzymes (Close and McArthur 2002). In the north, continuous light during a large part of the growing season may require high concentrations of photoprotective compounds, because temperatures during the nights frequently drop extremely low at a time when light intensity remains high. The decrease in the concentrations of apigenin and naringenin derivatives indicates that there may be a trade-off between these compounds in flavonoid synthesis. In the plant flavonoid pathway, apigenin and naringenin are synthesized before quercetin. In the south, flavonoid synthesis ends more frequently with apigenin and naringenin derivatives than in the north where the precursors for apigenin and naringenin are used especially for the synthesis of quercetin.

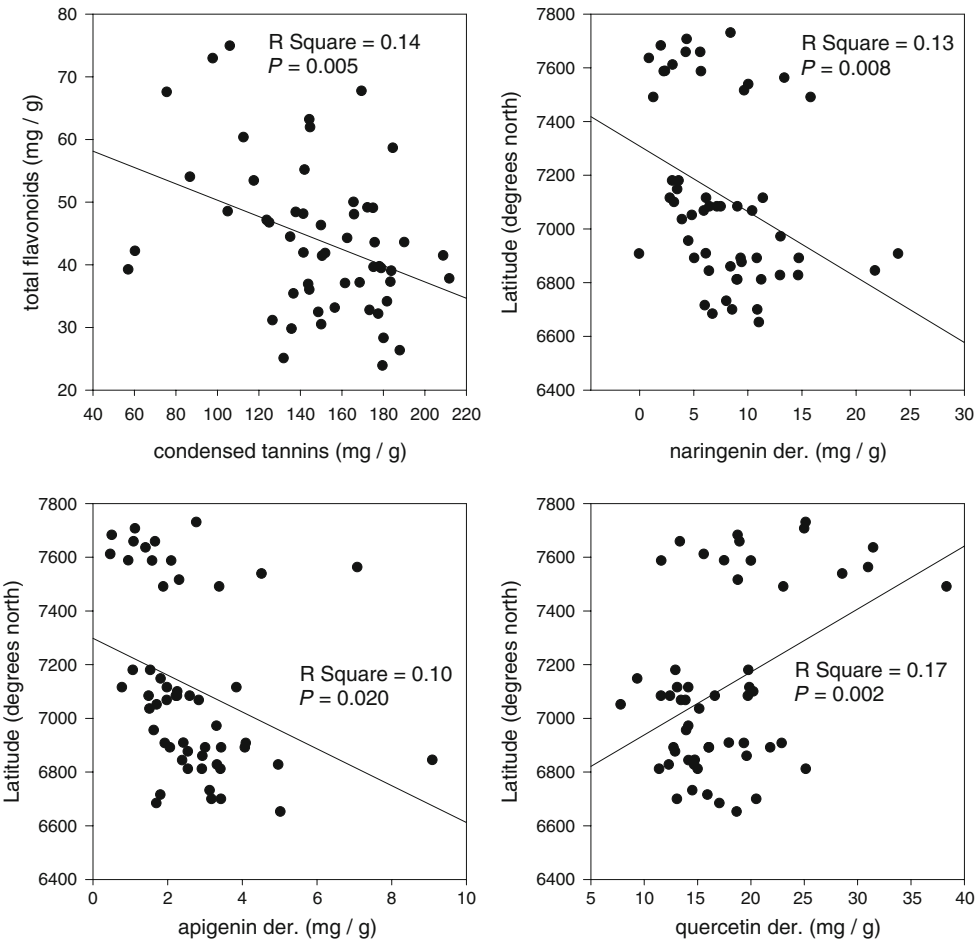

Fig. 4 The correlation between the concentration of quercetin, apigenin, and naringenin derivatives with latitude (degrees north) and the correlation between the total sum of flavonoids and condensed tannins in the north–south transect, *N*=54 study sites

There was no significant effect of latitude on the concentration of condensed tannins, which are considered to form the constitutive defense against herbivory in birch (Tuomi et al. 1990; Kaitaniemi et al. 1999; Mutikainen et al. 2000; Henriksson et al. 2003; Riipi et al. 2005). We are not aware of any study of latitudinal gradients in herbivory pressure on the white birch, but generally, invertebrate herbivory seems to increase (Tuomi et al. 1990). Mammalian browsing has been shown to reduce the concentrations of condensed tannins in birches (Danell et al. 1997; Lehtilä et al. 2000; Stark et al. 2007).

Despite the increase in photoprotective flavonoids towards high latitude and low temperature areas on a geographical scale, we found in our regional study that the sum of foliar flavonoids correlated positively with the temperature sum. In this area, the temperature sum is

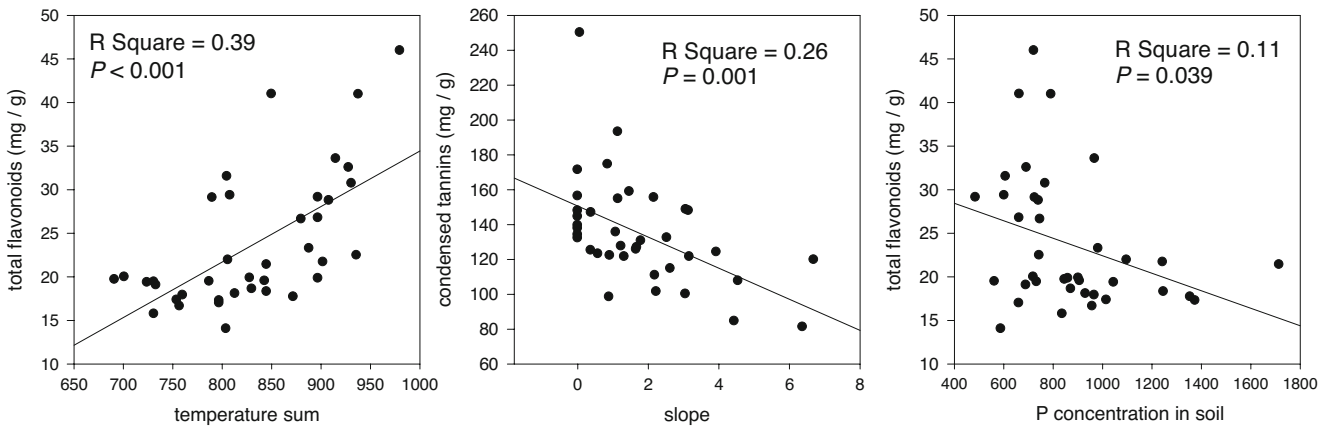

Fig. 5 The correlation between the concentration of total flavonoids and temperature sum and soil P concentration and the correlation between the concentration of condensed tannins and slope on a regional basis in the northern boreal zone, *N*=38 study sites

correlated strongly with altitude, which decreases towards the upland regions in northern and eastern Lapland. The positive correlation between flavonoid concentrations and the temperature sum in white birch is in line with an earlier study at high latitudes (Jonasson et al. 1986). The positive correlation is not necessarily in contrast with the latitudinal gradient with higher concentrations of quercetin derivatives in the north. The same substance group that showed higher concentrations in the north, and quercetin, did not respond positively to temperature, whereas the two substance groups that showed higher concentrations in the south, apigenin and naringenin, showed a positive correlation with the temperature sum on a regional basis and thus had parallel patterns with temperature on both geographical and regional scales.

The positive correlation between temperature and the concentration of phenolics agrees with experiments conducted at high latitudes (Graglia et al. 2001; Hansen et al. 2006). Jonasson et al. (1986) hypothesized that positive correlation between phenolic concentrations and temperature could be explained by the fact that the temperature effect on photosynthesis is proportionally higher than the temperature effect on nutrient supply rate. Therefore, availability of carbon for the synthesis of phenolic secondary compounds in northern ecosystems may increase with temperature, but due to limitation of available nutrients, only part of the surplus carbon can be allocated to new growth. Contrasting with areas in high latitudes, experimental warming in the south boreal areas has been shown to decrease the concentrations of phenolic compounds in birch and other boreal deciduous trees (Kuokkanen et al. 2001, 2003; Veteli et al. 2002). The role of temperature on the carbon allocation to phenolics under different light conditions is possibly a subject for future experimental investigations.

On a regional basis, we found a negative correlation between foliar phenolics and soil P concentrations in the geochemical database, which agrees with the inverse correlation between nutrient availability and leaf phenolic content shown by experimental fertilization (Mutikainen et al. 2000; Keski-Saari and Julkunen-Tiitto 2003). Antioxidative compounds have been shown to increase their turnover rates in conditions of high light (Polle and Rennenberg 1996) and nutrient limitation (Polle et al. 1992; Logan et al. 1999). When nutrient deficiency is limiting the synthesis of photosynthetic enzymes, the need for foliar photoprotection increases and thus, at any given light intensity, the degree of excess light at similar irradiance levels is relatively greater in nutrient-limited conditions (Close and McArthur 2002). Our study demonstrates that correlations between soil nutrient availability and plant phenolic concentrations can be observed at a landscape level.

Quercetin derivatives continue to be of great general interest because of their high antioxidant capacity and assumed effect of reducing the risk of oxidative stress-related chronic diseases in humans (Boots et al. 2007; Wach et al. 2007). Due to the high concentrations of quercetin, white birch foliage growing in the northern boreal zone would constitute a source of such natural products. On a regional basis, the total concentration of flavonoids was the highest in sites with a high temperature sum and a low P availability. Thus, our findings suggest that spatial data could be used as a tool for finding the optimal areas for collecting raw material for health-promoting natural products, thus bringing ecological knowledge to practice.

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References

ASADA, K. 1994. Production and action of active oxygen species in photosynthetic tissues, pp. 77–104, in C. H. Foyer, and P. M. Mullineaux (eds.). Causes of Photooxidative Stress and Amelioration of Defence Systems in PlantsCRC, Boca Raton.

AYRES, M. P., CLAUSEN, T. P., MACLEAN, S. F., REDMAN, A. M., and REICHARDT, P. B. 1997. Diversity of structure and antiherbivore activity in condensed tannins. *Ecology* 78:1696–1712.

BOOTS, A. W., LI, H., SCHINS, R. P. F., DUFFIN, R., HEEMSKERK, J. W. M., BAST, A., and HAENEN, G. R. M. M. 2007. The quercetin paradox. *Toxicol. Appl. Pharm.* 222:89–96.

BRYANT, J. P., CHAPIN, F. S. I. I. I., and KLEIN, D. R. 1983. Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* 40:357–368.

CLOSE, D. C., and MCARTHUR, C. 2002. Rethinking the role of many plant phenolics—protection from photodamage not herbivores? *Oikos* 99:166–172.

DANELL, K., HAUKIOJA, E., and HUSS-DANELL, K. 1997. Morphological and chemical responses of mountain birch leaves and shoots to winter browsing along a gradient of plant productivity. *Ecoscience* 4:296–303.

FLANAGAN, P. W., and VAN CLEVE, K. 1983. Nutrient cycling in relation to decomposition and organic-matter quality in taiga ecosystems. *Can. J. For. Res.* 13:795–817.

GRAGLIA, E., JULKUNEN-TIITTO, R., SHAVER, G., SCHMIDT, I. K., JONASSON, S., and MICHELSEN, A. 2001. Environmental control and intersite variations of phenolics in *Betula nana* in tundra ecosystems. *New Phytol.* 151:227–236.

HAMILTON, J. G., ZANGERL, A. R., DELUCIA, E. H., and BERENBAUM, M. R. 2001. The carbon–nutrient balance hypothesis: its rise and fall. *Ecol. Lett.* 4:86–95.

HANSEN, A. H., JONASSON, S., MICHELSEN, A., and JULKUNEN-TIITTO, R. 2006. Long-term experimental warming, shading and nutrient addition affect the concentration of phenolic compounds

in arctic–alpine deciduous and evergreen dwarf shrubs. *Oecologia* 147:1–11.

HENRIKSSON, J., HAUKIOJA, E., OSSIPOV, V., OSSIPOVA, S., SILLANPÄÄ, S., KAPARI, L., and PIHLAJA, K. 2003. Effects of host shaping on consumption and growth of the geometrid *Epirrita autumnata*: interactive roles of water, primary and secondary compounds. *Oikos* 103:3–16.

HILTUNEN, R. 1975. Variation and inheritance of some monoterpenes in *Pinus silvestris*. *Planta Med.* 28:315–323.

JONASSON, S., BRYANT, J. P., CHAPIN, F. S. III, and ANDERSON, M. 1986. Plant phenols and nutrients in relation to variations in climate and rodent grazing. *Am. Nat.* 128:394–408.

JONES, C. G., and HARTLEY, S. E. 1999. A protein competition model of phenolic allocation. *Oikos* 86:27–44.

JULKUNEN-TIITTO, R. 1985. Phenolic constituents in the leaves of northern willows: methods for the analysis of certain phenolics. *J. Agr. Food Chem.* 33:213–217.

KAITANIEMI, P., NEUVONEN, S., and NYYSSÖNEN, T. 1999. Effects of cumulative defoliations on growth, reproduction, and insect resistance in mountain birch. *Ecology* 80:524–532.

KALELA, A. 1958. Suomen metsäkasvillisuusvyöhykkeet ja Ragnar Hult (In Finnish). *Terra* 70:1–17.

KESKI-SAARI, S., and JULKUNEN-TIITTO, R. 2003. Resource allocation in different parts of juvenile mountain birch plants: effect of nitrogen supply on seedling phenolics and growth. *Physiol. Plant.* 117:1–13.

KESKI-SAARI, S., PUSENIUS, J., and JULKUNEN-TIITTO, R. 2005. Phenolic compounds in seedlings of *Betula pubescens* and *B. pendula* are affected by enhanced UVB radiation and different nitrogen regimens during early ontogeny. *Glob. Chang. Biol.* 11:1180–1194.

KORICHEVA, J., LARSSON, S., HAUKIOJA, E., and KEINÄNEN, M. 1998. Regulation of woody plant secondary metabolism by resource availability: hypothesis testing by means of meta-analysis. *Oikos* 83:212–226.

KUOKKANEN, K., JULKUNEN-TIITTO, R., KEINÄNEN, M., NIEMELÄ, P., and TAHVANAINEN, J. 2001. The effect of elevated CO_2 and temperature on the secondary chemistry of *Betula pendula* seedlings. *Trees* 15:378–384.

KUOKKANEN, K., YAN, S., and NIEMELÄ, P. 2003. Effects of elevated CO_2 and temperature on the leaf chemistry of birch *Betula pendula* (Roth) and the feeding behaviour of the weevil *Phyllobius maculicornis*. *Agr. Forest Entomol.* 5:209–217.

LAITINEN, M.-L., JULKUNEN-TIITTO, R., and ROUSI, M. 2000. Variation in phenolic compounds within a birch (*Betula pendula*) population. *J. Chem. Ecol.* 26:1609–1621.

LAITINEN, M.-L., JULKUNEN-TIITTO, R., and ROUSI, M. 2002. Foliar phenolic composition of European white birch during bud unfolding and leaf development. *Physiol. Plantarum* 114:450–460.

LAITINEN, M. -L., JULKUNEN-TIITTO, R., YAMAJI, K., HEINONEN, J., and ROUSI, M. 2004. Variation in birch bark secondary chemistry between and within clones: implications for herbivory by hares. *Oikos* 104:316–326.

LARACINE-PITTET, C., and LEBRETON, P. 1988. Flavonoid variability within *Pinus sylvestris*. *Phytochemistry* 27:2663–2666.

LARSON, R. A. 1988. The antioxidants of higher plants. *Phytochemistry* 27:969–978.

LEHTILÄ, K., HAUKIOJA, E., KAITANIEMI, P., and LAINE, K. A. 2000. Allocation of resources within mountain birch canopy after simulated winter browsing. *Oikos* 90:160–170.

LOGAN, B. A., DEMMIG-ADAMS, B., and ROSENSTIEL, T. N. 1999. Effect of nitrogen limitation on foliar antioxidants in relationship to other metabolic characteristics. *Planta* 209:213–220.

MARTZ, F., SUTINEN, M. -L., DEROME, K., WINGSLE, G., JULKUNEN-TIITTO, R., and TURUNEN, M. 2007. Effects of ultraviolet (UV) exclusion on the seasonal concentration of photosynthetic and UV-screening pigments in Scots pine needles. *Glob. Chang. Biol.* 13:252–265.

MATTSON, W. J., JULKUNEN-TIITTO, R., and HERMS, D. A. 2005. CO_2 enrichment and carbon partitioning to phenolics: do plant responses accord better with the protein competition or the growth-differentiation balance models? *Oikos* 111:337–347.

MUTIKAINEN, P., WALLS, M., OVASKA, J., KEINÄNEN, M., JULKUNEN-TIITTO, R., and VAPAAVUORI, E. 2000. Herbivore resistance in *Betula pendula*: effect of fertilization, defoliation, and plant genotype. *Ecology* 81:49–65.

NERG, A., KAINULAINEN, P., VUORINEN, M., HANSO, M., HOLOPAINEN, J. K., and KURKELA, T. 1994. Seasonal and geographical variation of terpenes, resin acids and total phenolics in nursery growns seedlings of Scots pine (*Pinus sylvestris* L.). *New Phytol.* 128:703–713.

OJANSUU, R., and HENTTONEN, H. 1983. Estimation of local values of monthly mean temperature, effective temperature sum and precipitation sum from the measurements made by the Finnish Meteorological Office. *Silva Fennica* 17:143–160.

POLLE, A., and RENNENBERG, H. 1996. Photooxidative stress in trees, pp. 199–217, in C. H. Foyer, and P. M. Mullineaux (eds.). Causes of Photooxidative Stress and Amelioration of Defense Systems in PlantsCRC, Boca Raton.

POLLE, A., MÖSSNANG, M., VON SCHÖNBORN, A., SLADKOVIC, R., and RENNENBERG, H. 1992. Field studies on Norway spruce trees at high altitudes. I. Mineral, pigment and soluble protein contents of the needles as affected by climate and pollution. *New Phytol.* 121:89–97.

PORTER, L. J., HRSTICH, L. C., and CHAN, B. G. 1986. The conversion of procyanidins and pordelphinidins to cyanidin and delphinidins. *Phytochemistry* 25:223–230.

RAUHA, J.-P., REMES, S., HEINONEN, M., HOPIA, A., KÄHKÖNEN, M., KUJALA, T., PIHLAJA, K., VUORELA, H., and VUORELA, P. 2000. Antimicrobial effects of Finnish plant extracts containing flavonoids and other phenolic compounds. *Int. J. Food Microbiol.* 56:3–12.

RICE-EVANS, C. A., MILLER, N. J., and PAGANGA, G. 1996. Structure–antioxidant activity relationships of flavonoids and phenolic acids. *Free Radic. Biol. Med.* 20:933–956.

RICE-EVANS, C. A., MILLER, N. J., and PAGANGA, G. 1997. Antioxidant properties of phenolic compounds. *Trends Plant Sci.* 2:152–159.

RIIPI, M., OSSIPOV, V., LEMPA, K., HAUKIOJA, E., KORICHEVA, J., OSSIPOVA, S., and PIHLAJA, K. 2002. Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? *Oecologia* 130:380–390.

RIIPI, M., LEMPA, K., HAUKIOJA, E., OSSIPOV, V., and PIHLAJA, K. 2005. Effects of simulated winter browsing on mountain birch foliar chemistry and on the performance of insect herbivores. *Oikos* 111:221–234.

RUOHOMÄKI, K., CHAPIN, F. S. III, HAUKIOJA, E., NEUVONEN, S., and SUOMELA, J. 1996. Delayed inducible resistance in mountain birch in response to fertilization and shade. *Ecology* 77:2302–2311.

SALMINEN, J. P., OSSIPOV, V., HAUKIOJA, E., and PIHLAJA, K. 2001. Seasonal variation in the content of hydrolysable tannins in leaves of *Betula pubescens*. *Phytochemistry* 57:15–22.

STAMP, N. 2003. Out of the quagmire of plant defence hypotheses. *Q. Rev. Biol.* 78:23–55.

STARK, S., JULKUNEN-TIITTO, R., and KUMPULA, J. 2007. Ecological role of reindeer summer browsing in the mountain birch (*Betula pubescens* ssp. *czerepanovii*) forests: effects on plant defense, litter decomposition, and soil nutrient cycling. *Oecologia* 151:486–498.

TEGELBERG, R., JULKUNEN-TIITTO, R., and APHALO, P. J. 2001. The effects of long-term elevated UV-B on the growth and phenolics of field-grown silver birch (*Betula pendula*). *Glob. Chang. Biol.* 7:839–848.

THEIS, N., and LERDAU, M. 2003. The evolution of function in plant secondary metabolites. *Int. J. Plant Sci.* 163:S93–S102.

TUOMI, J., NIEMELÄ, P., and SIREN, S. 1990. The Panglossian paradigm and delayed inducible accumulation of foliar phenolics in mountain birch. *Oikos* 59:399–410.

VETELI, T. O., KUOKKANEN, K., JULKUNEN-TIITTO, R., ROININEN, H., and TAHVANAINEN, J. 2002. Effects of elevated CO_2 and temperature on plant growth and herbivore defensive chemistry. *Glob. Chang. Biol.* 8:1240–1252.

WACH, A., PYRZYNSKA, K., and BIESAGA, M. 2007. Quercetin content in some food and herbal samples. *Food Chem.* 100:699–704.

WOJDYLO, A., OSZMIANSKI, J., and CZEMERYS, R. 2007. Antioxidant activity and phenolic compounds in 32 selected herbs. *Food Chem.* 105:940–949.

We have been studying such interactions using a *Pinus nigra–Diplodia pinea–Neodiprion sertifer* tripartite system (Bonello and Blodgett 2003; Eyles et al. 2007). *P. nigra* (Pinaceae; Austrian pine) are among the most commonly planted trees in North America where they suffer from *Diplodia* shoot blight and canker, which can kill stressed trees (Peterson 1977). *D. pinea* (Ascomycetes; formerly *Sphaeropsis sapinea*) inhabits the phloem of stems and branches. This pathogen will also infect other two- and three-needle pines, but *P. nigra* is the most susceptible pine in North America. *N. sertifer* (Hymenoptera; Diprionidae; European pine sawfly) feeds on 2-year-old needles of several pine species and readily defoliates *P. nigra* (D.A. Herms, personal observation). It is also an outbreak insect known to be sensitive to host quality (Larsson et al. 2000). In a given season, *P. nigra* can be attacked by both of these pests, indicating that host-mediated interactions between them may occur. Given their feeding locations, changes in host plant quality in response to one attacker that are systemic are more likely to impact the other than are local changes in quality. In a 2-year study, we showed that *D. pinea* infection of the stem of young *P. nigra* consistently induced systemic resistance against the same fungus in branches. Defoliation of branches by *N. sertifer* failed to systemically affect growth of this insect in undamaged branches in either year. In terms of cross-resistance, however, defoliation of branches by *N. sertifer* induced systemic resistance to *D. pinea* in an undamaged branch in one of the 2 years of the study. Conversely, *D. pinea* infection of the stem induced systemic resistance to *N. sertifer* growth on foliage from an undamaged branch in 1 year, while in the other year, systemic effects of both *D. pinea* infection and *N sertifer* feeding on *N. sertifer* survival on undamaged branches were significant, but depended upon soil fertility level (Eyles et al. 2007). These results indicated that systemic induced resistance to *D. pinea* in *P. nigra* by previous infection with the same fungus was consistent and, although reciprocal cross-resistance can occur in *P. nigra* in response to these attackers, the results can be asymmetric within a single year and variable among years.

Although regulatory mechanisms of defenses in conifers are poorly understood, conifers produce a suite of secondary metabolites in response to attack by insects and pathogens, notably phenolics and terpenoids (Franceschi et al. 2005). In a companion paper that focused on fungal resistance mechanisms in the phloem, we showed that, when examined as a group, phenolic glycosides and stilbenes increased systemically in the phloem of branches of young *P. nigra* in response to *D. pinea* infection of the stem and were positively correlated with resistance to *D. pinea* in those branches. On the other hand, terpenes were not inducible in the phloem by fungal infection as a group and were unrelated to fungal resistance. Only a single terpenoid, germacrene D, was systemically inducible in the phloem of branches by defoliation by *N. sertifer*, but it was unrelated to fungal resistance (Wallis et al. 2008). Less studied than secondary metabolites in conifers are pathogenesis-related (PR) and other defense-related proteins, which can be associated with insect and disease resistance in a variety of plants. In *Picea sitchensis*, a family of dirigent proteins that assist in lignan formation were locally induced by insect feeding, as were β-1,3-glucanases, chitinase, a protease inhibitor, and peroxidases (Ralph et al. 2006, 2007; Lippert et al. 2007). In *Pinus sylvestris*, chitinases were produced constitutively and were induced locally by endophytic microbes (Pirttilä et al. 2002), but not pathogenic fungi (Hodge et al. 1995). In *Pinus monticola*, chitinase was induced locally in needles by a pathogenic fungus (Liu et al. 2005), as was expression of a PR10 protein (Liu et al. 2003). In *P. sylvestris* and *Picea abies*, peroxidases and polyphenol oxidases were induced locally after attack by pathogenic fungi (e.g., Johansson et al. 2004). *P. abies* also produces chitinases, chitosanases, and glucanases locally after challenge with pathogenic fungi (e.g., Jøhnk et al. 2005). These studies revealed substantial variation in when and where induction of defense proteins may be expected to occur in conifers. However, these studies focused primarily on local induction. While certainly important in understanding local restrictions on disease spread or insect feeding, systemic changes in plant defenses have the potential to have broader effects on other interacting species. Despite mounting evidence of their importance, systemic induction of PR and other defense-related proteins has been little studied in conifers (but see Richard et al. 2000) and never in *P. nigra* in response to fungal or insect attack.

Environmental factors, such as soil fertility, are known to affect disease and insect resistance in plants. For example, resistance of red pine (*Pinus resinosa*) to *D. pinea* is decreased by high soil fertility (Blodgett et al. 2005), which can result in increased tree mortality (Stanosz et al. 2004). Such findings may relate to changes in host plant quality across soil environmental gradients. Although never studied in conifers, protein defenses in many herbaceous and woody plants can vary with soil fertility. Constitutive activity of trypsin inhibitors, peroxidases, chitinases, and β-1,3-glucanases increased in response to increased fertility in *Brassica napus*, *Arabidopsis thaliana*, and two *Citrus* species (Cipollini and Bergelson 2001; Cipollini 2002; Borowicz et al. 2003). Induced activity of trypsin inhibitors, papain inhibitors, and peroxidases were higher in plants with higher soil fertility (Bolter et al. 1998; Cipollini and Bergelson 2001; Cipollini 2002; Borowicz et al. 2003). In contrast, constitutive activities of trypsin inhibitors, chitinases, β-1,3-glucanases, and peroxidases in soybean

and tomato decreased with increasing fertility (Inbar et al. 2001; Vollmann et al. 2003). The equivocal nature of these results may be due to the different methods used to induce defenses (insect feeding, wounding, or exogenous hormone application). Furthermore, different methods of altering soil nutrients and different methods of sampling tissues may have yielded such varying results.

In this 3-year replicated study, we induced young Austrian pine trees with insect defoliation of branches or fungal inoculation of the main stem and monitored constitutive and induced activities of defensive proteins and levels of soluble proteins in both phloem and needles of uninfected or undefoliated branches. The effects of these treatments were examined across three soil fertility levels to examine the extent to which fertility modulated protein accumulation and the constitutive or inducible activities of defense proteins. We expected total protein levels and defense protein activities to increase with increasing fertility. In addition, we expected activities of defense proteins to be induced systemically by insect and fungal damage.

Methods and Materials

The *P. nigra–D. pinea–N. sertifer* system and our experimental design are described in detail in Eyles et al. (2007). Briefly, 4-year-old bare-root *P. nigra* saplings were grown outside in 21-L containers with a commercial nursery substrate (KB container mix, Kurtz Bros. Central Ohio, LLC) at the Ohio Agricultural Research and Development Center (Wooster, OH, USA). Plants were placed on a fertigation system at either low, intermediate, or high fertilization levels (30, 75, 150 ppm N; N/P/K 3:1:2) in the spring of 2004. Fertilization levels were chosen to represent recommended fertility regimes for containerized evergreen trees in forest nursery production (Eyles et al. 2007). After a year of conditioning on the fertilization regimes, four separate induction treatments were applied in the spring of 2005 (*D. pinea* infection, mock inoculation, defoliation by *N. sertifer*, and unwounded controls). The induction of fungal inoculations occurred 5 cm above the soil on the main stem where agar plugs containing *D. pinea* were placed in a 10-mm diameter wound made with a cork borer. Mock-inoculated trees received a 10-mm cork borer wound and a sterile agar plug. At the same time, a separate set of trees were induced by *N. sertifer* by allowing several third and fourth instar larvae to defoliate approximately 75% of the foliage on the tree (typically 2- and 3-year-old needles), while one branch in the top whorl of the tree remained protected by a mesh bag. This whorl was approximately 10 cm from the base of the trees, which averaged about 24 cm in height when induced. We replicated the study by growing a set of 6-year-old containerized trees grown under similar conditions and exposed to the same fertility treatments beginning in 2005 that were induced in the spring of 2006. The mock inoculation treatment was omitted in 2006 because it did not alter response variables significantly from those of control plants in 2005. The top whorl on trees used in the second study was about 40 cm from the base of the trees, which averaged about 63 cm in height. The fertility and induction treatments in both 2005 and 2006 were assigned to five spatial blocks with six to ten trees assigned to each fertility and induction treatment combination in each block. Further details on the induction procedure and our experimental design can be found in Eyles et al. (2007).

Foliage and branch phloem were sampled 16 days after induction treatments in 2005 and 21 days after induction treatments in 2006 (Eyles et al. 2007). While these timeframes are not necessarily optimal for capturing maximal increases in protein defenses after induction, they were timed to coincide with sampling for secondary metabolites (Wallis et al. 2008) and for challenge bioassays (Eyles et al. 2007). On trees induced by insect defoliation, foliage (second year needles) and branch phloem (hereafter phloem) were collected from a bagged branch from the top whorl. On trees induced by *D. pinea* infection of the stem, foliage and phloem were collected from a corresponding branch on the same whorl as that collected on insect-defoliated trees. Tissues were flash frozen immediately in liquid nitrogen and stored below −20°C until analysis. Tissues were first weighed, then ground with a mortar and pestle in liquid nitrogen. Needles were homogenized whole, while phloem was dissected from branch samples prior to homogenization. Soluble proteins were extracted from ground samples in ice-cold 0.01 M sodium phosphate buffer (pH 6.8) containing 5% *w*/*v* poly(vinylpolypyrrolidone). Guaiacol peroxidase (POD), polyphenol oxidase (PPO), chitinase (CHI), trypsin inhibitor (TI) activities, and total soluble protein content were assayed by using spectrophotometric and radial diffusion techniques as in Cipollini et al. (2004). β-1,3-glucanase (BGLU) activity was measured by following the hydrolysis of laminarin spectrophotometrically at 500 nm (modified from Abeles and Forrence 1970). Each sample for BGLU analysis was blanked twice, once with a blank containing extraction buffer and laminarin, and again with one containing extraction buffer and sample to account for color in the protein extracts. In 2005, activities of POD, PPO, and CHI were assessed in foliage and phloem, while TI and BGLU were only assessed in phloem due to limited amounts of available foliage. In 2006, activities of POD, PPO, CHI, and BGLU were measured in both tissues. Levels of TI were not assessed in 2006, since only very low levels were detected in phloem in 2005. Activities of the proteins were

expressed both per unit of extracted biomass and per unit extracted protein. Patterns were generally similar when expressed either way, so only activities expressed per unit extracted protein are reported. Total soluble protein contents were expressed per unit fresh weight of extracted biomass.

Data were transformed as necessary and analyzed for treatment effects using analysis of variance (SAS Version 9.1, SAS Institute). Factors in the model included fertility level, induction treatment, and their interactions. Block effects were examined and were negligible, so the degrees of freedom associated with this factor were included in the error degrees of freedom. Means were separated using Bonferroni comparisons. Only statistical results deemed significant at α=0.05 are presented in the descriptions below, although all data are shown in the figures.

Results

Interactions between fertility level and induction treatment were not significant for total protein content or for the activity of any protein. In no case did total soluble protein levels increase with increasing fertility or did activities of any defensive protein decrease with increasing fertility. We thus focus descriptions on the significant main effects of induction treatment and fertility, although nonsignificant patterns are also shown on the figures.

Across induction treatments, total soluble protein content of needles decreased with increasing fertility in both years of the study (Fig. 1a; 2005-fertility: $F_{2,169}$=6.10, P=0.003; 2006-fertility: $F_{2,111}$=7.47, P<0.001). Across induction treatments, total soluble protein contents in phloem were about ten times lower than in needles and tended to decrease with increasing fertility in 2005 (Fig. 1b; fertility: $F_{2,135}$=2.94, P=0.056). Across fertility levels in 2005, total soluble protein levels in phloem were 40% higher in trees induced with the fungus than in either control trees or those receiving a mock inoculation (Fig. 1b; induction: $F_{3,135}$= 5.05, P=0.002).

Across induction treatments, POD activity in needles increased with increasing fertility in 2005 (Fig. 2a; fertility: $F_{2,141}$=13.70, P<0.001). Across fertility levels, POD activity was almost 90% lower in needles of insect-induced trees than in control trees in 2006 (Fig. 2a; induction: $F_{2,100}$=6.72, P=0.002). In phloem, POD activities were about 400 times higher than in needles (Fig. 2). As in needles, POD activity in phloem in 2005 increased with increasing fertility across induction treatments, although differences were not as pronounced as those in needles (Fig. 2b; fertility: $F_{2,140}$= 4.65, P=0.011).

Across induction treatments, PPO activity in needles increased with increasing fertility in 2005 (Fig. 3a; 2005-needles-fertility: $F_{2,158}$=3.24, P=0.042). Activities in phloem were about ten times higher than in needles in 2005, but still lowest in the lowest fertility treatment with the effect nearly significant (Fig. 3b; 2005-phloem-fertility: $F_{2,138}$=3.03, P= 0.051). Across fertility levels, PPO levels in phloem were 40% higher in trees induced by insect feeding than in unwounded trees in 2005 (induction: $F_{3,133}$=4.28, P=0.006). Neither mock inoculation nor fungal attack affected PPO levels in phloem in 2005 (Fig. 3b).

Across induction treatments, CHI activity increased with increasing fertility in needles in 2006 and in phloem in both years (Fig. 4; 2006-needles-fertility: $F_{2,107}$=3.08, P=0.050; 2005-phloem-fertility: $F_{2,139}$=3.09, P=0.049; 2006-phloem-fertility: $F_{2,109}$=6.62, P=0.002). In 2005, CHI levels in phloem were about five times higher than in needles, and in 2006, levels in phloem were about ten times higher than in

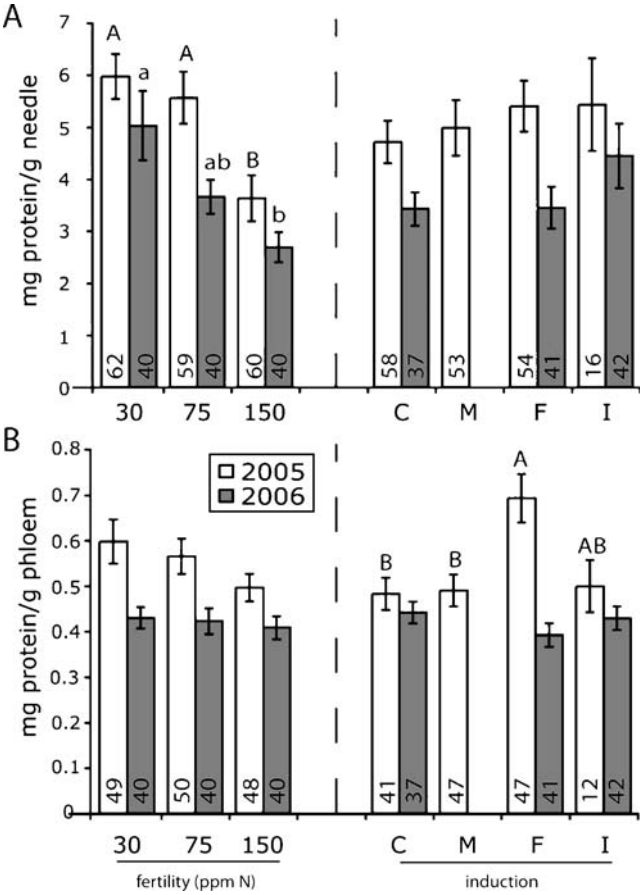

Fig. 1 Total soluble protein content of *P. nigra* tissues, means (milligrams soluble protein per gram fresh weight)±1 SE. *Bars with the same letter* were not significantly different using Bonferroni comparisons at P=0.05. If no letters are shown, results were not significantly different. Years were analyzed separately. *Numbers in bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug for 2005 only, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*. **a** Total soluble protein content of needles. **b** Total soluble protein content of phloem

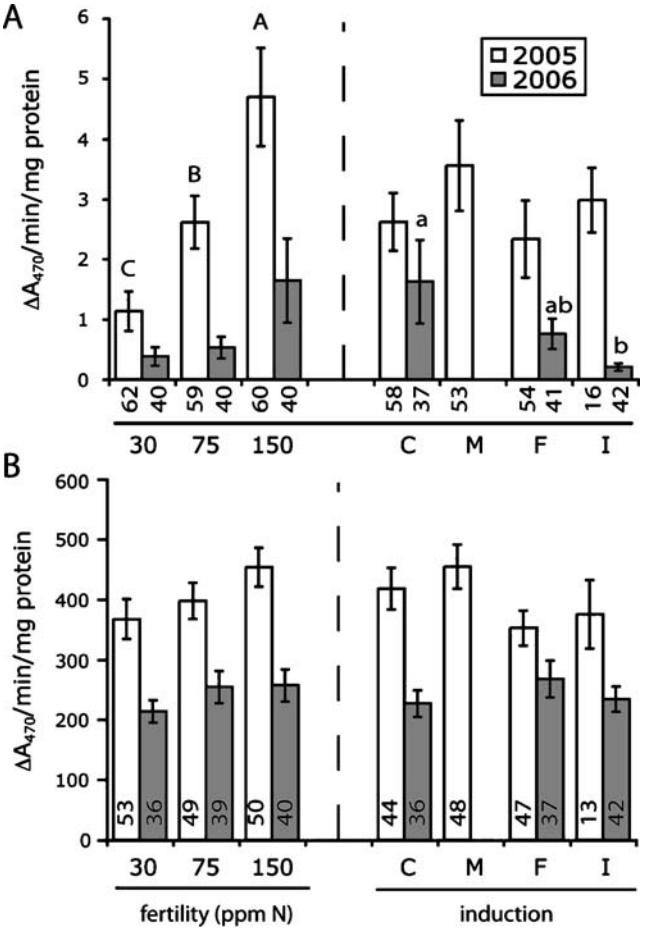

Fig. 2 Peroxidase activity in *P. nigra* tissues, means (ΔA_{470} per minute per milligram soluble protein)±1 SE. *Bars with the same letter* were not significantly different using Bonferroni comparisons at P=0.05. If no letters are shown, results were not significantly different. Years were analyzed separately. *Numbers in or immediately under bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug for 2005 only, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*. **a** Activity in needles. **b** Activity in phloem

needles (Fig. 4). Across fertility levels, no induction treatment altered CHI levels from those seen in controls in phloem in 2005, although levels were lower in trees induced by fungal attack than in trees receiving a mock inoculation (Fig. 4b; induction: $F_{3,139}$=3.07, P=0.030).

Activity of β-1,3-glucanase was very low in phloem in 2005 (Fig. 5). Across induction treatments, BGLU activity in needles in 2006 increased with increasing fertility (Fig. 5a; fertility: $F_{2,105}$=8.13, P<0.001). As for other defense proteins, BGLU activities were higher in phloem than in needles in 2006 (Fig. 5).

Trypsin inhibitor activity was only assessed in phloem in 2005 and was not significantly affected by any treatments. Levels detected were so low that TI was not measured in 2006 (Fig. 6).

Discussion

In 2005, we observed systemic induction of PPO in the phloem of undamaged branches of *P. nigra* trees that had been damaged by *N. sertifer* feeding on needles of other branches in the same whorl. Such systemic effects of insect feeding have never been demonstrated in conifer species, and they implicate long-distance signaling in the phloem. Systemic induction of PPO in upper leaves of poplar by damage to lower leaves has been demonstrated (Haruta et al. 2001), but systemic induction of this enzyme in the phloem of woody plants has not been studied to our knowledge. Despite systemic induction of PPO, defoliation by *N. sertifer* did not lead to systemic resistance to *N. sertifer* in the timeframe of our induction and challenge treatments (Eyles et al. 2007). In contrast, POD activity was

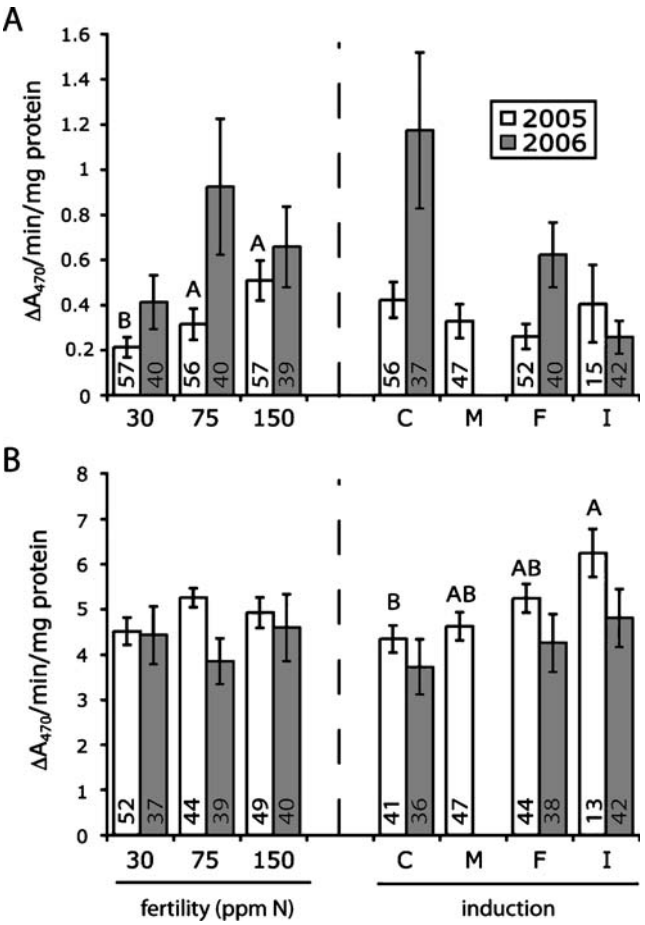

Fig. 3 Polyphenol oxidase activity in *P. nigra* tissues, means (ΔA_{470} per minute per milligram soluble protein)±1 SE. *Bars with the same letter* were not significantly different using Bonferroni comparisons at P=0.05. If no letters are shown, results were not significantly different. Years were analyzed separately. *Numbers in bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug for 2005 only, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*. **a** Activity in needles. **b** Activity in phloem

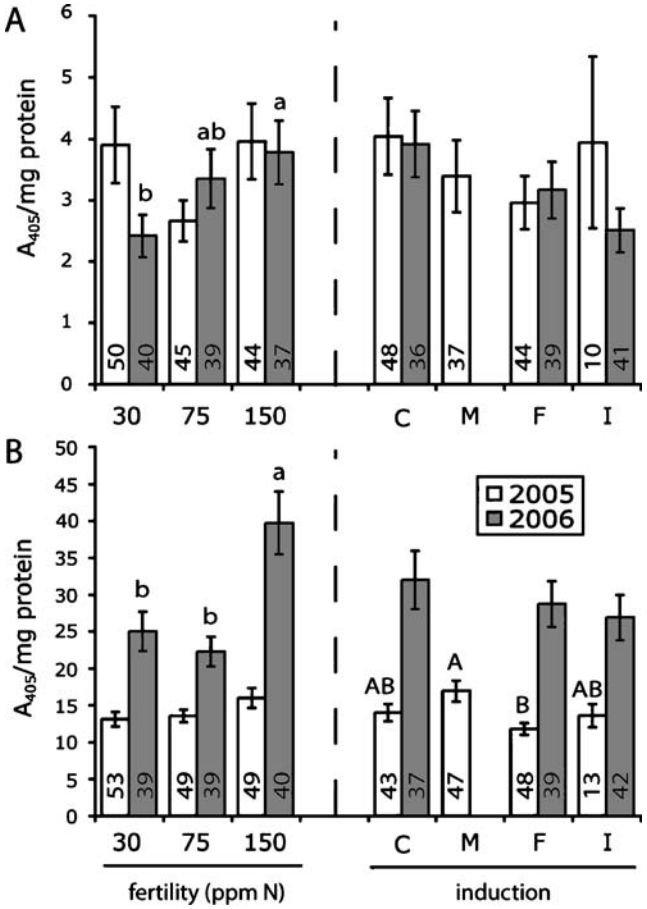

Fig. 4 Chitinase activity in *P. nigra* tissues, means (A_{405} per milligram soluble protein)±1 SE. *Bars with the same letter* were not significantly different using Bonferroni comparisons at P=0.05. If no letters are shown, results were not significantly different. Years were analyzed separately. *Numbers in bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug for 2005 only, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*. **a** Activity in needles. **b** Activity in phloem

systemically suppressed in needles by insect feeding on other branches in 2006. Suppression of POD could be due to resource constraints or may reflect an ability of the insect to directly suppress the plant's defense response through signal interactions (Musser et al. 2005). Inoculation with *D. pinea* had surprisingly little effect on systemic defense protein activities during the timeframe of our study in either year, and aside from effects on PPO and POD, we saw little systemic response to induction treatments in the other defense proteins that we studied. Activities of rapidly induced defense proteins often increase within a matter of hours to days, typically followed by a return to basal levels sometime thereafter, so our relatively delayed sampling may have limited our ability to observe maximal changes in protein activities. In addition, changes in these proteins induced locally to the site of damage may have been more noticeable than systemic changes. Some of the variation in plant responses to treatments among years may reflect age differences of the trees used in each year.

Despite the lack of effect on insect growth, insect induction induced systemic resistance to fungal growth in 2006 (Eyles et al. 2007). Conversely, fungal induction induced systemic resistance to fungal growth in both 2005 and 2006, and it induced systemic resistance to insect growth in 2005 (Eyles et al. 2007). Since fungal inoculation did not induce systemically any of the defensive proteins that we measured, other defenses (protein or nonprotein) must have been responsible for the fungal-induced effects on resistance. In the same trees as the current study, Wallis et al. (2008) found that phenolic glycosides and stilbenes increased systemically in the phloem of uninoculated

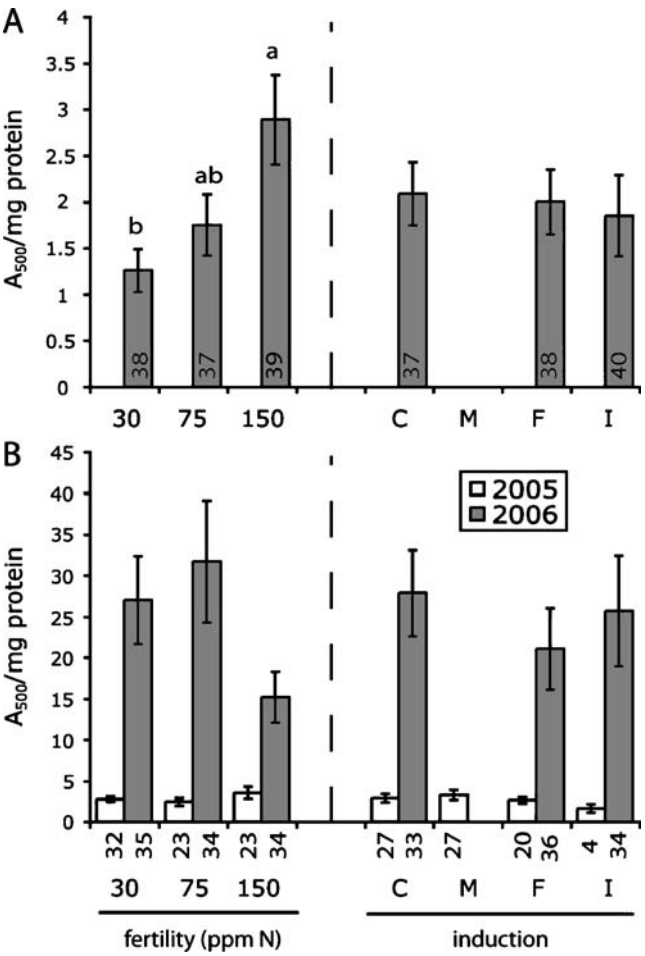

Fig. 5 β-1,3-glucanase activity in *P. nigra* tissues, means (A_{500} per milligram soluble protein)±1 SE. *Bars with the same letter* were not significantly different using Bonferroni comparisons at P=0.05. If no letters are shown, results were not significantly different. Years were analyzed separately. *Numbers in or immediately under bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug for 2005 only, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*. **a** Activity in needles. β-1,3-glucanase activity was not assayed in needles in 2005. **b** Activity in phloem

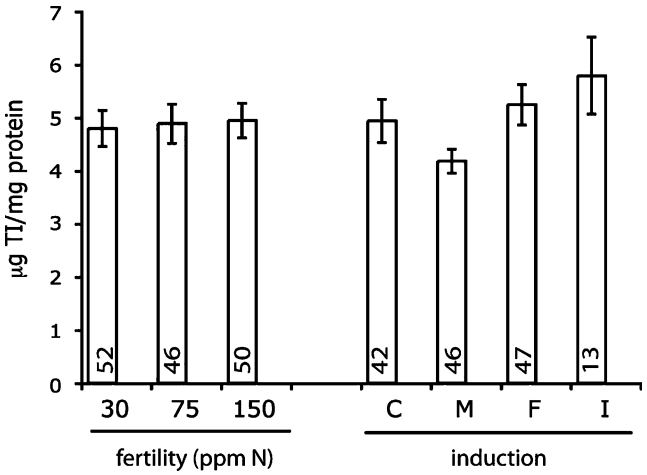

Fig. 6 Trypsin inhibitor levels in *P. nigra* phloem in 2005, means (micrograms trypsin inhibitor per milligram soluble protein)±1 SE. *Numbers in bars* indicate number of replicates. *C* control—untreated trees, *M* mock—trees challenged with a sterile agar plug, *F* fungus—trees inoculated with *D. pinea*, *I* insect—trees defoliated by *N. sertifer*

branches of *P. nigra* in response to infection of the stem and were positively correlated with resistance to *D. pinea* in those branches. In the current study, fungal inoculation induced systemic increases in total protein levels in the phloem, some of which with defensive roles may have contributed to resistance. Cross-resistance to pathogens induced by arthropod feeding has been observed in other studies, and in these examples, the inverse was also true (McIntyre et al. 1981; Karban et al. 1987; Inbar et al. 1998). These findings demonstrate that the outcome of host-mediated interactions between pests can be positive or negative and depends on environmental conditions as well as attacker identity.

More important than systemic induction, accumulation of total soluble proteins and the activity of most of the defense proteins studied here were mediated strongly by soil fertility. Declines in total soluble protein levels with increasing fertility contrasts with reports of either increases in total protein with increasing fertility (Borowicz et al. 2003; Vollmann et al. 2003) or constant total protein levels across fertility levels (Cipollini and Bergelson 2001). Although the results presented here were unexpected, they were consistent in both years of the study in trees of different ages and exposed to different ambient climatic conditions. Wallis et al. (2008) also documented decreases in total phenolics and concentrations of several individual phenolic metabolites in the trees in the high-fertility treatment in 2005. Assuming that our high-fertility treatment was not actually stressful to the plants, several factors may have contributed to this unexpected result. Protein concentrations were expressed per unit fresh weight of tissues, so if protein production rates stayed the same or declined as the trees grew, protein concentrations would decline due to dilution in greater amounts of tissue. Proteins may also have been present but less extractable for some reason in plants grown at high fertility. We also analyzed only soluble proteins, and insoluble structural proteins may respond differently to changes in fertility. In other studies, however, increases in protein levels with fertility were seen for total (soluble and insoluble) proteins (Vollmann et al. 2003), as well as for soluble proteins alone (Borowicz et al. 2003). This effect is unlikely to be due to the nature of the fertilizer, since Borowicz et al. (2003) also used a complete fertilizer and found increasing levels of total proteins with increasing fertility. Finally, the growth conditions used here may have been more conducive to production of certain metabolites that may have competed for substrate with nitrogen-rich proteins. Regardless of the mechanisms that control protein production in conifers, soil fertility clearly played an important role in this study.

In contrast to effects on total soluble protein levels, defense protein activities generally increased with increasing fertility, as has been seen in other studies (e.g., Bolter et al. 1998; Cipollini and Bergelson 2001). This was true whether the proteins were responsive to induction and whether their activities were expressed per unit protein or per unit biomass. This suggests that maintenance or increased production of some proteins is favored under increasing fertility levels, while levels of other proteins apparently decline. The finding that total protein levels declined while activities of some defense proteins increased suggests that, per unit biomass, tissues from plants in our high-fertility treatment should be of lower quality to herbivores and pathogens sensitive to such factors than tissues from our low-fertility treatment. However, in our studies, fertility had no direct effect on resistance to the fungus or the insect (Eyles et al. 2007, Wallis et al. 2008). This may be due to the fact that, as certain defenses increased with fertility, such as the activity of defensive proteins seen here, other defenses decreased with fertility, such as concentrations of total phenolics and individual phenolic metabolites shown by Wallis et al. (2008).

Trypsin inhibitors have been little studied in conifers, but are a well-known constitutive and inducible defense in many herbaceous and woody plants (e.g., Bolter et al. 1998; Cipollini and Bergelson 2001; Major and Constabel 2008). In 2005, we found only a low level of TI activity in phloem and we have found no activity in needles in other studies (D. Cipollini, unpublished data). While activities tended to increase with fertility in 2005, as with the other defensive proteins, TI activities were not affected significantly by any of our treatments. The restriction of TI activity in the phloem indicates that it could act against phloem feeding pests, probably wood boring insects or other phloem-inhabiting pathogens, but the low activity detected may limit its importance.

We have demonstrated systemic induction or suppression by insect feeding of PPO and POD in the phloem of branches of *P. nigra*. In contrast, *D. pinea* infection had no significant systemic effect on these proteins in needles or phloem, but it did induce systemic increases in total soluble protein accumulation in the phloem. Soil fertility strongly decreased concentrations of total soluble protein in needles and phloem, while it increased the activity of several defense proteins. Variation in concentration and activities of these proteins may be related to variation in pest attack and performance on *P. nigra* across natural fertility gradients in the field, but their impact on pests depends on correlated responses in other plant defenses.

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References

ABELES, F. B., and FORRENCE, L. E. 1970. Temporal and hormonal control of β-1.3-glucanase in *Phaseolus vulgaris* L. *Plant Physiol.* 45:395–400.

BLODGETT, J. T., HERMS, D. A., and BONELLO, P. 2005. Effects of fertilization on red pine defense chemistry and resistance to *Sphaeropsis sapinea*. *For. Ecol. Manag.* 208:373–382.

BOLTER, C. J., LATOSZEK-GREEN, M., and TENUTA, M. 1998. Dependence of methyl jasmonate- and wound-induced cysteine proteinase inhibitor activity on nitrogen concentration. *J. Plant Physiol.* 152:427–432.

BONELLO, P., and BLODGETT, J. T. 2003. *Pinus nigra–Sphaeropsis sapinea* as a model pathosystem to investigate local and systemic effects of fungal infection of pines. *Physiol. Mol. Plant Pathol.* 63:249–261.

BOROWICZ, V. A., ALBRECHT, U., and MAYER, R. T. 2003. Effects of nutrient supply on *Citrus* resistance to root herbivory by *Diaprepes abbreviatus* L. (Coleoptera: Curculionidae). *Environ. Entomol.* 32:1242–1250.

CIPOLLINI, D. F. 2002. Does competition magnify the fitness costs of induced responses in *Arabidopsis thaliana*? A manipulative approach. *Oecologia* 131:514–520.

CIPOLLINI, D. F., and BERGELSON, J. 2001. Plant density and nutrient availability constrain constitutive and wound-induced expression of trypsin inhibitors in *Brassica napus*. *J. Chem. Ecol.* 27:593–610.

CIPOLLINI, D. F., ENRIGHT, S., TRAW, M. B., and BERGELSON, J. 2004. Salicylic acid inhibits jasmonic acid-induced resistance of *Arabidopsis thaliana* to *Spodoptera exigua*. *Mol. Ecol.* 13:1643–1653.

EYLES, A., CHORBADJIAN, R., WALLIS, C., HANSEN, R., CIPOLLINI, D., HERMS, D., and BONELLO, P. 2007. Cross-induction of systemic induced resistance between an insect and a fungal pathogen in Austrian pine over a fertility gradient. *Oecologia* 153:365–374.

FRANCESCHI, V. R., KROKENE, P., CHRISTIANSEN, E., and KREKLING, T. 2005. Anatomical and chemical defenses of conifer bark against bark beetles and other pests. *New Phytol.* 167:353–376.

HARUTA, M., PEDERSEN, J. A., and CONSTABEL, C. P. 2001. Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. *Physiol. Plant.* 112:552–558.

HODGE, A., ALEXANDER, I. J., and GOODAY, G. W. 1995. Chitinolytic activities of *Eucalyptus pilularis* and *Pinus sylvestris* roots systems challenged with mycorrhizal and pathogenic fungi. *New Phytol.* 131:255–261.

INBAR, M., DOOSTDAR, H., and MAYER, R. T. 2001. Suitability of stressed and vigorous plants to various insect herbivores. *Oikos* 94:228–235.

INBAR, M., DOOSTDAR, H., S, onoda, R. M., LEIBEE, G. L., and MAYER, R. T. 1998. Elicitors of plant defensive systems reduce insect densities and disease incidence. *J. Chem. Ecol.* 24:135–149.

JOHANSSON, S. M., LUNDGREN, L. N., and ASIEGBU, F. O. 2004. Initial reactions in sapwood of Norway spruce and Scots pine after wounding and infection by *Heterobasidion parviporum* and *H. annosum*. *For. Pathol.* 34:197–210.

JØHNK, N., HIETALA, A. M., FOSSDAL, C. G., COLLINGE, D. B., and NEWMAN, M.-A. 2005. Defense-related genes expressed in Norway spruce roots after infection with the root rot pathogen *Ceratobasidium bicorne* (anamorph: *Rhizoctonia* sp.). *Tree Physiol.* 25:1533–1543.

KARBAN, R., ADAMCHAK, R., and SCHNATHORST, W. A. 1987. Induced resistance and interspecific competition between spider mites and a vascular wilt fungus. *Science* 234:678–680.

LARSSON, S., EKBOM, B., and BJÖRKMAN, C. 2000. Influence of plant quality on pine sawfly population dynamics. *Oikos* 89:440–450.

LIPPERT, D., CHOWRIRA, S., RALPH, S. G., ZHUANG, J., AESCHLIMAN, D., RITLAND, C., RITLAND, K., and BOHLMANN, J. 2007. Conifer defense against insects: proteome analysis of Sitka spruce (*Picea sitchensis*) bark induced by mechanical wounding or feeding by white pine weevils (*Pissodes strobii*). *Proteomics* 7:248–270.

LIU, J.-J., EKRAMODDOULLAH, A. K. M., and YU, X. 2003. Differential expression of multiple PR10 proteins in western white pine following wounding, fungal infection and cold-hardening. *Physiol. Plant.* 119:544–553.

LIU, J.-J., EKRAMODDOULLAH, A. K. M., and ZAMANI, A. 2005. A class IV chitinase is up-regulated by fungal infection and abiotic stresses and associated with slow-canker-growth resistance to *Cronartium ribicola* in Western White Pine (*Pinus monticola*). *Phytopathology* 95:284–291.

MAJOR, I. T., and CONSTABEL, C. P. 2008. Functional analysis of the Kunitz trypsin inhibitor family in poplar reveals biochemical diversity and multiplicity in defense against herbivores. *Plant Physiol.* 146:888–903.

MCINTYRE, J. L., DODDS, J. A., and HARE, J. D. 1981. Effects of localized infections of *Nicotiana tabacum* by tobacco mosaic virus on systemic resistance against diverse pathogens and an insect. *Phytopathology* 71:297–301.

MUSSER, R. O., CIPOLLINI, D. F., HUM-MUSSER, S. M., WILLIAMS, S. A., BROWN, J. K., and FELTON, G. W. 2005. Evidence that the caterpillar salivary enzyme glucose oxidase provides herbivore offense in *Solanaceous* plants. *Arch. Insect Biochem. Physiol.* 58:128–137.

PETERSON, G. W. 1977. Infection, epidemiology, and control of *Diplodia* blight of Austrian, ponderosa, and Scots pine. *Phytopathology* 67:511–514.

PIRTTILÄ, A. M., LAUKKANEN, H., and HOHTOLA, A. 2002. Chitinase production in pine callus (*Pinus sylvestris* L.): a defense reaction against endophytes? *Planta* 214:848–852.

RALPH, S. G., JANCSIK, S., and BOHLMANN, J. 2007. Dirigent proteins in conifer defense II: extended discovery, phylogeny, and constitutive and stress-induced gene expression in spruce (*Picea* spp.). *Phytochemistry* 68:1975–1991.

RALPH, S. G., YUEH, H., FRIEDMANN, M., AESCHLIMAN, D., ZEZNIK, J. A., NELSON, C. C., BUTTERFIELD, Y. S. N., KIRKPATRICK, R., LIU, J., JONES, S. J. M., MARRA, M. A., DOUGLAS, C. J., RITLAND, K., and BOHLMANN, J. 2006. Conifer defense against insects: microarray gene expression profiling of Sitka spruce (*Picea sitchensis*) induced by mechanical wounding or feeding by spruce budworms (*Choristoneura occidentalis*) or white pine weevils (*Pissodes strobi*) reveals large-scale changes of the host transcriptome. *Plant Cell Environ.* 29:1545–1570.

RICHARD, S., LAPOINTE, G., RUTLEDGE, R. G., and SÉGUIN, A. 2000. Induction of chalcone synthase expression in white spruce by wounding and jasmonate. *Plant Cell Physiol.* 41:982–987.

STANOSZ, G. R., TROBAUGH, J., GUTHMILLER, M. A., and STANOSZ, J. C. 2004. *Sphaeropsis* shoot blight and altered nutrition in red pine plantations treated with paper mill waste sludge. *For. Pathol.* 34:245–253.

VOLLMANN, J., GRAWUSGRUBER, H., WAGENTRISTL, H., WOHLESER, H., and MICHELE, P. 2003. Trypsin inhibitor activity of soybean as affected by genotype and fertilisation. *J. Sci. Food Agric.* 83:1581–1586.

WALLIS, C., EYLES, A., CHORBADJIAN, R., MCSPADDEN GARDENER, B., HANSEN, R., CIPOLLINI, D., HERMS, D. A., and BONELLO, P. 2008. Systemic induction of phloem secondary metabolism and its relationship to resistance to a canker pathogen in Austrian pine. *New Phytol.* 177:767–789.

Say) is a member of the gall midge family (Diptera: Cecidomyiidae), which contains many gall-forming species. Feeding by virulent Hessian fly larvae does not result in the development of a typical gall structure. Instead, the larvae induce young unexpanded leaves to develop a gall-like nutritive tissue, similar to the inner lining of a gall that lacks an outer region of tissue called a covering gall. Harris et al. (2006) examined the ultrastructure of virulent Hessian fly-induced nutritive tissue cells in host wheat plants (*Triticum aestivum* L. em Thell) and reported an accumulation of cellular organelles (mitochondria, proplastids, Golgi, rough endoplasmic reticulum) and a decrease in size of the nucleus. Cell wall thinning leading to rupture of nutritive tissue cells was observed and is presumed to be the mechanism by which the cell contents are made available to virulent Hessian fly larvae.

Interactions between Hessian fly larvae and their host wheat plants are characterized as incompatible when avirulent larvae are detected by the plant in an *R*-gene-mediated gene-for-gene recognition event that blocks them from establishing permanent feeding sites, thus resulting in larval death (Flor 1955; Hatchett and Gallun 1970; Kaloshian 2004). Conversely, compatible wheat–Hessian fly interactions occur when virulent larvae infest the host plant and successfully establish feeding sites among the leaf sheaths near the base of the plant (the crown). Macroscopic changes observed in susceptible plants include a darkening of green leaf color (Robinson et al. 1960) and stunted plant growth compared to uninfested plants (Cartwright et al. 1959). These characteristics of susceptible wheat plants promote the survival of virulent Hessian fly larvae by providing access to nutrients in an area of the plant where larvae are protected from natural enemies.

It is unclear whether susceptible wheat plants (1) fail to mount a defense response against virulent Hessian fly larvae, (2) mount only a partial defense response, or (3) are subject to active suppression of defense mechanisms as a result of virulent larval feeding. Tooker and De Moraes (2007) reported that susceptible wheat plants attacked by virulent Hessian fly larvae failed to produce volatile chemicals (indirect defense response) that could attract insect natural enemies, whereas feeding by larvae of the tobacco budworm (*Heliothis virescens* Fabricius), a generalist herbivore, caused them to emit significantly higher amounts of volatile chemicals. The authors noted that prior feeding by Hessian fly larvae changed plant volatile emissions following subsequent feeding by *H. virescens*, suggesting that virulent Hessian fly larvae may exert some control over indirect defense responses in host wheat plants (Tooker and De Moraes 2007).

Recent research has identified biochemical pathways and physiological mechanisms in wheat conferring resistance and susceptibility to Hessian fly (Puthoff et al. 2005; Sardesai et al. 2005; Giovanini et al. 2006, 2007; Subramanyam et al. 2006, 2008; Liu et al. 2007; Tooker and De Moraes 2007). Zhu et al. (2008) examined the nutrient content of Hessian fly-infested wheat plants and reported changes in carbon and nitrogen metabolism in the wheat line *H9*-Molly (contains the *H13* resistance gene) infested with the Hessian fly line *vH13* (virulent on *H9*-Molly wheat). Changes included a 36% increase in free carbon-containing compounds and a 46% increase in free nitrogen-containing compounds. Wheat metabolic pathways reported to be associated with these changes included glycolysis, the tricarboxylic acid cycle, the pentose phosphate pathway, and amino acid synthesis (Zhu et al. 2008).

Bronner (1992) reported that nutritive tissues lining cecidomyiid-induced galls accumulate "soluble amino products", presumed to be amides or amino acids, which were proposed to arise from either proteolysis in gall tissues or transport from other parts of the host plant. Free amino acids (nonprotein-bound amino acids) ingested by an insect carry the advantage of being absorbed directly without processing in the gut, such as is required for polypeptides. Some species of aphid are known to alter the nutritional quality of host wheat plants by increasing the abundance of plant free amino acids in phloem (Telang et al. 1999; Eleftherianos et al. 2006). Two aphid species that attack wheat, the greenbug (*Schizaphis graminum* Rondani) and the Russian wheat aphid (*Diuraphis noxia* Mordvilko), manipulate wheat plant physiology and enhance the free amino acid composition of phloem sap that they ingest (Sandström et al. 2000).

In the present study, results support the hypothesis that crown tissues in Hessian fly-susceptible wheat plants contain increased levels of free amino acids following feeding by virulent Hessian fly larvae. Although the physiological function of elevated free amino acids in susceptible wheat plants remains unclear, we propose that this increase could improve the nutritional quality of plant fluids ingested by virulent larvae. Alternatively, increased levels of free amino acids may be associated with physiological processes in the plant and have no direct effect on larval nutrition. A possible link between elevated levels of free proline in susceptible plants and host plant energy requirements during compatible wheat–Hessian fly interactions is discussed.

Methods and Materials

Plant Growth and Hessian Fly Infestation Hessian fly Biotype L was maintained as a purified laboratory stock by the USDA-ARS Crop Production and Pest Control Research Unit in West Lafayette, IN, USA and was used for all infestations throughout the present study. Biotype L was collected as a wild population from an Indiana wheat field

and is virulent to four standard wheat differential lines, each containing one of the following Hessian fly-resistance genes: *H3*, *H5*, *H6*, and the two-gene combination *H7H8*. The laboratory Hessian fly line *vH13*, which is mentioned in association with other research, differs from Biotype L in that *vH13* was artificially selected in the lab, from an unrelated fly stock, to be virulent on wheat containing the *H13* resistance gene. Two near-isogenic winter wheat lines (Patterson et al. 1994), Newton (susceptible to Biotype L; compatible interaction) and *H9*-Iris (resistant to Biotype L due to the *H9* resistance gene; incompatible interaction), were used for all experiments. Ten wheat seeds were planted in each 10-cm plastic pot and infested at the two-leaf stage with Hessian fly Biotype L (five females and two males per pot) as previously described (Giovanini et al. 2006). Control plants were treated identically except that they received no flies. Following infestation, plants were dissected daily to determine when larvae reached the base of the crown (aerial portion of a seedling above the roots and below the ligule of the first leaf). To verify that the predicted interactions occurred, plants grown in parallel to those used for experimentation were compared 14 days after egg hatch, demonstrating that compatible interactions resulted in stunted plants containing living larvae and incompatible resulted in unstunted plants containing only dead first instars. The four wheat differential lines (resistance genes *H3*, *H5*, *H6*, and *H7H8*) were included as controls to verify the genotype of Biotype L. Two sets of tissue (biological replicates) were grown simultaneously with plants for free amino acid analysis and RNA isolation in each replicate.

Isolation, Identification, and Quantification of Wheat Free Amino Acids Crown tissues from infested and uninfested wheat seedlings were collected for free amino acid analysis 1, 4, and 7 days after Hessian fly egg hatch. Two biological replicates of each treatment (ten plants pooled per replicate) were harvested. Larvae were removed, from where they had settled at the base of leaf three, by washing with 0.2 µm filtered water after pealing back outer leaves. Then the basal 1 cm of crown tissue from each plant was collected and pooled to determine grams fresh weight (gfw) per sample. Samples were stored in liquid nitrogen until free amino acid isolation was carried out (on the same day) by using the methods of Lam et al. (2003).

Dried samples were stored at −80°C prior to shipment on dry ice to the Molecular Structure Facility at the University of California, Davis. Free amino acid analysis was conducted with a Beckman 6300 amino acid analyzer (Beckman Coulter, Fullerton, CA, USA) with a lithium citrate buffer system (Ozols 1990). Prior to analysis, samples were acidified with sulfosalicylic acid to remove intact proteins. Free amino acids were separated by ion-exchange chromatography (Rossomando 1990) and analyzed by using a post-column ninhydrin reaction detection system (Macchi et al. 2000). Samples for free amino acid analysis were dissolved in lithium citrate buffer containing aminoethylcysteine (AEcys) as an internal standard. AEcys was used to correct for any variation in the operating conditions of the Beckman 6300 amino acid analyzer. Authentic standards for amino acid identification were utilized (Pickering #012006P physiological standard mixture, Beckman Coulter). Amino acids were identified using Beckman 32 Karat software (version 5.0). In Table 2, ammonia refers to ammonia salts (detected as ammonium ion, NH_4^+) and ethanolamine is an organic chemical compound detected in our experiments because it is a primary amine and contains an amino group that reacts with ninhydrin (Macchi et al. 2000).

Concentration data for amino acids and other detected compounds were used to calculate nanomoles per gram fresh weight plant tissue (nmol/gfw) for each chemical. Based on the methods used to separate and identify free amino acids, cysteine and tryptophan could not be assayed. Quantification of these chemicals would have required different analytical methods. For each treatment, the fold-change in concentration for a given chemical (shown in Table 1) was calculated by dividing the concentration (nonlogarithm transformed) in the Hessian fly-infested treatment by the concentration in the corresponding uninfested controls (i.e., for each chemical and at each time point, average values for uninfested *H9*-Iris and Newton controls were used as divisors to calculate fold-change of corresponding infested samples). Reported fold-changes represent two biological replicates.

RNA Isolation and cDNA Synthesis The basal 1-cm of crown tissues from infested and uninfested wheat seedlings were collected into liquid nitrogen as two biological replicates per treatment (ten plants per replicate) for RNA extraction 1, 4, and 7 days after Hessian fly egg hatch. These collections were made at the same time as those used for amino acid analysis. Larvae were not removed from plants. RNA isolation and cDNA synthesis followed the methods of Giovanini et al. (2006).

Primers for Quantitative Real-time PCR Our interest in five of the nine wheat sequences used for qRT-PCR resulted from an Affymetrix GeneChip® Wheat Genome Array experiment recently carried out by our laboratory (C. Williams, unpublished). Probe sets corresponding to Hessian fly-responsive genes included histidine amino acid transporter (GenBank accession Ta.12339.1.S1_at), prephenate dehydratase (Ta.9122.1.S1_at), proline transporter (Ta.30603.1.S1_s_at), pyrroline-5-carboxylate synthetase (Ta.7091.1.S1_at), and transmembrane amino acid transporter (Ta.27343.1.S1_at). Annotation of the Affymetrix

Table 1 Wheat gene-specific primers used in quantitative real-time PCR

Gene name (*T. aestivum*)	Accession	Primer sequence
Alanine aminotransferase (glutamic acid synthesis)	AJ606028	(F) 5′ TGTCGTCGTTCCTGGATCTG
		(R) 5′ TGGATATGATTGCAGGGATTTTG
Glutamine-dependent asparagine synthetase (glutamic acid synthesis)	AY621539	(F) 5′ TGAAGGGCCTGAATGATGATT
		(R) 5′ CCCTGCTTGCTGGAGTAGAGA
Histidine amino acid transporter	BT009250	(F) 5′ GGATTCCAAGACATACAAATTCTACTCA
		(R) 5′ TCGTGGTAGGCATCGTAAATGAT
Ornithine aminotransferase (glutamic acid synthesis)	AF022915	(F) 5′ GGCACGGAGGCAAATGAG
		(R) 5′ AGTGAAATAATGTCATGGGAACCA
Prephenate dehydratase (Phenylalanine synthesis)	BE419015	(F) 5′ GAGCTACCCGATGGACATGAC
		(R) 5′ TCGATACCTCTAGGGAGCTACGA
Proline transporter	BQ162277	(F) 5′ GCTTCTCTGAGGAGTGAGGATAGTTT
		(R) 5′ TAAATGAAGGTAAGGCATGCTACATC
Pyrroline-5-carboxylate synthetase (proline synthesis)	AF022914	(F) 5′ GCACCCTCGAATTTGTTGATG
		(R) 5′ ACAATCTGTGTGTGCACTTCCAT
Transmembrane amino acid transporter	BT009014	(F) 5′ TCGGCGAGCTCAAGGAGTAC
		(R) 5′ TGTGCCGCTGTGCTACTCA
Ubiquitin	X56803	(F) 5′ GGTGTCTCCGGTATCCTCCAA
		(R) 5′ TGCTCCACACCAGCAGAAGT

probe set sequences was carried out with HarvEST software (http://harvest.ucr.edu; Wheat 1 Array, version 1.52). Additional functional annotation was performed by using two web-based resources: the International Union of Biochemistry and Molecular Biology Enzyme Nomenclature website (www.chem.qmul.ac.uk/iubmb), and MetaCyc, a database of experimentally elucidated metabolic pathways (http://metacyc.org; Caspi et al. 2006). The Affymetrix target sequence was used to design qRT-PCR primers (Table 1) with Primer Express software (Applied Biosystems, Foster City, CA, USA). The protocol for qRT-PCR was as described in Giovanini et al. (2006). Following qRT-PCR, a melt curve analysis verified primer specificity by confirming the presence of a single PCR product. mRNA levels were calculated by using the standard curve serial dilution method described in the ABI "User Bulletin 2: ABI PRISM 7700 Sequence Detection System". The threshold cycle (C_T) for each dilution was plotted against its cDNA concentration (with an arbitrary starting quantity of 1 for the undiluted sample) and a regression equation generated Arbitrary Expression Values (AEV) for the experimental samples. Fold-changes reported in the text were calculated by dividing the AEV (nonlogarithm transformed) of a Hessian fly-infested treatment by the AEV of the corresponding uninfested control at the same time point. Fold-changes reported in the text and mRNA abundance data (AEV) shown in Fig. 2 represent two independent experiments (two biological replicates), and all samples were subjected to qRT-PCR × 3 (three technical replicates).

Statistical Analysis Experimental results were analyzed by using SAS software version 9.1.3 (SAS Institute Inc., Cary, NC, USA). Significance of the free amino acid concentration data and qRT-PCR data was determined by analysis of variance (ANOVA) using the PROC MIXED procedure of SAS. For analysis of free amino acid concentration data, the three main effects included in the analysis models were infestation status (Hessian fly-infested, -uninfested), interaction type (compatible, incompatible), and experimental time point. All interactions of main effects were included in the analysis models as predictor variables. Three-factor ANOVA models were fitted to the amino acid concentration data, with logarithm transformed concentration being the response variable for each type of amino acid. The LSMEANS statement for the three-way interaction was used to conduct pairwise comparisons of the mean amino acid concentration between Hessian fly-infested and -uninfested. For analysis of qRT-PCR data, two-factor ANOVA models were fitted with logarithm transformed qRT-PCR AEV data, which served as the response variable. ANOVA models were fitted to interaction type and gene type, and pairwise comparisons of the mean mRNA abundance levels were conducted using the LSMEANS statement for the two-way interaction. All P-values for amino acid and qRT-PCR data were adjusted by the Bonferroni correction procedure with respect to the total number of experimental time points and the total number of genes tested. The adjusted P values were considered statistically significant if $P<0.05$.

Results

Accumulation of Standard Free Amino Acids in Wheat Crown Tissues Although changes in free amino acid levels

were detected in both susceptible and resistant wheat plants as early as 1 day after egg hatch, these changes were not significantly different compared to uninfested controls (Table 2). Four days after egg hatch, significant increases in free amino acid abundance were observed in susceptible plants and included alanine (ala) (adjusted *P*-values from Bonferroni correction: $P=0.003$), glutamic acid (glu) ($P=0.009$), glycine (gly) ($P=0.046$), phenylalanine (phe) ($P=0.044$), proline (pro) ($P<0.001$), and serine (ser) ($P=0.046$). In resistant plants at the same time point, only ser ($P=0.002$) was significantly more abundant compared to uninfested controls. At the last time point assayed, 7 days after egg hatch, fewer significant changes in amino acid abundance were observed in susceptible plants compared to the previous 4-day time point, with only the aromatic amino acids phe ($P<0.001$) and tyrosine (tyr) ($P=0.001$) being more abundant compared to uninfested controls (Table 2).

Accumulation of Other Nitrogen-Containing Products in Wheat Crown Tissues Our study also quantified α-amino-*n*-butyric acid (AABA), ammonia, ethanolamine, γ-aminobutyric acid (GABA), 1-methylhistidine, and ornithine (orn). Compared to uninfested controls, no significant differences in the abundance of these chemicals were detected, but some trends were observed. For example, AABA was present at detectable levels only in control and resistant plants 1 and 4 days after egg hatch (Table 2). Ammonia, a normal product of amino acid metabolism,

Table 2 Fold-change of chemical concentration in Hessian fly-infested wheat compared to uninfested controls

Chemical	1 day after egg hatch		4 days after egg hatch		7 days after egg hatch	
	Sus.[a]	Res.[b]	Sus.	Res.	Sus.	Res.
Ala	1.55[c]±0.16	1.64±0.11	**2.18±0.02**	1.23±0.12	1.09±0.22	1.07±0.23
Arg[d]	1.18±0.15	1.14±0.15	1.53±0.65	−1.78±0.32	1.28±0.26	1.02±0.55
Asn	−1.43±0.40	1.09±0.39	1.34±0.84	−1.55±0.03	−2.04±0.85	−2.04±1.16
Asp	1.27±0.03	1.09±0.04	1.37±0.01	−1.15±0.23	−1.38±0.02	−1.14±0.12
Glu	1.19±0.07	1.14±0.01	**1.61±0.20**	−1.04±0.13	−1.27±0.07	−1.19±0.02
Gln	1.07±0.29	1.21±0.21	1.74±0.52	1.32±0.17	−2.34±0.74	1.09±0.20
Gly	1.08±0.16	1.25±0.13	**1.68±0.38**	1.25±0.09	1.01±0.16	−1.16±0.001
His[d]	−1.10±0.42	1.97±0.18	2.15±0.10	−1.30±0.01	1.40±0.05	−1.32±0.50
Ile[d]	1.07±0.17	1.22±0.06	−1.44±0.05	−1.55±0.21	−1.54±0.32	−1.52±0.70
Leu[d]	−1.15±0.31	1.09±0.10	−1.58±0.07	−1.76±0.19	−1.24±0.20	−1.40±0.74
Lys[d]	1.12±0.36	1.14±0.12	1.14±0.10	−1.94±0.39	1.00±0.02	−1.37±0.80
Met[d]	1.43±0.12	1.91±0.12	1.95±0.68	1.01±0.12	−1.41±0.03	−1.24±0.30
Phe[d]	1.22±0.30	1.80±0.03	**2.53±0.30**	−1.34±0.17	**4.93±0.37**	−1.55±0.31
Pro	1.37±0.13	1.12±0.13	**4.79±0.95**	−1.66±0.23	−1.44±0.82	−1.47±0.04
Ser	1.26±0.28	1.16±0.06	**2.02±0.08**	**1.80±0.26**	−1.27±0.22	1.12±0.16
Thr[d]	1.16±0.25	1.14±0.02	1.10±0.08	1.19±0.11	−1.91±0.21	−1.09±0.25
Tyr	1.08±0.15	1.33±0.09	1.66±0.37	−1.28±0.30	**3.75±0.50**	−1.37±0.50
Val[d]	1.18±0.22	1.21±0.05	1.02±0.09	−1.20±0.08	−1.24±0.23	−1.31±0.36
AABA[e]	nd[f]	1.24[g]	nd	1.41[g]	nd	nd
Ammonia[h]	1.70±0.47	−1.23±0.39	1.13±0.25	1.29±0.79	−1.35±0.33	1.77±1.01
Ethanolamine[i]	−1.20[g]	1.43±0.26	−1.31±0.03	1.10±0.27	−1.58±0.25	−1.03[g]
GABA[j]	−1.20±0.21	2.12±0.37	−1.15±0.33	−1.56±0.04	1.74±0.62	−1.71±0.09
1-methylhistidine	nd	nd	Detected[k]	nd	nd	nd
Orn	nd	Detected[l]	Detected[k]	nd	Detected[m]	Detected[l]

[a] Susceptible Newton wheat + Hessian fly Biotype L
[b] Resistant *H9*-Iris wheat + Hessian fly Biotype L
[c] Mean fold-change ±SD (calculated from nonlogarithm transformed amino acid concentration)
[d] Essential amino acid for insects (must be received in the diet)
[e] *AABA* alpha-amino-*n*-butyric acid
[f] nd not detected in treatment or control
[g] Detected in one replicate only
[h] Ammonia salts detected as ammonium ion, NH_4^+
[i] A primary amine detected due to the presence of an amino group
[j] *GABA* gamma-aminobutyric acid
[k] Detected in susceptible plants but not in control (both replicates)
[l] Detected in resistant plants but not in control (one replicate)
[m] Detected in susceptible plants but not in control (one replicate).
Significant differences between infested and uninfested control samples are shown in bold (significance level threshold = $P<0.05$).

showed a nonsignificant trend for decreased abundance over time in susceptible plants and increasing abundance in resistant plants. Both 1-methylhistidine and orn were present at relatively low levels in some samples but were not detectable in uninfested controls at the same time points (consequently fold-changes could not be calculated). 1-Methylhistidine was detected only in susceptible plants 4 days after egg hatch, and although orn was detected at low concentration in several treatments and time points tested, the only time it was detected in both replicates of a given treatment was 4 days after egg hatch in susceptible plants.

Concentration of Individual Free Amino Acids in Wheat Crown Tissues In addition to comparing fold-change of free amino acids, we ranked by concentration the amino acids that had significant fold-change increases (Table 2) to determine whether these changes were among the most abundant or among the less frequently occurring amino acids. We were particularly interested in glu and pro due to their proposed roles as an energy source for the plants during times of stress. In susceptible plants 4 days after egg hatch, glu was the most abundant of 18 standard amino acids measured (Table 3), and pro was the eighth most abundant but was present at a concentration lower than the treatment average. In resistant plants 4 days after egg hatch, ser was the only free amino acid with a significant increase in fold-change (Table 2), and it was the third most abundant, being present at a concentration higher than the treatment average (Table 3). The significant fold-change increases in phe and its precursor tyr in susceptible plants 7 days after egg hatch were of interest due to the role of these amino acids in hardening of insect cuticle; phe was present at a concentration slightly higher than the treatment average, while the concentration of tyr was less than the treatment average (Table 3).

Accumulation of mRNA Encoded by Wheat Genes Involved in Amino Acid Synthesis At the same time points used to assay free amino acid levels, quantitative real-time PCR (qRT-PCR) was used to measure the abundance of mRNA encoded by wheat genes involved in amino acid synthesis (Fig. 1). mRNA encoding alanine aminotransferase (synthesis of glu, a precursor of pro) was more abundant compared to controls one (1.81-fold, $P<0.001$), and 4 days (1.59-fold, $P=0.002$) after egg hatch in susceptible plants (Fig. 2A) and corresponded to increased free glu in susceptible plants 4 days after egg hatch (Table 2). mRNAs encoding ornithine aminotransferase (conversion of orn to glu) and glutamine-dependent asparagine synthetase (conversion of gln to glu) were each more abundant compared to controls 1 day after egg hatch in susceptible plants (ornithine aminotransferase: 1.54-fold, $P<0.001$; glutamine-dependent asparagine synthetase: 1.82-fold, $P=0.006$; data not shown) and preceded a significant increase in free glu in susceptible plants at the next time point tested (4 days after egg hatch; Table 2).

Wheat mRNA encoding pyrroline-5-carboxylate synthetase (catalyzes synthesis of pro from glu; Hare and Cress 1997) was more abundant in susceptible plants than in controls, 1 day (1.62-fold, $P<0.001$) and 4 days (2.58-fold, $P<0.001$) after egg hatch (Fig. 2B) and was associated with elevated free pro, which showed the largest fold-increase above control levels of any free amino acid measured 4 days after egg hatch (4.79-fold; Table 2). mRNA encoding prephenate dehydratase (synthesis of the aromatic amino acid phe) increased in susceptible plants compared to

Table 3 Concentration of amino acids found to be significantly more abundant in Hessian fly-infested wheat compared to uninfested controls

Amino acid	Day[a]	Host plant phenotype	Average concentration[b]	Concentration[c]	Rank[d]
Ala	Four	Susceptible[e]	1,316.1±1,585.8	1,829.3±14.8	6
Glu	Four	Susceptible	1,316.1±1,585.8	4,978.2±634.5	1
Gly	Four	Susceptible	1,316.1±1,585.8	248.7±56.3	13
Phe	Four	Susceptible	1,316.1±1,585.8	362.3±42.5	11
Phe	Seven	Susceptible	738.6±770.5	821.1±61.4	7
Pro	Four	Susceptible	1,316.1±1,585.8	708.5±220.8	8
Ser	Four	Susceptible	1,316.1±1,585.8	2,742.1±102.8	4
Ser	Four	Resistant[f]	857.4±1,108.2	2,435.6±350.8	3
Tyr	Seven	Susceptible	738.6±770.5	294.9±39.2	10

[a] Number of days after Hessian fly egg hatch
[b] Average concentration ±SD of all 18 standard amino acids assayed for a given treatment (expressed as nanomoles per gram fresh plant tissue weight)
[c] Concentration of individual amino acids ±SD (expressed as nanomoles per gram fresh plant tissue weight)
[d] Rank of individual amino acid concentration within a given treatment. Rank of 1=most abundant amino acid in a treatment; rank of 18=least abundant amino acid in a treatment
[e] Susceptible Newton wheat + Hessian fly Biotype L
[f] Resistant *H9*-Iris wheat + Hessian fly Biotype L

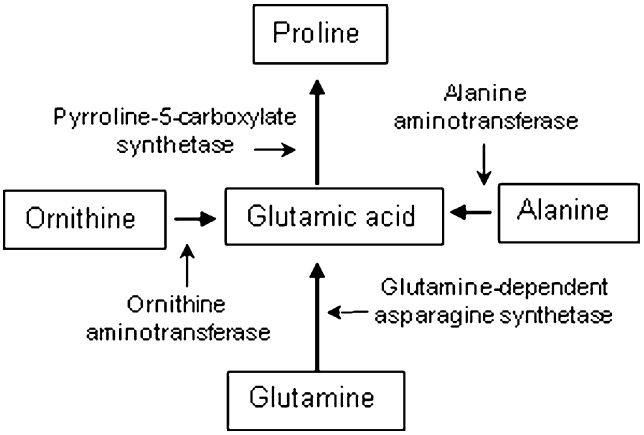

Fig. 1 Amino acid pathways leading to synthesis of glutamic acid and proline. Selected biosynthetic enzymes involved in the pathways are also shown (the abundance of mRNA encoding these enzymes was measured using qRT-PCR)

controls 4 days after egg hatch (1.79-fold, $P<0.001$; Fig. 2C) and corresponded to increased free phe in susceptible plants on days 4 and 7 (Table 2).

Accumulation of mRNA Encoded by Wheat Genes Involved in Amino Acid Transport In addition to de novo synthesis of free amino acids, the concentration of these chemicals may be increased at larval feeding sites by transport from other tissues. mRNA encoding a pro transporter was significantly more abundant in susceptible plants than in controls, 4 days (1.75-fold, $P<0.001$) and 7 days (2.05-fold, $P<0.001$) after egg hatch (Fig. 2D) and corresponded to increased free pro in susceptible plants 4 days after egg hatch (Table 2). In susceptible plants at all time points tested, mRNA encoding a transmembrane amino acid transporter was more abundant compared to uninfested controls (day 1: 2.92-fold, $P<0.001$; day 4: 7.03-fold, $P<0.001$; day 7: 3.53-fold, $P<0.001$; Fig. 2E). While mRNA encoding a histidine transporter was significantly more abundant than in controls, 4 days (6.74-fold, $P<0.001$) and 7 days (5.83-fold, $P<0.001$) after egg hatch in susceptible plants (Fig. 2F), free his levels were not significantly higher in susceptible plants compared to uninfested controls (Table 2).

Discussion

Biotype L larvae caused greater increases for susceptible than for resistant plants in wheat free amino acids and mRNAs that encode enzymes for amino acid synthesis or

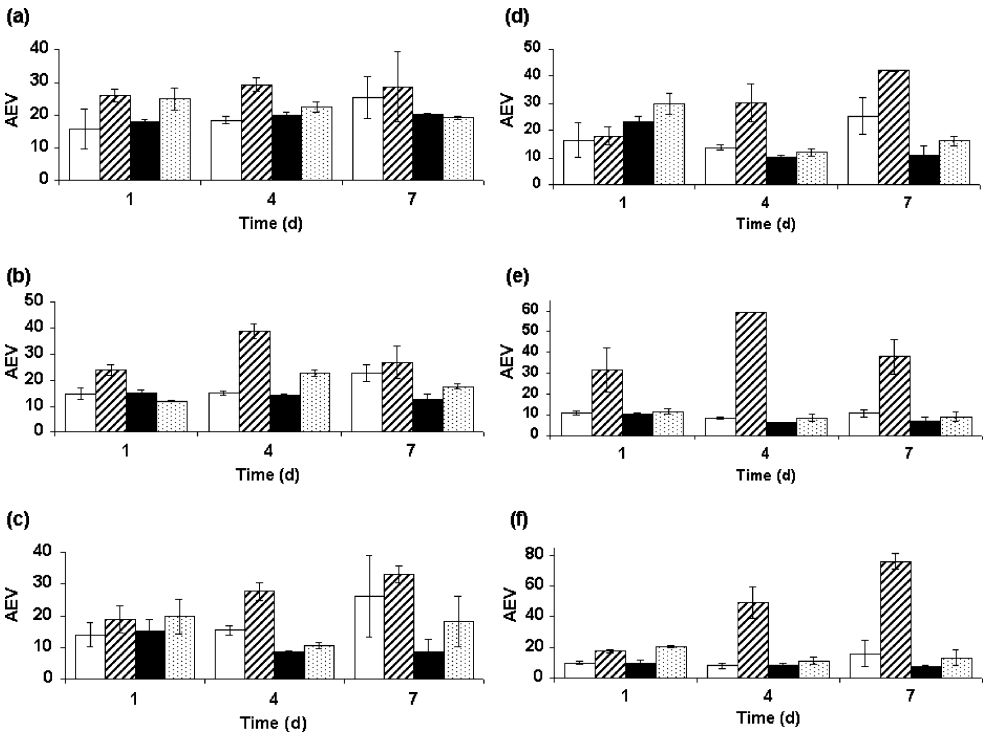

Fig. 2 mRNA levels for wheat genes involved in amino acid synthesis and transport. mRNA levels were quantified by qRT-PCR 1, 4, and 7 days after Hessian fly Biotype L egg hatch. *Bars* represent the mean arbitrary expression value (AEV)±SD (nonlogarithm transformed) from two independent experiments. AEV data are shown for Newton uninfested wheat (*white bar*), Biotype L-infested Newton susceptible wheat (*bar with diagonal lines*), *H9*-Iris uninfested wheat (*black bar*), and Biotype L-infested *H9*-Iris resistant wheat (*bar with dots*). Plants used for isolation of total RNA for qRT-PCR were grown in parallel to plants used for free amino acid analysis. **a** Alanine aminotransferase (glutamate synthesis). **b** Pyrroline-5-carboxylate synthetase (proline synthesis). **c** Prephenate dehydratase (phenylalanine synthesis). **d** Proline transporter. **e** Transmembrane amino acid transporter. **f** Histidine amino acid transporter

transport. This manipulation of free amino acid abundance may result in improved nutritional quality of plant fluids ingested by virulent larvae. Gall-forming insects are known to manipulate their host plants and produce new cell-types and structures as well as changes in source-sink relationships that are beneficial to larval development. Likewise, alterations in host plant gene expression and the production of chemicals, such as polyamines and leaf waxes, are among the responses of susceptible wheat when under attack by virulent Hessian fly larvae (Williams et al., unpublished). Alternatively, these increases in amino acid abundance may be a side effect that does not impact larval nutrition directly, but may be associated with the changing physiological processes in the susceptible plant as it adapts to accommodate the Hessian fly larvae.

Among the amino acids that became more abundant in susceptible plants were three essential amino acids [his, methionine (met), and phe] that insects cannot synthesize and must be received in their diet. Sandström et al. (2000) reported that feeding by the greenbug (*S. graminum*) caused an increase in host wheat plant production of these same three plus six additional essential free amino acids. Russian wheat aphid (*D. noxia*) feeding on wheat resulted in higher levels of the essential amino acids arginine (arg), his, and lysine (lys) (Sandström et al. 2000). Our studies with Hessian fly larvae on susceptible wheat detected significant increases in five nonessential amino acids [alanine (ala), aspartate (asp), glu, glycine (gly), and ser] that were not found to become more abundant following feeding by either *S. graminum* or *D. noxia*. Thus, wheat plants respond differently to attack by insects from diverse feeding guilds.

Once the wheat nutritive tissue was established and Hessian fly larvae were in their sessile second-instar, the greatest accumulation occurred for tyr, its precursor phe and mRNAs that encode enzymes involved in their synthesis. Tyrosine is important for immature insects because it is a precursor for formation of polyphenols and quinones that lead to darkening and hardening of insect cuticles (Heady et al. 1982) at pupation (Dindo et al. 2006). The high levels of phe and tyr that we observed 7 days after egg hatch occurred about 2 days before melanization would have started (McColloch 1923) just prior to pupation. In plants, plastids appear to be a primary site for synthesis of aromatic amino acids (Gruys and Sikorski 1999). Interestingly, Harris et al. (2006) noted an increased abundance of proplastids in Hessian fly-induced nutritive tissue cells.

Proline may be rapidly synthesized, transported, and degraded to provide energy for plant functions induced by Hessian fly larvae in susceptible wheat. Amino acids that can be converted into pro accumulated to high levels 4 days after egg hatch; glu (pro precursor, Fig. 1) was present in the highest concentration of any amino acid, and several amino acids capable of being converted to glu (ala, gln, his, and orn) were more abundant than in controls (Tables 2, 3). Increased abundance of mRNA encoding enzymes involved in synthesis of glu and pro (Fig. 2; alanine aminotransferase, pyrroline-5-carboxylate synthetase) suggested that increased free pro resulted from de novo amino acid synthesis and did not arise simply from proteolysis. At the same time, large fold-change increases in pro were detected, yet its concentration was relatively low suggesting that it was being transported or metabolized (4 days after egg hatch at the peak of nutritive tissue formation). Our qRT-PCR results showing increased abundance of mRNA encoding a proline transporter (Fig. 2) suggested translocation of accumulated pro, but the lack of high pro concentration suggested that it was being metabolized. In plants, biosynthetic routes that contribute to pro synthesis are regulated at both the gene expression and enzyme activity level (Verma and Zhang 1999).

Several published reports highlight possible functions for free pro in plants. Free pro accumulates in plants undergoing biotic or abiotic stress (Rhodes et al. 1999), followed poststress by rapid catabolism, which may support mitochondrial oxidative phosphorylation and generate ATP for repair of stress-induced damage (Hare and Cress 1997). Meon et al. (1978) reported that free pro levels increased in tomato roots (*Solanum lycopersicum* L.) following infection by *Agrobacterium tumefaciens*, suggesting that metabolic demands from giant cell and gall formation were met by translocating free pro. Oxidation of one molecule of pro yields approximately 30 ATP equivalents, making its accumulation an effective means by which to store energy that can be rapidly mobilized in the plant (Hare and Cress 1997). mRNAs associated with the tricarboxylic acid cycle become more abundant in Hessian fly-susceptible wheat (Zhu et al. 2008). Increased levels of tricarboxylic acid cycle intermediates could stimulate production of NADH and lead to greater production of ATP through the action of the electron transport chain, to meet energetic demands encountered during compatible wheat–Hessian fly interactions.

The possibility that free pro is ingested and used directly by virulent Hessian fly larvae cannot be ruled out. Plant pro can function as an insect-feeding stimulant (Behmer and Joern 1994; Carter et al. 2006) and also serves as an insect energy metabolite (Bursell 1981). Evidence suggests that oxidation of free pro in insect hemolymph leads to increased energy production through the formation of tricarboxylic acid cycle intermediates (Sacktor and Childress 1967; Bursell 1981; Gäde and Auerswald 2002). The involvement of biosynthetic pathways that lead to production of pro and a concomitant increase in free pro levels we observed in susceptible wheat could serve as a direct energy source for developing larvae and improve the nutritional quality of their diet.

The compatible interaction in which virulent Hessian fly larvae manipulate the development and physiology of susceptible wheat is only recently starting to be understood at the molecular level. However, we have demonstrated that larvae induce increased production of free amino acids that are essential for insect development. In addition, increased synthesis and transport of pro may provide energy directly to larvae as well as supporting plant processes such as nutritive tissue formation that deliver nutrients to developing larvae.

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References

BEHMER, S. T., and JOERN, A. 1994. The influence of proline on diet selection: sex-specific feeding preferences by the grasshoppers *Ageneotettix deorum* and *Phoetaliotes nebrascensis* (Orthoptera: Acrididae). *Oecologia* 98:76–82.

BRONNER, R. 1992. The role of nutritive cells in the nutrition of cynipids and cecidomyiids, pp. 118–140, in J. D. Shorthouse, and O. Rohfritsch (eds.). Biology of Insect-Induced Galls Oxford University Press, Oxford.

BURSELL, E. 1981. The role of proline in energy metabolism, pp. 135–154, in G. H. Downer (ed.). Energy Metabolism in Insects Plenum Press, New York.

CARTER, C., SHAFIR, S., YEHONATAN, L., PALMER, R. G., and THORNBURG, R. 2006. A novel role for proline in plant floral nectars. *Naturwissenschaften* 93:72–79.

CARTWRIGHT, W. B., CALDWELL, R. M., and COMPTON, L. E. 1959. Responses of resistant and susceptible wheats to Hessian fly attack. *Agron. J.* 51:529–531.

CASPI, R., FOERSTER, H., FULCHER, C. A., HOPKINSON, R., INGRAHAM, J., KAIPA, P., KRUMMENACKER, M., PALEY, S., PICK, J., RHEE, S. Y., TISSIER, C., ZHANG, P., and KARP, P. D. 2006. MetaCyc: a multiorganism database of metabolic pathways and enzymes. *Nuc. Acids Res.* 34:D511–D516(database issue).

DINDO, M. L., GRENIER, S., SIGHINOLFI, L., and BARONIO, P. 2006. Biological and biochemical differences between in vitro- and in vivo-reared *Exorista larvarum*. *Entomol. Exp. Appl.* 120:167–174.

ELEFTHERIANOS, I., VAMVATSIKOS, P., WARD, D., and GRAVANIS, F. 2006. Changes in the levels of plant total phenols and free amino acids induced by two cereal aphids and effects on aphid fecundity. *J. Appl. Entomol.* 130:15–19.

FLOR, H. H. 1955. Host–parasite interaction in flax rust—its genetics and other implications. *Phytopathology* 45:680–685.

GÄDE, G., and AUERSWALD, L. 2002. Beetle's choice—proline for energy output: control by AKHs. *Comp. Biochem. Physiol. B* 132:117–129.

GIOVANINI, M. P., PUTHOFF, D. P., NEMACHECK, J. A., MITTAPALLI, O., SALTZMANN, K. D., OHM, H. W., SHUKLE, R. H., and WILLIAMS, C. E. 2006. Gene-for-gene defense of wheat against the Hessian fly lacks a classical oxidative burst. *Mol. Plant-Microbe Interact* 19:1023–1033.

Giovanini, M. P., Saltzmann, K. D., Puthoff, D. P., Gonzalo, M., Ohm, H. W., and Williams, C. E. 2007. A novel wheat gene encoding a putative chitin-binding lectin is associated with resistance against Hessian fly. *Mol. Plant Pathol* 8:69–82.

GRUYS, K. J., and SIKORSKI, J. A. 1999. Inhibitors of tryptophan, phenylalanine, and tyrosine biosynthesis as herbicides, pp. 357–384, in B. K. Singh (ed.). Plant Amino Acids: Biochemistry and Biotechnology. New York: Marcel Dekker.

HARE, P. D., and CRESS, W. A. 1997. Metabolic implications of stress-induced proline accumulation in plants. *Plant Growth Regul.* 21:79–102.

HARRIS, M. O., STUART, J. J., MOHAN, M., NAIR, S., LAMB, R. J., and ROHFRITSCH, O. 2003. Grasses and gall midges: plant defense and insect adaptation. *Annu. Rev. Entomol.* 48:549–577.

HARRIS, M. O., FREEMAN, T. P., ROHFRITSCH, O., ANDERSON, K. G., PAYNE, S. A., and MOORE, J. A. 2006. Virulent Hessian fly (Diptera: Cecidomyiidae) larvae induce a nutritive tissue during compatible interactions with wheat. *Ann. Entomol. Soc. Am.* 99:305–316.

HATCHETT, J. H., and GALLUN, R. L. 1970. Genetics of the ability of the Hessian fly, *Mayetiola destructor*, to survive on wheat having different genes for resistance. *Ann. Entomol. Soc. Am.* 63:1400–1407.

HEADY, S. E., LAMBERT, R. G., and COVELL, C. V. Jr. 1982. Determination of free amino acids in larval insect and gall tissues of the goldenrod, *Solidago canadensis* L. *Comp. Biochem. Physiol. B* 73:641–644.

KALOSHIAN, I. 2004. Gene-for-gene disease resistance: Bridging insect pest and pathogen defense. *J. Chem. Ecol.* 30:2419–2438.

LAM, H.-M., WONG, P., CHAN, H.-K., YAM, K.-M., CHEN, L., CHOW, C.-M., and CORUZZI, G. M. 2003. Overexpression of the *ASN1* gene enhances nitrogen status in seeds of *Arabidopsis*. *Plant Physiol.* 132:926–935.

LIU, X., BAI, J., HUANG, L., ZHU, L., LIU, X., WENG, N., REESE, J. C., HARRIS, M., STUART, J. J., and CHEN, M.-S. 2007. Gene expression of different wheat genotypes during attack by virulent and avirulent Hessian fly (*Mayetiola destructor*) larvae. *J. Chem. Ecol.* 33:2171–2194.

MACCHI, F. D., SHEN, F. J., KECK, R. G., and HARRIS, R. J. 2000. Amino acid analysis, using postcolumn ninhydrin detection, in a biotechnology laboratory, pp. 9–30, in C. Cooper, N. Packer, and K. Williams (eds.). Methods in Molecular Biology, Vol. 159: Amino Acid Analysis Protocols Humana Press, Totawa, NJ.

MCCOLLOCH, J. W. 1923. The Hessian fly in Kansas. *Kans. Agr. Exp. Sta. Tech. Bul.* 11:96.

MEON, S., FISHER, J. M., and WALLACE, H. R. 1978. Changes in free proline following infection of plants with either *Meloidogyne javanica* or *Agrobacterium tumefaciens*. *Physiol. Plant Pathol.* 12:251–256.

OZOLS, J. 1990. Amino acid analysispp. 587–601, in J. N. Abelson, M. I. Simon, and M. P. Deutscher (eds.). Methods in Enzymology, Vol. 182: Guide to Protein Purification Academic Press, San Diego.

PATTERSON, F. L., MAAS, F. B. III, FOSTER, J. E., RATCLIFFE, R. H., CAMBRON, S. E., SAFRANSKI, G., TAYLOR, P. L., and OHM, H. W. 1994. Registration of eight Hessian fly resistant common winter wheat germplasm lines (Carol, Erin, Flynn, Iris, Joy, Karen, Lola, and Molly). *Crop Sci.* 34:315–316.

PUTHOFF, D. P., SARDESAI, N., SUBRAMANYAM, S., NEMACHECK, J. A., and WILLIAMS, C. E. 2005. *Hfr-2*, a wheat cytolytic toxin-like gene, is up-regulated by virulent Hessian fly larval feeding. *Mol. Plant Pathol.* 6:411–423.

RHODES, D., VERSLUES, P. E., and SHARP, R. E. 1999. Role of amino acids in abiotic stress resistance, pp. 319–356, in B. K. Singh

(ed.). Plant Amino Acids: Biochemistry and Biotechnology Marcel Dekker, New York.

ROBINSON, R. J., MILLER, B. S., MILLER, H. L., MUSSMAN, H. C., JOHNSON, J. A., and JONES, E. 1960. Chloroplast number in leaves of normal wheat plants and those infested with Hessian fly or treated with maleic hydrazine. *J. Econom. Entomol.* 53:560–566.

ROSSOMANDO, E. F. 1990. Ion-Exchange Chromatography, pp. 309–316, in J. N. Abelson, M. I. Simon, and M. P. Deutscher (eds.). Methods in Enzymology, Vol. 182: Guide to Protein Purification Academic Press, San Diego.

SACKTOR, B., and CHILDRESS, C. C. 1967. Metabolism of proline in insect flight muscle and its significance in stimulating the oxidation of pyruvate. *Arch. Biochem. Biophys.* 120:583–588.

SARDESAI, N., SUBRAMANYAM, S., NEMACHECK, J., and WILLIAMS, C. W. 2005. Modulation of defense-response gene expression in wheat during Hessian fly larval feeding. *J. Plant Interact.* 1:39–50.

SANDSTRÖM, J., TELANG, A., and MORAN, N. A. 2000. Nutritional enhancement of host plants by aphids – a comparison of three aphid species on grasses. *J. Insect Physiol.* 46:33–40.

SUBRAMANYAM, S., SARDESAI, N., PUTHOFF, D., MEYER, J. M., NEMACHECK, J. A., GONZALO, M., and WILLIAMS, C. W. 2006. Expression of two wheat defense-response genes, *Hfr-1* and *Wci-1*, under biotic and abiotic stresses. *Plant Sci.* 170:90–103.

SUBRAMANYAM, S., SMITH, D. F., CLEMENS, J. C., WEBB, M. A., SARDESAI, N., and WILLIAMS, C. W. 2008. Hessian fly larvae induce production of HFR-1, a high mannose N-glycan-specific wheat lectin. *Plant Physiol.* 147, July 2008.

TELANG, A., SANDSTRÖM, J., DYRESON, E., and MORAN, N. A. 1999. Feeding damage by *Diuraphis noxia* results in nutritionally enhanced phloem diet. *Entomol. Exp. Appl.* 91:403–412.

TOOKER, J. F., and DE MORAES, C. M. 2007. Feeding by Hessian fly [*Mayetiola destructor* (Say)] larvae does not induce plant indirect defenses. *Ecol. Entomol.* 32:153–161.

VERMA, D. P. S., and ZHANG, C.-S. 1999. Regulation of proline and arginine biosynthesis in plants, pp. 249–265, in B. K. Singh (ed.). Plant Amino Acids: Biochemistry and Biotechnology Marcel Dekker, New York.

ZHU, L., LIU, X., LIU, X., JEANNOTTE, R., REESE, J. C., HARRIS, M., STUART, J. J., and CHEN, M.-S. 2008. Hessian fly (*Mayetiola destructor*) attack causes a dramatic shift in carbon and nitrogen metabolism in wheat. *Mol. Plant-Microbe Interact.* 21:70–78.

1992) and soil organic matter dynamics (Veen et al. 1989), so that changes in rhizodeposition patterns and amounts could have profound effects on overall soil properties and ultimately affect the survival of the plant (Widmer et al. 2001). Some workers (Klein et al. 1988, Grayston et al. 1996, Merbach et al. 1999) have suggested that there may be a release of C-rich exudates by some plants as an adaptation to stimulate microbial activity (Lynch and Whipps 1990, Mawdsley and Bardgett 1997).

Defoliation (removal of parts of the shoot) is a common feature in the life span of many pasture species with effects on both above-ground productivity and below-ground turnover of roots (Milchunas and Lauenroth 1993; McNaughton et al. 1998). In grasses such as *Lolium perenne* L., the ability to grow after cutting or grazing is important to persistence, although species differ in their responses to defoliation. Frequently, defoliation and grazing practices cause a change in both metabolism and the pattern of C allocation within grasses (Macduff and Jackson 1992; Johansson 1993; De Visser et al. 1997), resulting in a reduction of nutrient uptake and an increase in exudation of organic C from roots (Deane-Drummond 1986; Paterson and Sim 1999). Frequent defoliation of perennial ryegrass (*Lolium perenne* L.) resulted in lower water-soluble carbohydrate reserves and less developed root systems than in plants defoliated less frequently, with frequent defoliation in spring reducing survival over summer (Fulkerson and Slack 1994). There are also interactions between the carbon balance of the plant and that of other nutrients. For example, defoliation of *Lolium perenne* L. inhibited nitrate uptake, and absorption rates increased only after the whole plant carbon balance was re-established (Clement et al. 1978). Similarly Macduff and Jackson (1992) noted a three-fold increase in nitrate efflux from roots of defoliated plants compared to those of non-defoliated plants.

Several studies of root exudates have concentrated on the analysis of the individual monosaccharides and the more complex polysaccharide groups (Jones and Darrah 1992; Morvan-Bertrand et al. 1999; Osborn et al. 1999). Identification of individual sugars poses several analytical problems. Greenaway et al. (1987) developed an acetylation technique to allow the detection of small amounts of carbohydrates by gas chromatography-mass spectrometry (GC-MS). Jones and Darrah (1992) used this technique to analyze the sugars at the root tip and in root exudates of maize (*Zea mays* L.). Glucose, mannose, and galactose were the major sugars present, with traces of fructose and trehalose. Osborn et al. (1999) developed a more sensitive method for analyzing sugars in the mucilage of maize that involved the hydrolysis of the polysaccharides to their constituent monosaccharides followed by subsequent identification using GC. The major monosaccharide component identified was glucose but, as with earlier techniques (e.g., Oades 1967), some peaks remained unidentified.

The aims of this study were to collect and quantitatively analyze water soluble carbohydrates in root exudates of perennial ryegrass, and to investigate the effects of defoliation on their composition. A microlysimeter system was designed in which plants were grown from seed, and exudates were collected while the plants grew. Preliminary work demonstrated that the concentration of sugars in exudates was below the detection limit of liquid chromatography using Dionex columns, so GC-MS was employed for analysis (HPLC-MS being unavailable). The resulting study combined derivatization techniques developed by Osborn et al. (1999) with revised GC-MS procedures to analyze sugars in perennial ryegrass (*Lolium perenne* L.) exudates. Full details of the GC-MS analysis are given by Clayton (2003).

Methods and Materials

Growth of Plants and Collection of Exudates Seeds of *Lolium perenne* L. were sterilized in a petri dish containing 20 ml of sterile water, covered, and agitated for 1 min. The seeds were then soaked in 75% ethanol for 1 min, the excess solution decanted, and 20 ml 1% sodium hypochlorite solution was added for 5 min. Seeds were soaked again in 75% ethanol for 30 sec, drained, and rinsed immediately, twice with sterile water to remove any trace of the sodium hypochlorite solution. Seeds were then left to stand in sterile water to imbibe for a further 30 min before transfer to a second sterile petri dish. Five seeds from each petri dish were removed with the use of sterile tweezers, and planted into each of eight microlysimeters (50 ml syringe barrel) filled with sterilized sand (Murray et al. 1996). Each microlysimeter was housed in a separate growth chamber constructed from a Perspex cylinder (250 mm tall and 150 mm diam) with Perspex plates fitted to the top and bottom (Murray et al. 2002). The apparatus (all tubing, taps, and mycrolysimeters) was made with autoclavable materials. All items, even though packaged as sterile, were autoclaved before use. Nutrients (Hoagland and Arnon 1950) were pre-mixed, and frozen until required, and both the container and nutrient solution were autoclaved before use. Filters were fitted to both the "air in" and "nutrient out" tubes of the nutrient container to maintain sterility.

Individual growth chambers were placed inside a controlled temperature laboratory with a light/dark temperature of 18/14°C, a 16 hr photoperiod, and a photon flux density of approximately 350µmol m^{-2} sec^{-1} at the plant level. The five plants in each chamber were supplied with nutrient solution at a rate of 1 ml min^{-1} for 15 min, three times daily by using a peristaltic pump. Air, filtered through

a 500 μm filter, was pumped through the chambers at a rate of 250 ml min^{-1}. Plants were allowed to grow for 28 d to produce established plants, and then the drained nutrient solution containing exudates was collected from the individual chambers at 3 sampling times (08:00; 16:00; 24:00 hr) each day for a further 27 d period (referred to as days after establishment, dae). The solutions were immediately frozen to limit any microbial activity and subsequently freeze-dried and stored in a cool dry, dark environment for future analysis. At 8 dae, four chambers were randomly selected and all plants within them defoliated to 4 cm above the height of the sand; this height was chosen to leave some photosynthesizing leaf area after defoliation. Thereafter, the same plants were again defoliated at 10, 12, 15, 18, and 24 dae. As the experiment progressed, the time span increased between each cut as growth became less vigorous.

Analysis and Identification of Sugars The method developed by Osborn et al. (1999) that involves simultaneous hydrolysis and acetylation of the freeze-dried root exudate was used. Dulcitol (galactitol) was added at 2 μg as a standard to each flask before samples were acetylated. Dulcitol was chosen because preliminary studies showed that it was not present in any of the exudates, possessed a longer retention time than the other sugars present, and was identified easily. A mixture of acetic anhydride-glacial acetic acid-sulfuric acid (10:10:1) was refluxed with the freeze-dried exudate in a round bottom flask placed in a water bath at 40°C for 3 hr. The reaction mixture was then cooled in an ice bath before 5 ml of distilled water was added, drop wise, and 20 ml of diethyl ether was used to extract the resulting aqueous phase. The organic phases were then washed with saturated K_2CO_3 solution, dried over magnesium sulfate, and filtered. The solution was evaporated under reduced pressure at 60°C to give an oily product ready for GC-MS analysis.

Analyses were carried out on a Hewlett Packard 5890 GC (Hewlett Packard 5890) fitted with a Restek Rtx-50 column (15 m long, 0.25 mm i.d., 0.1 μm coating) with helium as the carrier gas at an inlet pressure of 34 kPa, coupled to a Hewlett Packard 5970 Mass Selective Detector. At injection, the initial column temperature was set to 50°C and held for three min before increasing to 250°C at a rate of 15°C min^{-1}, and the final temperature was held for 5 min. The temperature of the injection head was 260°C.

The monosaccharide carbohydrate groups were identified from their mass spectra and retention times. Detection was enhanced by running samples in selected-ion mode. The fragment ion at m/z 115 was a particularly useful marker, present in the mass spectra of all the various sugar types and, coupled with the retention time, made detection of monosaccharide sugars easier because peaks could be distinguished from background contaminants. Sugars such as the pentoses and deoxyhexoses had similar acetylated molecular weights because they had the same number of OH groups prior to acetylation. Analysis of the results was thus made easier when sugars were grouped according to the number of OH groups present prior to acetylation rather than the number of carbon atoms.

Commercially available sugars were used for comparison of retention times and mass spectra to the detected sugars. Samples of 15 sugars (including the dulcitol standard) were individually weighed to four decimal places, then dissolved in deionized water, freeze-dried and acetylated. This mixture of derivatized monosaccharide sugars produced an array of GC peaks with various retention times corresponding to the component sugars (see Clayton 2003). By examining the mass spectral fragmentation pattern and the retention time, it was possible to characterize and confirm the identity of each GC peak. Individual sugars found in exudates were identified by comparison of retention times and mass spectra with those of the individual, derivatized commercial sugars.

Quantification of Sugars Three replicate flasks, each containing known weights of the 15 standard sugars, were acetylated and injected into the GC-MS. The peak area for each sugar was determined. The ratios of peak areas and amounts of each sugar to the dulcitol standard were calculated. This allowed calculation of a calibration factor, which was used to convert areas of sugar peaks in the exudate samples to quantities. Individual sugars were grouped into six types, and a mean calibration factor for each group was used to calculate the quantity of each sugar present. The calibration factor was <1 for the 4 OH (erythritol and threitol) and 5 OH (arabitol and xylitol) alcohol sugars, but >1 for all other groups (see Clayton 2003).

Statistical Analysis Some of the data were analyzed with the statistical package GenStat Release 6.1 using the program Bartlett 3-group regression method (Sokal and Rohlf 1981) that was designed to predict functional relationships between two variables (X and Y) using only one sample. The procedure estimates the functional relationship by calculating the slope (b) of the X variate. Defoliated values were plotted against non-defoliated values, and the slope (b) and 95% confidence levels were calculated (b_1 5% standard error below the slope of regression line; and b_2 5% standard error above the slope of regression line). If b_1 and b_2 were both <1, the effect of defoliation was significant at the 5% confidence level and less sugar was produced, whereas if b_1 and b_2 were both >1, the effect of defoliation was to enhance sugar production significantly (5% confidence level). Our "null" hypothesis was that the slope of values from defoliated plants when plotted against values from non-defoliated

plants would be 1 if defoliation had no effect on sugar exudation.

Results

Detection of Sugars Preliminary analysis of individual microlysimeters produced chromatograms with only a few variable sugar peaks present, so solutions from all microlysimeters were combined (8 prior to defoliation and 4 each of defoliated and non-defoliated thereafter) at each sampling time to facilitate analysis. The gas chromatograms showed predominantly one anomer (one peak) for each monosaccharide. Although it was sometimes possible to identify both anomers (two peaks), this was rare because the background noise masked many smaller peaks. The technique of acetylating OH groups on the carbon rings of sugar or at the end of the molecule resulted in a longer retention time the greater the number of OH groups present (Table 1). By using a combination of retention times and the library of fragmentation ions, it was possible to detect six major sugar types based on the number of carbon atoms present and the number of OH groups present prior to acetylation. This permitted the identification of several aldehyde sugars and alcohol sugars not previously documented as being present in root exudates.

Early samples (1 and 2 dae) contained an array of sugars, but peak intensities were low compared to the dulcitol standard (Fig. 1, peak G), though by 6 dae, with increased plant growth, many of the peaks were larger than the standard. At 1 dae, there were few alcohol sugars present, but by 6 dae they were some of the major peaks found (Fig. 1). At this early stage of sampling, it was not possible to detect any differences among sugars collected in the light and dark phases.

Shortly after defoliation commenced (8–12 dae), there were no obvious differences between defoliated and non-defoliated plants; aldehyde and alcohol groups were present with gradually increasing intensity. At 13 dae, a rapid change in alcohol sugars occurred in both defoliated and non-defoliated plants, as the peaks associated with 4 C, 4 OH (Fig. 2a and b, peak A) and 6 C, 6 OH alcohol sugars (Fig. 2a and b, peak F) almost disappeared, while the 5 C, 5 OH alcohol peaks (Fig. 2a and b, peak D) also were greatly reduced in size. Between 13 and 27 dae, the 4 C, 4 OH alcohol sugar (Fig. 2c and d, peak A) never reappeared in either the non-defoliated or defoliated plants. However, there was a gradual recovery of both 5 C, 5 OH and 6 C, 6 OH, alcohol sugars in defoliated plants with time (Fig. 2d, peaks D and F).

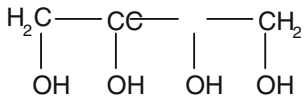
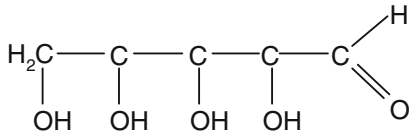
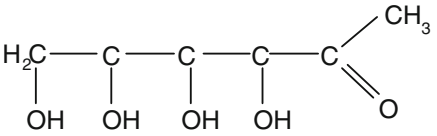
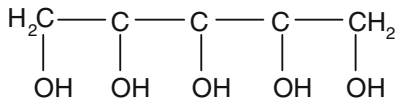
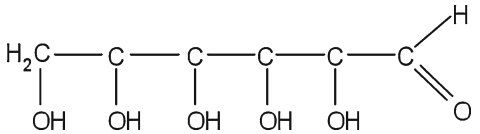
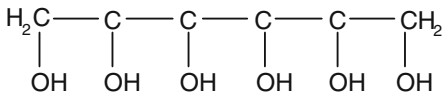
In contrast to the alcohol sugars, there was no sudden decrease in peak intensity after 13 dae for the 5 C, 4 OH, 6 C 4 OH, and 6 C, 5 OH aldehyde sugars (Fig. 2, peaks B, C and E). The 5 C, 4 OH, and 6 C, 4 OH aldehyde sugars increased in both non-defoliated and defoliated exudates, and there was no obvious effect of defoliation. This was also the pattern for the more complex 6 C, 5 OH aldehyde sugars, so that for the first few weeks of sampling, peak intensities increased for both non-defoliated and defoliated plants. However, by 24 dae and thereafter, comparison of peak intensities with the internal dulcitol standard showed that defoliation caused an increase in 6 C, 5 OH aldehyde sugars released in root exudates (Fig. 2d, peak E), though not of 5 C, 4 OH and 6 C, 4 OH aldehyde sugars (Fig. 2d, peaks B and C). Over the 27 d period of defoliation, the aldehyde sugars became the major sugar components of the root exudates.

Identification and Quantification of Sugars By using the library of retention times and mass spectra prepared from derivatized, commercially available sugars, it was possible to name 11 of the individual peaks of the chromatograms. Peak areas were measured and normalized with respect to the dulcitol standard, and the previously determined calibration factor for groups of sugars was applied to give estimates of the quantities of each sugar in the exudates. Mass was recorded for each of 11 named peaks at each sampling point, and cumulative exudation recorded. To make the presentation of the results easier, sugars with similar cumulative patterns were consolidated into three groups. The first group, where defoliation enhanced sugar amounts; the second, where defoliation had no effect; and the third, where there was a detrimental effect.

The first group contains the 4 C, 4 OH alcohol ‘type’ 1 group sugars, erythritol and threitol, and the 5 C 5 OH alcohol ‘type’ 4 group sugar xylitol (Fig. 3). Clear differences between non-defoliated and defoliated plants were evident from the initial period of defoliation onwards. Changes occurred earliest in threitol (7 dae) but were more pronounced in the erythritol and xylitol sugars, especially at 11 dae, and resulted in enhanced exudation of these sugars in defoliated plants.

For the second group of sugars, although patterns were slightly different among the individual sugars, there was no overall enhancement or detrimental effect on sugar amounts resulting from defoliation. Of the two 5 C, 4 OH aldehyde ‘type’ 2 group sugars, xylose was present in much greater amounts than ribose (Fig. 3d and e). Cumulative amounts of xylose were slightly greater in non-defoliated plants, but followed a similar pattern for both treatments. This contrasted with ribose, which was cumulatively greater from the defoliated samples until the later stages of the 27 d period. The only identifiable sugar of the 6 C, 4 OH aldehyde ‘type’ 3 was fucose (Fig. 3f). Again, there was little evidence of an effect of defoliation over the 27 d period, but the pattern of production appeared slightly more

Table 1 Retention times for the six fragmentation types of sugars found in root exudates from *Lolium perenne*

9-10 min	4C, 4OH alcohols (e.g. erthritol, threitol)  Fragmentation Type 1.	Derivatize to tetracetates
11-12 min	5C, 4OH aldehydes (e.g. arabinose, xylose, ribose)  Fragmentation Type 2.	(pentoses) Derivatize to tetracetates
11-12 min	6C, 4OH aldehydes (e.g. rhamnose, fucose)  Fragmentation Type 3.	(deoxyhexoses) Derivatize to tetracetates
12-13 min	5C, 5OH alcohols (e.g. arabitol, xylotol)  Fragmentation Type 4.	Derivatize to pentacetates
14 min	6C, 5OH aldehydes (e.g. galactose, glucose, mannose)  Fragmentation Type 5.	(hexoses) Derivatize to pentacetates
15-16 min	6C, 6OH alcohols (e.g. galactitol, glucitol, mannitol)  Fragmentation Type 6.	Derivatize to hexacetates

erratic for the non-defoliated plants. Galactose (a 6 C, 5 OH aldehyde 'type' 5 group sugar) exudation also showed no overall effect of defoliation although cumulative release was higher in defoliated plants until 21 dae, after which the non-defoliated weight was slightly higher (Fig. 3g).

The third group, showed a detrimental effect of defoliation on the amounts of sugar exuded when compared to non-defoliated plants although the extent of the effect was different among sugars. For glucose (6 C, 5 OH alcohol 'type' 5 sugar), the quantity released from non-defoliated plants was considerably higher than that from defoliated plants from 17 dae onwards (Fig. 3h). Similar patterns were also found for arabitol (5 C 5 OH alcohol 'type' 4 sugar) and mannitol (6 C, 6 OH alcohol 'type' 6 sugar) where there was initially no influence of defoliation, but there was a sudden increase in amounts in the non-defoliated samples 22 dae (Fig. 3i and j).

Amounts of mannose were smaller compared with the other two named 6 C, 5 OH aldehyde 'Type' 5 group sugars (glucose and galactose). Cumulative weights followed a similar pattern of sugar exudation for both defoliated and non-defoliated plants until 16 dae, after which almost no more mannose was produced by defoliated plants. In non-defoliated plants, the cumulative weight of mannose was still increasing at the end of the 27 d period (Fig. 3k).

The bulking of samples meant the loss of replication, but the Bartlett 3-group regression method (Sokal and Rohlf 1981) allowed statistical testing of the responses of

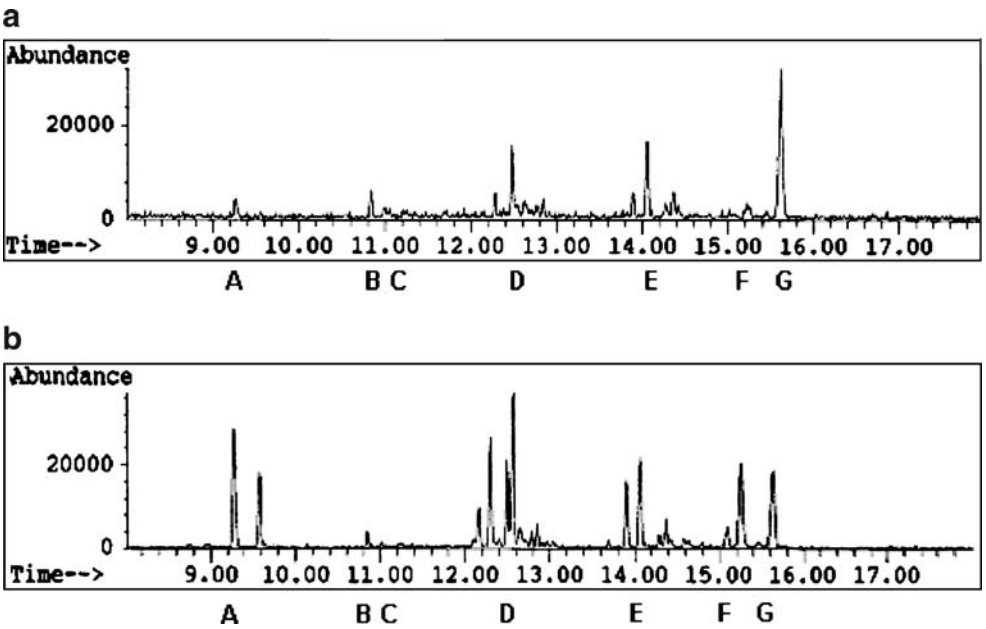


Fig. 1 Typical chromatograms showing the increase in alcohol sugars between 1 dae (a) and 6 dae (b). The letters on the x axes show: **A**, 4 C 4 OH alcohol sugars (MS fragmentation pattern type 1 – see Table 1 for a fuller description of fragmentation patterns); **B**, 5 C 4 OH aldehyde sugars (MS fragmentation pattern type 2); **C**, 6 C 4 OH aldehyde sugars (MS fragmentation pattern type 3); **D**, 5 C 5 OH alcohol sugars (MS fragmentation pattern type 4); **E**, 6 C 5 OH aldehyde sugars (MS fragmentation pattern type 5); **F**, 6 C 6 OH alcohol sugars (MS fragmentation pattern type 6); **G**, dulcitol standard (also MS fragmentation pattern type 6)

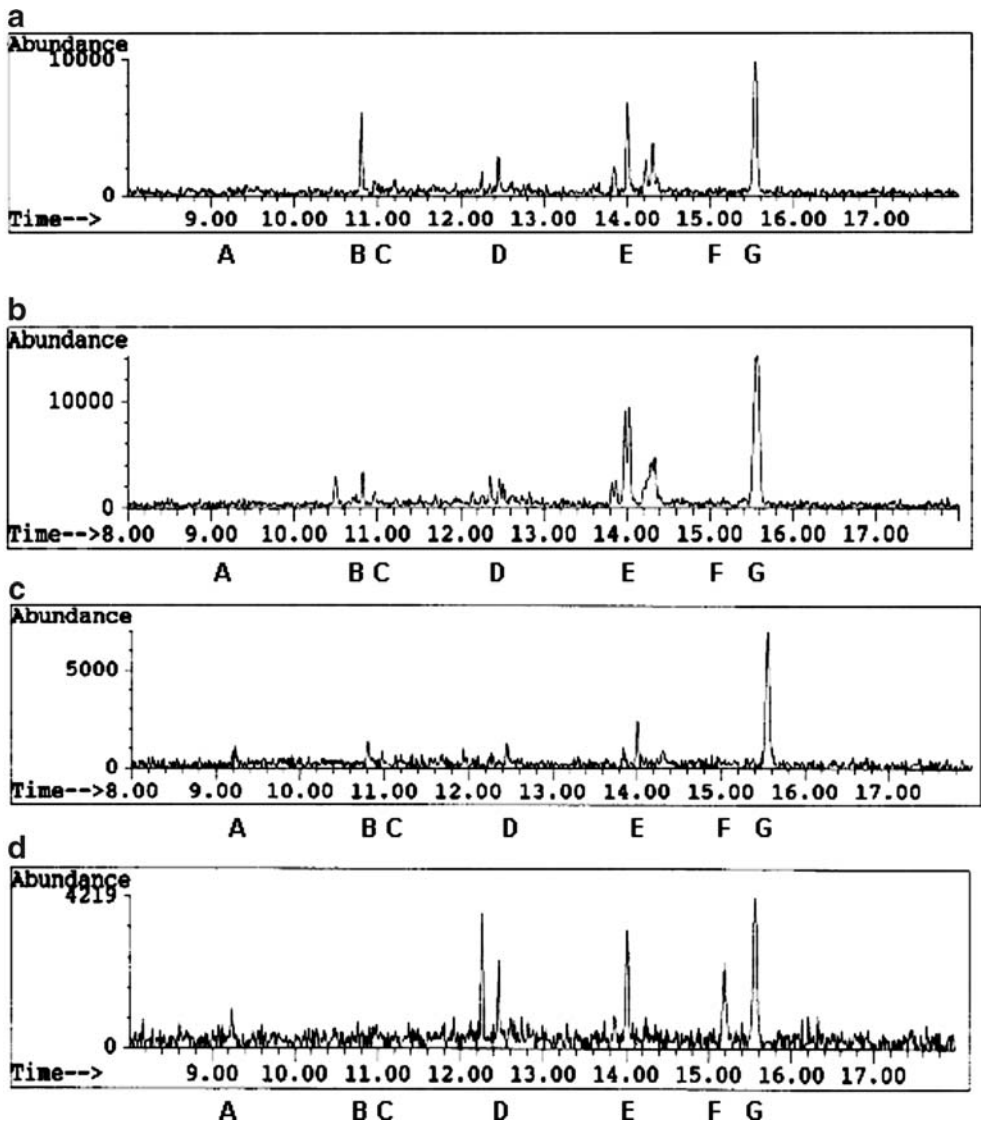


Fig. 2 Typical chromatograms showing the sugars recorded in root exudates at 13 dae (a, non-defoliated and b, defoliated plants) and 27 dae (c, non-defoliated and d, defoliated plants). The letters on the x axes are as in Fig. 1 and indicate where a peak would be expected if that sugar were present in the exudate

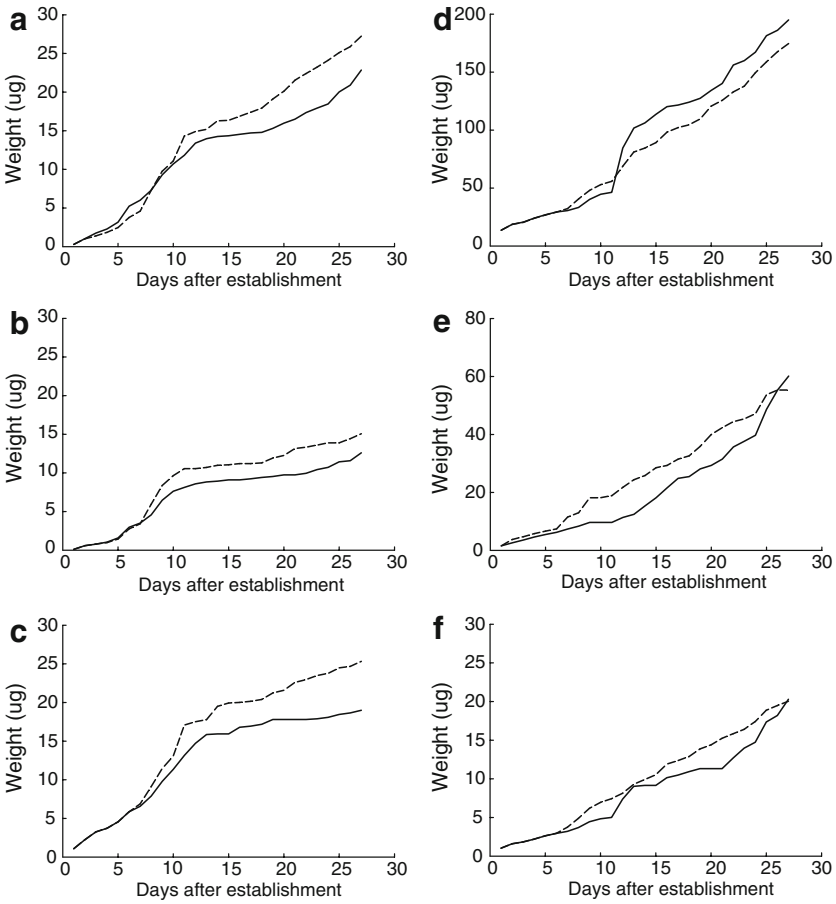


Fig. 3 Cumulative weights of sugars present in non-defoliated (solid line) and defoliated (dashed line) plant root exudates over the 27 d period: a) erythritol, b) threitol, c) xylitol, d) xylose, e) ribose, f) fucose, g) galactose, h) glucose, i) arabitol, j) mannitol, and k) mannose. The defoliation commenced at 8 dae and was repeated at 10, 12, 15, 18 and 24 dae

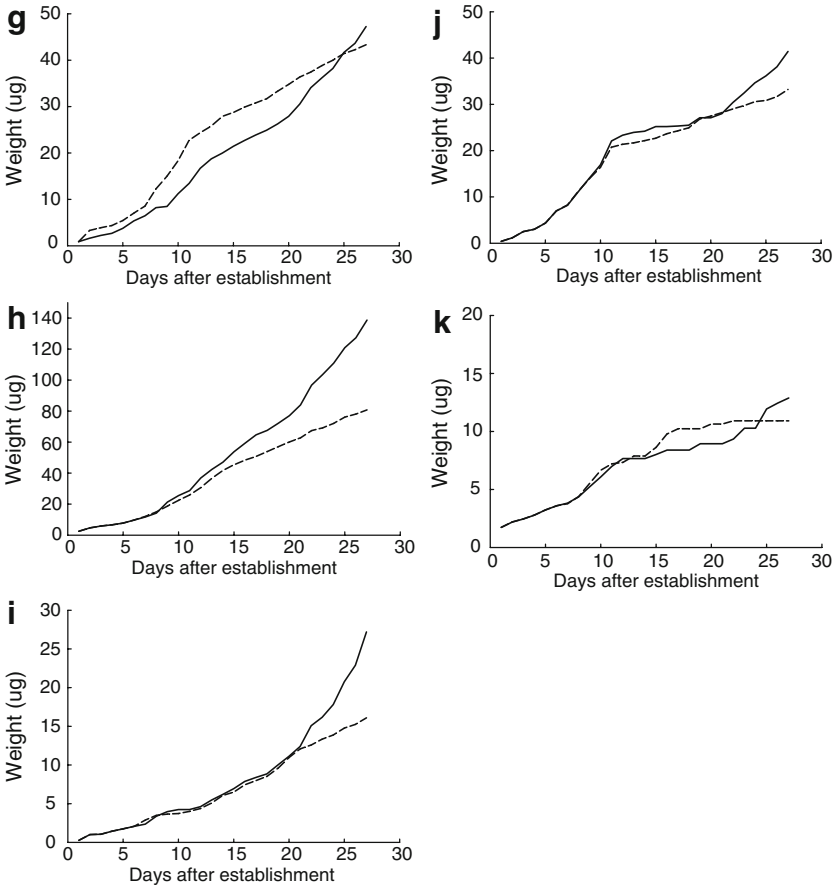
individual sugars to defoliation at each sampling time (08:00; 16:00; and 24:00 hr) over the period of defoliation (8 to 27 dae; Table 2). This analysis highlighted not only large variations in response to defoliation among individual sugars, but also variation within sugar groups. Defoliation reduced exudation of glucose at all sampling times (*b*: 0.6698; 0.5697; 0.6844), whereas for xylitol, amounts were enhanced at all sample times (*b*: 1.282; 1.780; 1.174). For all the other nine identifiable sugars, the effect of defoliation was more varied. For xylose, mannose, and mannitol, amounts were reduced in some of the defoliated exudate samples, but not at all sampling times. Ribose showed a similar but opposite pattern. At the 8:00 sample time, there were greater amounts of sugar from exudates of defoliated plants than from non-defoliated plants (b= 1.518), with no overall effects at 16:00 (b=1.111) and 24:00 (b=0.9023). For erythritol, threitol, fucose, and arabitol, there was a variable pattern of sugar exudation. While at one sampling time there was an enhancing effect due to defoliation, at another there was a detrimental effect. Because of these variable patterns, multiple testing corrections were not applied to the significance values.

Overall, xylose was the sugar exuded in the highest amount, and mannose the least, with glucose demonstrating the largest difference between non-defoliated and defoliated treatments and fucose the smallest difference. The 4 C, 4 OH alcohol group sugars (erythritol and threitol) was the only group present in exudates in greater amounts in defoliated samples than non-defoliated plants. There were differences within sugar groups, so that in the 5 C, 5 OH alcohol sugars, amounts of xylitol in exudates were slightly higher for defoliated plants, but the opposite was found for arabitol. For the aldehydes sugars, the amounts were generally greater in the non-defoliated plants.

Discussion

Root exudation is a key process in the transfer of C into the soil, and influences the composition and activities of soil microbial communities especially in processes of organic matter decomposition and nutrient cycling (Paterson 2003). The effect of defoliation of grasses has been found in some studies to increase the exudation of soluble compounds (Macduff and Jackson 1992, Morvan-Bertrand et al. 1999, Paterson and Sim 1999), and as a consequence, to enhance microbial activity over short periods, resulting in increased microbial biomass (Holland 1995, Mawdsley and Bardgett

Fig. 3 (continued)



1997) and culturable bacteria in the rhizosphere (Mawdsley and Bardgett 1997). However, other studies have indicated the opposite, with defoliation having little or no effect on overall microbial activity and microbial biomass (Wardle et al. 1997, Mikola et al. 2001).

In the present study, there was a slight increase in the total amount of sugars detected in the root exudates of defoliated compared to non-defoliated plants after the initial defoliation. This observation confirms those of Paterson and Sim (1999) who measured increased exudation of soluble compounds from *Lolium perenne* L. for periods of 3–5 d following cutting. Similarly, Paterson et al. (2005) measured enhanced C exudation from roots of *Festuca rubra* 0–2 d after defoliation but, as in the present study, this initial effect was subsequently reversed. The transient elevation of exudation following defoliation may reflect depletion of C and N storage compounds during this period (Paterson et al. 2005).

Mawdsley and Bardgett (1997) reported that continuous defoliation reduced C exudation by 14% in ryegrass and 4% in clover. Here, continual defoliation reduced the total quantity of sugars exuded by 16%, although the degree of change seemed to depend upon the type of sugar. The more complex 6 C, 5 OH aldehyde group sugars, especially glucose, showed changes in exudation patterns due to defoliation earlier and to a greater extent, than the 5 C, 4 OH (xlyose and ribose) and 6 C, 4 OH (fucose) aldehyde groups. Results showed that even sugars within the same group 'type' had different responses to defoliation. The overall amounts of arabitol and xylitol (5 C, 5 OH alcohol 'type' 4 group) sugars were approximately the same, although the cumulative patterns of sugar exudation were different. The 4 C, 4 OH alcohol sugars (erythritol and threitol) were the only group, along with xylitol (5 C 5 OH alcohol group), that was higher for defoliated plants than for non-defoliated plants throughout the 27 d experiment. Erythritol and threitol showed no sign of a detrimental effect of defoliation although the Bartlett 3-group regression method (Sokal and Rohlf, 1981) highlighted differences between the individual sugars. Both erythritol and threitol had a period where there was a significant effect of defoliation on sugar release that was not readily seen in the cumulative graphs.

Because sterile microcosms were used in this study, some care is required in the extrapolation of the results to field systems. The plants were grown in sterile sand (Hodge et al. 1996; Paterson et al. 1999), because under natural growth conditions in soil, root exudates are rapidly used by microbes and their chemical nature altered (Haller and Stolp 1985). The present study combined analysis that used

Table 2 Slope values of monosaccharide sugars generated using the bartlett 3-group regression method (Sokal and Rohlf 1981)

Sugars	Sample time (h)	95% confidence interval Low (b_1)	High (b_2)	Slope (b)
	8	0.7541	0.9947	0.8728
Erythritol	16	1.487	1.812	1.631
	24	1.515	1.865	1.662
	8	1.148	1.402	1.269
Threitol	16	0.4944	0.6218	0.553
	24	1.053	1.336	1.179
	8	0.8683	1.025	0.9461
Xylose	16	0.4944	0.6218	0.553
	24	0.9732	1.109	1.041
	8	1.232	1.881	1.518
Ribose	16	0.9326	1.361	1.111
	24	0.7585	1.122	0.9023
	8	1.160	1.666	1.417
Fucose	16	0.5115	0.6797	0.5968
	24	1.388	2.308	1.768
	8	1.017	1.258	1.123
Arabitol	16	0.5671	0.7551	0.6401
	24	0.5408	0.8042	0.6616
	8	1.112	1.458	1.282
Xylitol	16	1.503	2.102	1.780
	24	1.019	1.333	1.174
	8	0.7383	0.8772	0.8014
Galactose	16	1.132	1.528	1.303
	24	0.8874	1.064	0.9697
	8	0.6323	0.7177	0.6698
Glucose	16	0.5319	0.6165	0.5697
	24	0.6300	0.7469	0.6844
	8	0.5096	0.8750	0.6819
Mannose	16	0.8444	1.278	1.019
	24	0.4679	0.6980	0.5766
	8	0.7447	0.8934	0.8193
Mannitol	16	0.7198	0.9286	0.8155
	24	0.8339	1.057	0.9382

Values demonstrating that defoliation was detrimental to exudation are highlighted in dark grey while those demonstrating that defoliation enhanced exudation are highlighted in light grey. Values with no significant effect are not highlighted

GC-MS, a system used in previous studies (Svenningsson et al. 1990; Morvan-Bertrand et al. 1999), with an acetylation method (Osborn et al. 1999) to improve the range of sugars detectable in single-ion mode. Previous work identified several aldehyde sugars present in the root exudates of *Lolium perenne* plants (Jones and Darrah 1992; Morvan-Bertrand et al. 1999), although not the range detected by using the method developed in this work. Although it was not possible to identify every peak, 11 individual sugars were identified comprising 6 aldehyde sugars (consistent with earlier work reported by Jones and Darrah 1992) and 5 alcohol sugars not previously documented.

The acetylation technique could be used only to identify the monosaccharide components of root exudates. Although the majority of sugars were probably free monosaccharides, any polysaccharides present in the exudates would have been hydrolyzed to their constituent monosaccharide compounds (Osborn et al. 1999). For example, the disaccharide sucrose would have been acetylated to fructose (Guerrand et al. 1996; Morvan-Bertrand et al. 1999). However, as there was no evidence of fructose in any of the root exudate samples, it suggests that although sucrose is often a major transport sugar in grasses (Pollock et al. 2003), it was not exuded in any great amount from the roots of these *L. perenne* plants. These results agree with those of Jones and Darrah (1992) who also failed to detect sucrose and only 1.5% fructose in the total sugars exuded from root tips of *Zea mays*. Read and Gregory (1997) also found total sugars in the mucilage of *Zea mays* to be dominated by glucose.

The presence of a large number of monosaccharides in the exudate samples, especially glucose and xylose, has significant implications for the turnover of carbon in the rhizosphere, as such monosaccharides would be broken down rapidly in the presence of soil microbes (Killham 1994; Paterson 2003). Little is known about the effects of specific sugars on microbial communities and whether changes in the composition of sugars in exudates following defoliation would have differential effects on those communities. Moreover, soil microbes are likely to increase the net loss of root C through their activity as a C-sink (partially offset in the present study by the frequent flushing out of the exudates) and because of production of metabolites that increase root permeability (Meharg and Killham 1995).

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References

CLAYTON, S. J. 2003. Carbon Reallocation in Grasslands Under Stress. Ph.D. Thesis. The University of Reading, Reading.

CLEMENT, C. R., HOPPER, M. J., JONES, L. H. P., and LEAFE, E. L. 1978. The uptake of nitrate by *Lolium perenne* from flowing nutrient solution 11. Effect of light, defoliation, and relationship to CO_2 flux. *J. Exp. Bot.* 29:1173–1183.

DE VISSER, R. D., VIANDEN, H., and SCHNYDER, H. 1997. Kinetics and relative significance of remobilized and current C and N incorporation in leaf and root growth zones of *Lolium perenne* after defoliation: assessment by ^{13}C and ^{15}N steady-state labelling. *Plant, Cell Environ.* 20:37–46.

DEANE-DRUMMOND, C. E. 1986. Nitrate uptake into *Pisum sativum* L. cv. Feltham first seedlings: commonality with nitrate uptake into *Chara corallina* and *Hordeum vulgare* through a substrate cycling model. *Plant, Cell Environ.* 9:41–48.

FULKERSON, W. J., and SLACK, K. 1994. Leaf number as a criterion for determining defoliation time for *Lolium perenne*.1. Effect of water-soluble carbohydrates and senescence. *Grass Forage Sci.* 49:373–377.

GRAYSTON, S. J., VAUGHAN, D., and JONES, D. 1996. Rhizosphere carbon flow in trees, in comparison with annual plants: the importance of root exudation and its impact on microbial activity and nutrient availability. *Appl. Soil Ecol.* 5:29–56.

GREENAWAY, W., SCAYSBROOK, T., and WHATLEY, F. R. 1987. The analysis of bud exudate of *populus x euramericana*, and of propolis, by gas chromatography. *Proc. Roy. Soc. London* 232:249–272.

GUERRAND, D., PRUD'HOMME, M. P., and BOUCAUD, J. 1996. Fructan metabolism in expanding leaves, mature leaf sheaths and mature leaf blades of *Lolium perenne.* Fructan synthesis, fructosyltransferase and invertase activities. *New Phytol.* 134:205–214.

HALLER, T., and STOLP, H. 1985. Quantitative estimation of root exudation of maize plants. *Plant Soil* 86:207–216.

HOAGLAND, D. R. and ARNON, D. I. 1950. The water culture method for growing plants without soil. California Agricultural Experimental Circular No. 347.

HODGE, A., GRAYSTON, S. J., and ORD, B. G. 1996. A novel method for characterisation and quantification of plant root exudates. *Plant Soil* 184:97–104.

HOLLAND, J. N. 1995. Effects of above-ground herivory on microbial biomass in conventional and no-tillage agrosystems. *Appl. Soil Ecol.* 2:275–279.

JOHANSSON, G. 1993. Carbon distribution in grass (*Festuca pratensis* L.) during regrowth after cutting-utilization of stored and newly assimilated carbon. *Plant Soil* 15:11–20.

JONES, D. L., and DARRAH, P. R. 1992. Re-sorption of organic components by roots of *Zea mays*. L and its consequences in the rhizosphere. *Plant Soil* 143:259–266.

KILLHAM, K. 1994. Soil Ecology. Cambridge University Press, Cambridge.

KILLHAM, K., and YEOMANS, C. 2001. Rhizosphere carbon flow measurement and implications: from isotopes to reporter genes. *Plant Soil* 232:91–96.

KLEIN, D. A., FREDERICK, B. A., BIONDINI, M., and TRLICA, M. J. 1988. Rhizosphere microorganism effects on soluble amino acids, sugars and organic acids in the root zone of *Agropyron cristatum*, *A. smithii* and *Bouteloua gracilis*. *Plant Soil* 110:19–25.

LYNCH, J. M., and WHIPPS, J. M. 1990. Substrate flow in the rhizosphere. *Plant Soil* 129:1–10.

MACDUFF, J. H., and JACKSON, S. B. 1992. Influx and efflux of nitrate and ammonium in Italian ryegrass and white clover roots: comparisons between effects of darkness and defoliation. *J. Exp. Bot.* 249:525–535.

MAWDSLEY, J. L., and BARDGETT, R. D. 1997. Continuous defoliation of perennial ryegrass (*Lolium perenne*) and white clover (*Trifolium repens*) and associated changes in the composition and activity of the microbial population of an upland grassland soil. *Biol Fertil. Soils* 24:52–58.

MCNAUGHTON, S. J., BANYIKWA, F. F., and MCNAUGHTON, M. M. 1998. Root biomass and productivity in a grazing ecosystem: Serengeti. *Ecol.* 79:587–592.

MEHARG, A. A., and KILLHAM, K. 1995. Loss of exudates from roots of perennial ryegrass with a range of microorganisms. *Plant Soil* 170:345–349.

MERBACH, W., MIRUS, E., KNOF, G., REMUS, R., RUPPEL, S., RUSSOW, R., GRANSEE, A., and SCHULZE, J. 1999. Release of carbon and nitrogen compounds by plant roots and their possible ecological importance. *J Plant Nutr. Soil Sci.* 162:373–383.

MIKOLA, J., YEATES, G. W., WARDLE, D. A., BARKER, G. M., and BONNER, K. I. 2001. Response of soil food-web structure to defoliation of different plant species combination in an experimental grassland community. *Soil Biol. Biochem.* 33:205–214.

MILCHUNAS, D. G., and LAUENROTH, W. K. 1993. Quantitative effects of grazing on vegetation and soils over a global range of environments. *Ecol. Monographs* 63:327–366.

MORVAN-BERTRAND, A., PAVIS, A. N., BOUCAUD, J., and PRUD'HOMME, M. P. 1999. Partitioning of reserve and newly assimilated carbon in roots and leaf tissues of *Lolium perenne* during regrowth after defoliation: by 13 C steady state labelling and carbohydrate analysis. *Plant Cell Environ.* 22:1097–1108.

MURRAY, P. J., HATCH, D. J., and CLIQUET, J. B. 1996. Impact of insect root herbivory on the growth and nitrogen and carbon contents of white clover (*Trifolium repens*) seedlings. *Canad. J. Bot.* 74:1591–1595.

MURRAY, P. J., DAWSON, L. A., and GRAYSTON, S. J. 2002. Influence of root herbivory on growth response and carbon assimilation by white clover plants. *Appl. Soil Ecol.* 20:97–105.

OADES, J. M. 1967. Gas-liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *J. Chromatog.* 28:246–252.

OSBORN, H. M. I., LOCHEY, F., MOSLEY, L., and READ, D. 1999. Analysis of polysaccharides and monosaccharides in the root

mucilage of maize (*Zea mays* L.) by gas chromatography. *J. Chromatog. A* 831:267–276.

OSTLE, N., WHITELEY, A. S., BAILEY, M. J., SLEEP, D., INESON, P., and MANEFIELD, M. 2003. Active microbial RNA turnover in a grassland soil estimated using a $^{13}CO_2$ spike. *Soil Biol. Biochem.* 35:877–885.

PATERSON, E. 2003. Importance of rhizodeposition in the coupling of plant and microbial productivity. *Europ. J. Soil Sci.* 54:741–750.

PATERSON, E., and SIM, A. 1999. Rhizodeposition and C-partitioning of *Lolium perenne* in axenic culture affected by nitrogen and defoliation. *Plant Soil* 216:155–164.

PATERSON, E., HODGE, A., THORNTON, B., MILLARD, P., and KILLHAM, K. 1999. Carbon partitioning and rhziosphere C-flow in *Lolium perenne* as affected by CO_2 concentration, irradiance and below-ground conditions. *Global Change Biol.* 5:669–678.

PATERSON, E., THORNTON, B., MIDWOOD, A. J., and SIM, A. 2005. Defoliation alters the relative contributions of recent and non-recent assimilate to root exudation from *Festuca rubra*. *Plant Cell Environ.* 28:1525–1533.

POLLOCK, C., FARRAR, J., TOMAS, D., GALLAGHER, J., LU, C., and KOROLEVA, O. 2003. Balancing supply and demand: the spatial regulation of carbon metabolism in grass and cereal leaves. *J. Exp. Bot.* 54:489–494.

READ, D. B., and GREGORY, P. J. 1997. Surface tension and viscosity of axenic maize and lupin root mucilages. *New Phytol.* 137:623–628.

SOKAL, R. R., and ROHLF, F. J. 1981. Biometry. New York, W H Freeman and Company.

SVENNINGSSON, H., SUNDIN, P., and LILJENBERG, C. 1990. Lipids, carbohydrates and amino acids exuded from the axenic roots of rape seedlings exposed to water-deficit stress. *Plant Cell Environ.* 13:155–162.

VEEN, J. A. V., MERCKX, R., and GEIJN, S. C. 1989. Plant and soil related controls of the flow of carbon from roots through the soil microbial biomass. *Plant Soil* 115:179–188.

WARDLE, D. A., BONNER, K. I., and NICHOLSON, K. S. 1997. Biodiversity and plant litter: experimental evidence which does not support the view that enhanced species richness improves ecosystem function. *Oikos* 79:247–258.

WIDMER, F., FLIESSBACH, A., LACZKO, E., SCHULZE-AURICH, J., and ZEYER, J. 2001. Assessing soil biological characteristics: a comparison of bulk soil community DNA-, PLFA-, and Biolog TM-analyses. *Soil Biol. Biochem.* 33:1029–1036.

elusive but may involve differences in innate immune responses (Woodhams et al. 2007a). An additional hypothesis is that a mutualism exists between the amphibian host and cutaneous antifungal bacteria symbionts and is effective on some individuals and species, but not in others (Fig. 1; Harris et al. 2006; Lauer et al. 2007, 2008; Woodhams et al. 2007b).

We suspected that the inhibitory effects of the antifungal symbionts are due to secondary bacterial metabolites as opposed to resource competition. Previously, we tested the hypothesis of inhibitory metabolite production with a strain of *Lysobacter gummosus* isolated from the skin of the salamander *P. cinereus*. The bacterium inhibited the fungus in challenge assays and produced the antifungal metabolite 2,4-diacetylphloroglucinol in vitro at concentrations that could inhibit the pathogen (Brucker et al. 2008).

Continuing our investigations of antifungal metabolites, a bacterium closely related to *Janthinobacterium lividum* based on DNA sequence similarity was also examined. This bacterium is commonly found on *P. cinereus* as well as the four-toed salamander, *Hemidactylium scutatum*, and it strongly inhibited pathogenic fungi in vitro (Lauer et al. 2007). We hypothesized that it, too, produces antifungal metabolites that act to reduce or prevent colonization of *B. dendrobatidis* on amphibians. Furthermore, we sampled the skins of wild salamanders to determine whether antifungal metabolites were present in inhibitory concentrations.

Methods and Materials

Isolation and Culturing of J. lividum As per published method (Harris et al. 2006; Lauer et al. 2007, 2008; Brucker et al. 2008), individuals of the salamander species *P. cinereus* were rinsed twice to remove transient bacteria and swabbed with sterile swabs. Pure bacterial cultures were obtained, genomic DNA was extracted, and a portion of the 16S rRNA gene was amplified by using 357F and 907R primers (Lauer et al. 2007). DNA obtained from the pure culture of *J. lividum* was sequenced by Agencourt (Beverly, MA, USA). A consensus sequence of approximately 1,400 bp was obtained by aligning the forward and reverse sequences' amplicons. The sequence was compared with the reference organisms by BLAST search (http://www.ncbi.nlm.nih.gov/blast) using the GenBank database in order to confirm the identification of *J. lividum* (99% match).

Co-cultures of *J. lividum* and *B. dendrobatidis* (JEL 215 strain) were grown on 1% tryptone agar plates and incubated for 48 h. The plates were checked for the presence of a zone of fungal inhibition around the bacterial colony to reconfirm the bacterium's antifungal properties (Lauer et al. 2007). Additionally, 100 ml co-cultures were made in liquid 1% tryptone medium to obtain adequate amounts of metabolites for chemical analysis (Brucker et al. 2008). Concurrently, co-cultures were compared to monocultures of the organisms to discriminate metabolites that are not produced by *J. lividum* (unpublished data). To inoculate the cultures, *J. lividum* was transferred from agar plates into 100 ml of 1% tryptone and

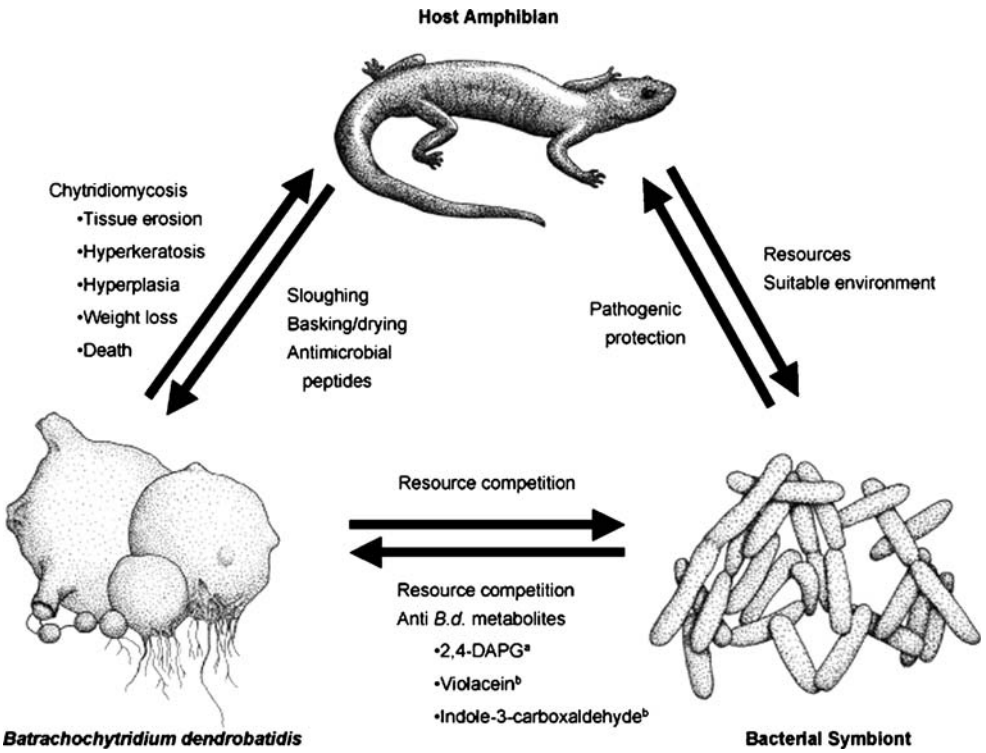

Fig. 1 Model of amphibian-bacteria-fungi interactions. The *arrows* indicate response or causation between the organisms. [a]Antifungal metabolite produced by *L. gummosus*. [b]Antifungal metabolite produced by *J. lividum*

1 ml of a solution containing zoospores (the motile infective stage of *B. dendrobatidis*; $1–2\times10^6$ zoospores/ml). All cultures were incubated for 72 h in a Lab-Line incubator shaker at room temperature (~23°C). After incubation, cultures were centrifuged at 5,000 rpm for 12 min. The supernatant was kept frozen until organic extraction; the pellet was discarded.

Organic Extraction and Antifungal Metabolite Isolation To extract organic metabolites from the cultures, the supernatant was thawed and extracted ×4 with a 1/3 volume of ethyl acetate (EtOAc). The combined organic layers were dried over Na_2SO_4, filtered, and evaporated in vacuo. The crude samples (3–15 mg) were kept frozen at −20°C, under nitrogen, until further analysis. A total of eight samples were extracted.

Each crude sample was dissolved in high performance liquid chromatography (HPLC)-grade methanol (2–3 ml). RP-HPLC analysis (Agilent Technologies, 1200 series, Wilmington, DE, USA) was then used to determine the retention times of the antifungal standards as well as to analyze the components of bacterial samples. The HPLC diode array detector was programmed to record absorbance at 220, 270, and 310 nm. Samples were injected (50 μl) into the HPLC equipped with a C18 reverse phase column (5 μm; 4.6×150 mm; Agilent Technologies, Wilmington, DE, USA) and eluted at 1 ml/min. The initial eluent, 10% acetonitrile/water (*v*/*v*, both acidified with 0.1% acetic acid), ran for 2 min. This was followed by a linear gradient to 100% acetonitrile (acidified with 0.1% acetic acid), over an 18-min period. This final solvent was eluted for another 3 min. Fractions were collected, and those that demonstrated antifungal activity were purified further and identified.

Nuclear Magnetic Resonance and High Resolution Mass Spectrometry Analysis of Antifungal Metabolites ^{1}H nuclear magnetic resonance (NMR) spectra were performed in $CDCl_3$ (indole-3-carboxaldehyde) and DMSO-d_6 with an aliquot of $CDCl_3$ (violacein) on a Bruker Advance 600 MHz NMR spectrophotometer. Samples were sent to the Mass Spectrometry Facility at Harvard University (Cambridge, MA, USA) for high resolution mass spectrometry (HR-MS) analysis (Agilent 6210 Time-of-flight LC/MS, ESI source, positive mode).

Confirmation of Antifungal Metabolites and Their Inhibitory Activity To determine the minimum inhibitory concentration (MIC) of the compounds required to inhibit the growth of *B. dendrobatidis*, an *in vitro* assay was performed according to the procedures previously detailed (Brucker et al. 2008). In brief, concentration of metabolites was varied in 96-well microtiter plates, and fungal growth was determined by measuring optical density. The positive control wells contained the fungus with the solvent DMSO; the negative control wells contained heat-killed zoospores. An exception to our previous published procedure was the reduction of the solvent DMSO to 1% by volume. Indole-3-carboxaldehyde treatments and controls were replicated 16 times, while the violacein assay treatments and controls were replicated eight times.

Statistical Analysis A Dunnett's *t* test was used to test the null hypothesis of "no inhibition" by comparing the amount of fungal growth in each metabolite concentration treatment to the positive control treatment that had fungi without metabolites. Pair-wise comparisons were made with Tukey's HSD procedure, which holds experiment-wide error at a maximum of 0.01.

Extraction of Metabolites from Skin Samples Seven wild-caught *P. cinereus* were collected from the James Madison University Arboretum (Harrisonburg, VA, USA) during the months of November and December of 2007. Each individual was handled separately with sterilized gloves and housed overnight, in separate sterile containers at 14° C. Salamanders were euthanized with gaseous CO_2. A 2.3–4.4-cm^2 portion of skin was excised from the shoulders to the hips, and the surface area of the skin was determined by using the program ImageTool 3.0 (distributed by the University of Texas Health Science Center at San Antonio, Texas. May 2002). The skin components were then extracted with HPLC-grade methanol (4×5 ml). The organic solvent was evaporated, and the resulting sample was then constituted with 200 μl of HPLC-grade methanol before being injected onto the HPLC, as per above. Approximate concentrations were determined by computing a standard curve of the metabolites. Animals were collected by permit from the Virginia Division of Game and Inland Fisheries. Our animal care protocol was approved by JMU's Animal Care and Use Committee.

Sampling for Naturally Occurring J. lividum Sampling and analysis of cutaneous bacteria follow methods published in detail by us elsewhere (Lauer et al. 2007). Briefly, transient bacteria were removed from salamander skin by rinsing them twice in sterile water and then swabbing their ventral and lateral sides with a wet sterile cotton swab (Harris et al. 2006; Lauer et al. 2007, 2008). The presence of *J. lividum* was tested by using polymerase chain reaction or denaturing gradient gel electrophoresis (DGGE; see published methods (Lauer et al. 2007)). Typically one bacterial species is indicated by one band (sequence type) on DGGE gels, although heterogeneity in ribosomal RNA copy number in some species can cause a species to appear as two or more bands. In one lane, we placed the amplification products from a

pure culture of *J. lividum*. Other lanes of the DGGE contained a culture-independent assay of bacterial diversity on salamander skins. If one band in these lanes migrated to the same position as the band from the pure culture of *J. lividum*, then we concluded that *J. lividum* was present on the skin.

Evaluating Mucus Depth of P. cinereus The mucous depth of *P. cinerus* was calculated by using three salamanders collected from local populations in order to determine metabolite concentration on salamander skins. The salamanders were sedated and euthanized using carbon dioxide. Skin samples were removed using a double-headed scalpel, which was assembled by using two size 10 scalpel blades taped parallel to each other. The resulting double-headed scalpel had a parallel 1–1.5 mm span between the blades and was used to cut and remove three 1–1.5-mm thick cross-sections, per salamander, at three locations: on the tail, above the hind legs, and below the fore limbs. Scalpel blades were replaced between each cut. Each cross-sectional sample was rotated 90° and immediately laid transversely onto a glass microscope slide.

To avoid distortion of the sample from the weight of the cover slip, two platforms were cemented on either side of the sample comprised of 7–10 cover slips, with a final cover slip spanning the sample field. Drops of a 1% saline solution were added to the sample to prevent dehydration of the sample. Two to three drops of methylene blue were added to the saline solution to provide contrast for the background and translucent mucus layer.

The slide was placed on an inverse microscope within half an hour of construction. Five out of the nine slides had undamaged, viable, cross-sectional samples. From these five samples, the depth of the mucus layer was measured at five random locations per sample, using microscopy measurement software (Nikon Imaging Systems Elements, Tokyo, Japan).

Results

Compounds in the culture medium produced by *J. lividum* that were fractioned around 9.07 min and 11.15 min via HPLC ("Electronic Supplementary Material" SI Fig. 1) inhibited fungal growth. Further HPLC purification of these bioactive fractions led to the isolation of two antifungal metabolites whose structures were identified as violacein and indole-3-carboxaldehyde (Fig. 2) via NMR and HR-MS analysis ("Electronic Supplementary Material" SI Table 1).

Optical density was used to assess fungal growth, and inhibitory growth assays against *B. dendrobatidis* were performed to determine the MIC for each of the metabolites. Increasing concentrations of violacein and indole-3-carboxaldehyde reduced fungal growth (violacein: $F=73.32$, $df=1$, 180, $P<0.001$; indole-3-carboxaldehyde: $F=211.59$, $df=1$, 84, $P<0.001$; Fig. 3). We determined MICs of 1.8 μM for violacein and 68.9 μM for indole-3-carboxaldehyde (Fig. 3).

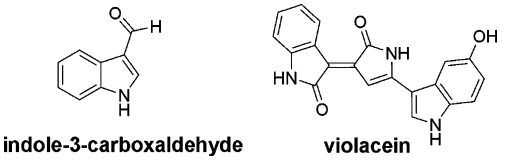

Fig. 2 Chemical structures of indole-3-carboxaldehyde and violacein, the two antifungal metabolites produced by *J. lividum* isolated from both the redbacked salamander (*P. cinereus*) and the four-toed salamander (*H. scutatum*)

We tested whether the metabolites identified from laboratory cultures of *J. lividum* could be detected directly on the salamanders in the wild and if their concentrations were sufficient to inhibit *B. dendrobatidis*. Of seven wild-caught salamanders, three housed the bacterium *J. lividum* as indicated by an analysis of DGGE ("Electronic Supplementary Material" SI Fig. 2). One individual had indole-3-carboxaldehyde on its skin, and three others had violacein. After determining an average depth of *P. cinereus* skin mucus (37 μm; see "Electronic Supplementary Material" SI Table 2), the volume of mucus on each individual and concentrations of the metabolites were calculated ("Electronic Supplementary Material" SI Table 2). The concentration of indole-3-carboxaldehyde on one wild-caught *P. cinereus* was estimated to be 51 μM (Table 1). The wild-caught salamanders displayed violacein exhibited concentrations of 207, 10, and 4 μM on their respective skins.

Discussion

A symbiotic relationship between cutaneous bacteria and a host amphibian is plausible; metazoans evolved with bacteria and mutualistic associations are common in animals (McFall-Ngai 1999). A fascinating example of cutaneous bacteria that benefit their host can be found in leaf cutter ants, *Acromyrmex* (Currie et al. 2006). The farming ants house bacteria, *Pseudonocardia*, which feed in the hosts' cuticle, and in return produce metabolites that deter an unwanted fungal species, *Escoviopsis*, from invading their subterranean garden (Currie et al. 2006). Our studies indicate that a similar mutualism might exist in amphibians, although whether associations will be found between specific amphibians species and specific bacterial species remains an open question (Harris et al. 2006; Lauer et al. 2007, 2008; Woodhams et al. 2007b; Brucker et al. 2008; Fig. 1).

The bacterium *J. lividum* is likely to be a mutualistic partner with red-backed salamanders. The two metabolites produced by *J. lividum* and found on salamander skin, indole-3-carboxaldehyde and violacein (Fig. 2; "Electronic

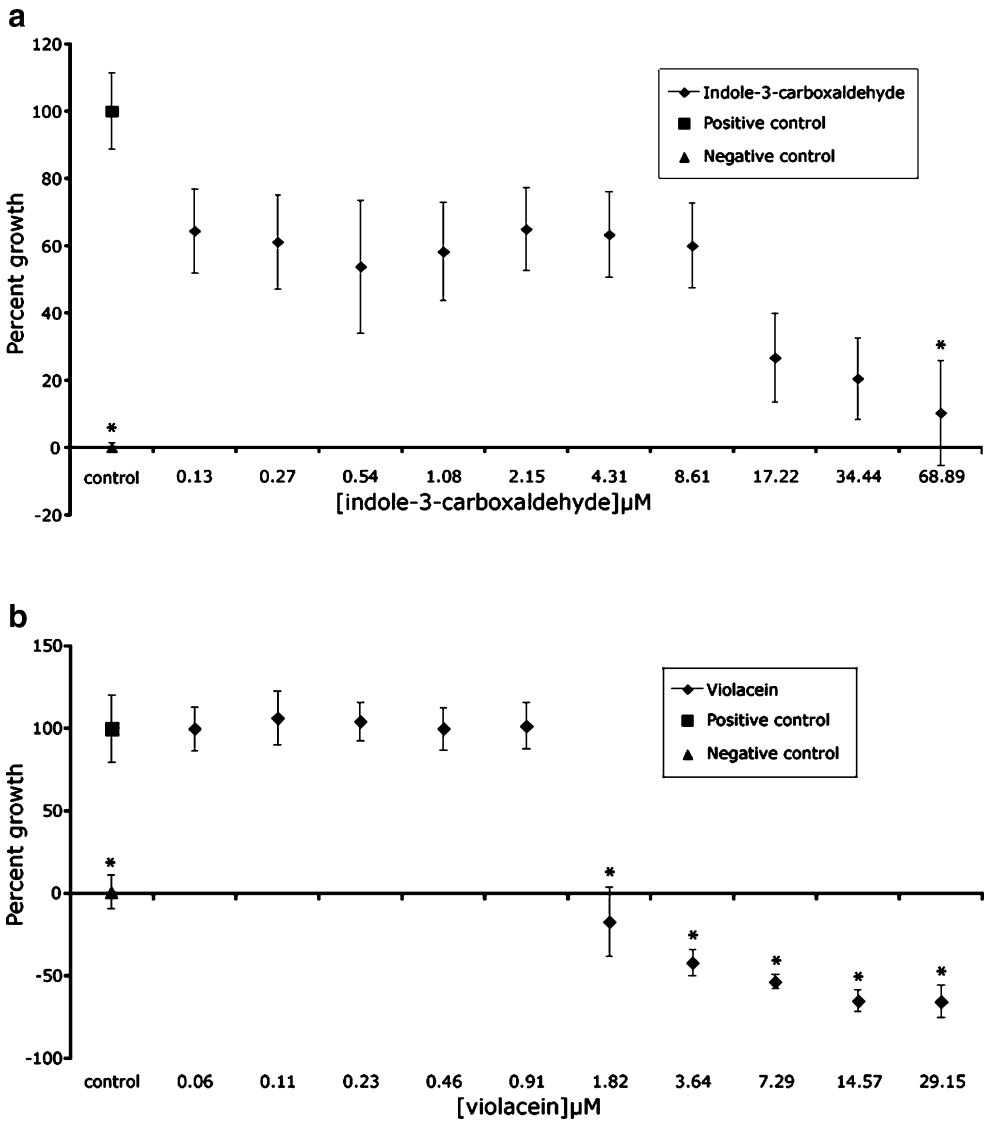

Fig. 3 Inhibition of growth in *B. dendrobatidis* due to the presence of **a** indole-3-carboxaldehyde or **b** violacein over a 7-day period. Percent growth is computed as difference in optical density between days 0 and 7 for each concentration compared to controls. *Error bars* indicate standard deviation of mean difference. *Asterisk* Significant difference from positive control, Dunnett's test

Supplementary Material" SI Table 1), are known agents of chemical defense although indole-3-carboxaldehyde is not a reported product of *J. lividum*. The fungus *Epichloë festucae* employs indole-3-carboxaldehyde in its endophytic relationship with many different host plants, defending the host against fungal pathogens (Yue et al. 2000). This metabolite also has antibacterial characteristics against several human pathogens in vitro (Gutierrez-Lugo et al. 2005). Violacein biosynthesis is a cell density-dependent factor, controlled via quorum sensing for the bacterial species *Chromobacterium violaceum.* Violacein is a known metabolite of *J. lividum* as well as several marine surface-associated bacteria (Durán and Menck 2001; Matz et al. 2008). Violacein has a broad bioactivity profile including antibacterial, antiviral, and antitumoral activity (Durán and Menck 2001). Neither compound, however, has been reported to protect vertebrates in the wild from an emerging infectious disease.

The concentrations of indole-3-carboxaldehyde and violacein on the skin of the salamanders (Table 1) approached or exceeded the MICs determined in vitro (Fig. 3). The concentration of indole-3-carboxaldehyde on the skin of one individual was just below the MIC required to completely inhibit *B. dendrobatidis* growth (51 vs. 68.9 μM). The violacein MIC was exceeded in all three salamanders that had violacein, with one individual having a concentration 114 times greater than the in vitro MIC. Comparatively, 2,4-diacetylphloroglucinol was not detected on the skin of the wild-caught salamanders, although there is a significant difference in concentrations required to inhibit *B. dendrobatidis* in vitro (MIC=136 μM; Brucker et al. 2008).

Concurrently, the bacterial diversity of the salamanders was examined with DGGE, specifically screening for *J. lividum* ("Electronic Supplementary Material" SI Fig. 1). Three of the four individuals that had indole-3-carboxaldehyde or violacein tested positive for *J. lividum.* This result

Table 1 Concentrations of indole-3-carboxaldehyde and violacein on skins of wild-caught *Plethodon cinereus*

Individual	Surface area of skin clipping (cm^2)	Volume of salamander mucus[a] (ml)	Area of indole-3-carboxaldehyde HPLC peak (mAU^2)	Mass of indole-3-carboxaldehyde on skin[b] (ng)	[indole-3-carboxaldehyde] on skin (μM)	Area of violacein HPLC peak (mAU^2)	Mass of violacein on skin[c] (ng)	[violacein] on skin (μM)	Detectable *J. lividum* through DGGE analysis
1[d]	3.137	1.16×10^{-5}	74	85	51	ND	ND	ND	+
2	2.311	8.55×10^{-6}	ND	ND	ND	ND	ND	ND	–
3	5.043	1.87×10^{-5}	ND	ND	ND	ND	ND	ND	–
4[d]	4.479	1.66×10^{-5}	ND	ND	ND	85	1148	207	+
5	4.005	1.48×10^{-5}	ND	ND	ND	3.6[d]	49	10	–
6[d]	3.167	1.17×10^{-5}	ND	ND	ND	1.1[d]	14	4	+
7	3.677	1.36×10^{-5}	ND	ND	ND	ND	ND	ND	–

ND not detected

[a] Depth estimated to be 37 μM; see "Electronic Supplementary Material"

[b] Calculated using the standard curve $y=5.0728\times$, ($R^2=0.994$) and the HPLC peak area of the sample in 200 μl of MeOH

[c] Calculated using the standard curve $y=25.143\times$, ($R^2=0.995$) and the HPLC peak area of the sample in 200 μl of MeOH

[d] Peak areas were estimated based on absorbance of light at 568 nm

does not exclude the possibility of low densities of *J. lividum* on the fourth individual, nor does it exclude the presence of alternative symbionts playing a similar role. *J. lividum* was not detected on the individuals lacking these metabolites. Together, these data suggest that the metabolites are related directly to the bacterial symbiont and further support the hypothesis that symbiotic bacteria play a significant role in host resistance.

Host salamanders have variations in the diversity of bacteria, both antifungal and non-antifungal, on their skin (Lauer et al. 2007, 2008), and this variation in bacteria populations may determine the type and amount of antifungal compounds available. We have evidence from laboratory experiments that amphibians with adequate microbially produced antifungal metabolites on their skin persist with *B. dendrobatidis* infection while those with low concentrations die (unpublished data). It is an open question why some amphibians do not have adequate antifungal metabolites on their skin, but perhaps there are costs as well as benefits to maintaining skin bacteria that produce such metabolites.

The presence or absence of beneficial bacteria and the antifungal metabolites they produce could account for the variation in infection and decline among populations and amphibian species. Some species house beneficial bacteria on their skin due to their physiology, behavior, environment, or any combination of the three. Physiologically, it is likely that there are strong selective pressures, such as antimicrobial peptides (Simmaco et al. 1998; Rollins-Smith et al. 2002) and glycoproteins (Ducklow and Mitchell 1979; Brizzi et al. 2002) on the skins of amphibians that influence the type of bacteria that are found there. Behaviorally, some amphibian species congregate densely, perhaps aiding in the spread of beneficial and pathogenic microbes; similarly, some care for their eggs and may, therefore, impart microbes to their embryos and young (Harris et al. 2006). Environmentally, factors such as plants, soils, and water can determine the type of bacteria available to the amphibian. In addition, anthropogenic effects may alter microbial species compositions (Belden and Harris 2007). These factors likely contribute to the differences observed in amphibian vulnerability to chytridiomycosis.

Our research suggests two modalities for amphibians affected by chytridiomycosis: (a) application of antifungal metabolites onto infected amphibians in laboratory environments; (b) application of probiotic bacteria that already exist on amphibians and that produce antifungal metabolites in natural environments.

The use of chemical treatments for chytridiomycosis is precedented in amphibian husbandry (Young et al. 2007) although they cannot provide long-term protection from the disease. However, the application of *J. lividum* itself as a bio-augmentation or probiotic treatment is potentially self-

propagating and may provide long-term protection from the disease as seen in agriculture (Haas and Degafo 2005). Should the anti *B. dendrobatidis* properties of the bacteria be extended to susceptible species, then it is possible that species declines due to the pathogen could be avoided. A study focusing on the beneficial antifungal bacteria of *R. muscosa* populations that are persisting with *B. dendrobatidis* has suggested that the entire population does not need to have beneficial bacteria to allow persistence (Woodhams et al. 2007b). By inoculating probiotic bacteria onto the amphibian host, *B. dendrobatidis* resistance may be self-perpetuating in the species and perhaps other amphibians through communal interactions, for example in hibernacula. This method could prove superior to the continual application of antifungal chemicals, which could have a negative effect on nontarget species such as beneficial fungi, is not self-perpetuating, and is economically difficult to sustain.

Further studies of the metabolites being produced by other antifungal bacteria from amphibians should yield a diverse suite of antifungal metabolites, perhaps including novel antifungal compounds that could be used in amphibian husbandry as well for treatment of human pathogens. Additional research is needed to understand the microbial ecology of amphibian skin and to use that understanding to develop strategies to manage chytridiomycosis in nature.

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References

BELDEN, L. K., and HARRIS, R. N. 2007. Infectious diseases in wildlife: the community ecology context. *Front. Ecol. Environ.* 5:533–539.

BERGER, L., SPEARE, R., DASZAK, P., GREEN, D. E., CUNNINGHAM, A. A., GOGGIN, C. L., SLOCOMBE, R., RAGAN, M. A., HYATT, A. D., MCDONALD, K. R., HINES, H. B., LIPS, K. R., MARANTELLI, G., and PARKES, H. 1998. Chytridiomycosis causes amphibian mortality associated with population declines in the rain forests of Australia and Central America. *Proc. Natl. Acad. Sci. U. S. A.* 95:9031–9036.

BRIGGS, C. J., VREDENBURG, V. T., KNAPP, R. A., and RACHOWICZ, L. J. 2005. Investigating the population-level effects of chytridiomycosis: an emerging infectious disease of amphibians. *Ecology* 86:3149–3159.

BRIZZI, R., DELFINO, G., and PELLEGRINI, R. 2002. Specialized mucous glands and their possible adaptive role in the males of some species of *Rana* (Amphibia, Anura). *J. Morphol.* 254:328–341.

BRUCKER, R. M., BAYLOR, C. M., WALTERS, R. L., LAUER, A., HARRIS, R. N., and MINBIOLE, K. P. C. 2008. The identification of 2,4-diacetylphloroglucinol as an antifungal metabolite produced by cutaneous bacteria of the salamander *Plethodon cinereus*. *J. Chem. Ecol.* 43:39–43.

CURRIE, C. R., POULSEN, M., MENDENHALL, J., BOOMSMA, J. J., and BILLEN, J. 2006. Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311:81–83.

DASZAK, P., STRIEBY, A., CUNNINGHAM, A. A., LONGCORE, J. E., BROWN, C. C., and PORTER, D. 2004. Experimental evidence that the bullfrog (*Rana catsbeiana*) is a potential carrier of chytridiomycosis, an emerging fungal disease of amphibians. *Herpetol. J.* 14:201–207.

DUCKLOW, H. W., and MITCHELL, R. 1979. Bacterial populations and adaptations in the mucus layers on living corals. *Limnol. Oceanogr.* 24:715–725.

DURÁN, N., and MENCK, C. F. M. 2001. *Chromobacterium violaceum*: A review of pharmacological and Industrial perspectives. *Crit. Rev. Microbiol.* 27:201–222.

GUTIERREZ-LUGO, M.-T., WOLDEMICHAEL, G. M., SINGH, M. P., SUAREZ, P. A., MAIESE, W. M., MONTENEGRO, G., and TIMMERMANN, B. N. 2005. Isolation of three new naturally occurring compounds from the culture of *Micromonospora* sp. P1068. *Nat. Prod. Res.* 19:645–652.

HAAS, D., and DEGAFO, G. 2005. Biological control of soil-borne pathogens by fluorescent *Pseudomonas*. *Nat. Rev. Microbiol.* 3:307–319.

HARRIS, R. N., JAMES, T. Y., LAUER, A., SIMON, M. A., and PATEL, A. 2006. The amphibian pathogen *Batrachochytrium dendrobatidis* is inhibited by the cutaneous bacteria of amphibian species. *EcoHealth* 3:53–56.

LAUER, A., SIMON, M. A., BANNING, J. L., ANDRÉ, E., DUNCAN, K., and HARRIS, R. N. 2007. Common cutaneous bacteria from the eastern red-backed salamander can inhibit pathogenic fungi. *Copeia* 3:630–640.

LAUER, A., SIMON, M. A., BANNING, J. L., LAM, B., and HARRIS, R. N. 2008. Diversity of cutaneous bacteria with antifungal activity isolated from the female four-toed salamanders. *Int. Soc. Microb. Ecol.* 2:145–157.

LIPS, K. R. 1999. Mass mortality and population declines of anurans at an upland site in western Panama. *Conserv. Biol.* 13:117–125.

LIPS, K. R., BREM, F., BRENES, R., REEVE, J. D., ALFORD, R. A., VOYLES, J., CAREY, C., LIVO, L., PRESSIER, A. P., and COLLINS, J. P. 2006. Emerging infectious disease and the loss of biodiversity in a Neotropical amphibian community. *Proc Natl. Acad. Sci. U. S. A.* 103:3165–3170.

MATZ, C., WEBB, J. S., SCHUPP, P. J., PHANG, S. Y., PENESYAN, A., EGAN, S., STEINBERG, P., and KJELLEBERG, S. 2008. Marine biofilm bacteria evade eukaryotic predation by targeted chemical defense. *Pub. Libr. Sci. U. S. A.* 7:3e2744.

MCFALL-NGAI, M. J. 1999. Consequences of evolving with bacterial symbionts: insights from the squid–*Vibrio* associations. *Annu. Rev. Ecol. Syst.* 30:235–256.

RACHOWICZ, L. J., KNAPP, R. A., MORGAN, J. A., STICE, M. J., VREDENBURG, V. T., PARKER, J. M., and BRIGGS, C. J. 2006. Emerging infectious disease as a proximate cause of amphibian mass mortality. *Ecology* 87:1671–1683.

ROLLINS-SMITH, L. A., REINERT, L. K., MIERA, V., and CONLON, J. M. 2002. Antimicrobial peptide defenses of the Tarahumara frog, *Rana tarahumarae*. Biochem. *Biophys. Res. Comm.* 297:361–367.

SIMMACO, M., MIGNOGNA, G., and BARRA, D. 1998. Antimicrobial peptides from amphibian skin: What do they tell us? *Biopolymers* 47:435–450.

STUART, S. N., CHANSON, J. S., COX, N. A., YOUNG, B. E., RODRIGUES, A. S. L., FISCHMAN, D. L., and WALLER, R. W.

2004. Status and trends of amphibian declines and extinctions wordwide. *Science* (*Wash*.) 306:1783–1786.

WOODHAMS, D. C., ARDIPRADJA, K., ALFORD, R. A., MARANTELLI, G., REINERT, L. K., and ROLLINS-SMITH, L. A. 2007a. Resistance to chytridiomycosis varies among amphibian species and is correlated with skin peptide defenses. *Anim. Conserv.* 10:409–417.

WOODHAMS, D. C., VREDENBURG, V. T., STICE, M. J., SIMON, M. A., BILLHEIMER, D., SHAKHTOUR, B., SHYR, Y., BRIGGS, C. J., ROLLINS-SMITH, L. A., and HARRIS, R. N. 2007b. Symbiotic bacteria contribute to innate immune defenses of the threatened mountain yellow-legged frog, *Rana muscosa*. *Biol. Conserv.* 138:390–398.

YOUNG, S., BERGER, L., and SPEAR, R. 2007. Amphibian chytridiomycosis: strategies for captive management and conservation. *Int. Zoo. Yb.* 41:85–95.

YUE, Q., MILLER, C. J., WHITE, J. F., and RICHARDSON, M. D. 2000. Isolation and characterization of fungal inhibitors from *Epichloe festucae*. *J. Agric. Food Chem.* 48:4687–4692.

of 1.5 larvae ml^{-1} in larval rearing water. Recently, Munga et al. (2006) compared oviposition choices of *An. gambiae* to (1) rainwater conditioned with different numbers (none, five or 50 in 200ml) of conspecific larvae but had been removed prior to assays and (2) rainwater with different densities of larvae (none, five, 40, 70 and 100 in 200ml). Fewer eggs were laid in rainwater conditioned with larvae than in unconditioned rainwater. In the presence of different densities of larvae, more eggs were laid in rainwater that had the fewest or no larvae. Additionally, the greatest number of eggs were laid in rainwater that contained the lowest concentration of larvae. These authors proposed that *An. gambiae* females were not influenced by the presence of conspecific larvae, but by other habitat characteristics associated with food quality and quantity, such as algal and microbial populations (Munga et al. 2006). In our previous studies on *An. gambiae*, we found that more eggs were laid in water from natural anopheline habitats than in distilled water or water from natural culicine habitats (Sumba et al. 2004a), and that volatile emissions associated with microbial populations from these habitats mediate this preference (Sumba et al. 2004b). We suggested that production of intra-specific cues from eggs or immature stages of the insect may occur only in habitats favorable for the optimal development of larvae and that rearing water (McCrae 1984) and rainwater (Munga et al. 2006) do not contain the full range of chemical signals present in natural anopheline larval habitats.

The present study was undertaken with two objectives in mind: (1) to compare *An. gambiae* oviposition levels in water collected from natural anopheline larval habitats with those in distilled water by using different densities of conspecific eggs or larvae and (2) to establish whether or not intra-specific olfactory signals mediate any of the observed effects.

Methods and Materials

Mosquitoes An. gambiae s.s. Mbita strain larvae were initially collected from anopheline pools at Mbita Point, Suba District, western Kenya, and reared in a screenhouse (11.5×7.1×4.4 m) (Seyoum et al. 2002) at a density of about 500 larvae in 3 l of water obtained from a natural ground pool containing predominantly anopheline larvae. Average temperature in the screenhouse was 29±2°C during the day and 24±2°C during the night, and RH ranged from 57±4% (day) to 72±5% (night). These conditions approximated the natural conditions prevailing in the Suba district in western Kenya. Larvae were fed daily on tetramin fish food (Seyoum et al. 2002). Adult mosquitoes were kept in standard 30×30×30 cm cages in an adult insectary at 27±2°C, 65–70% RH, a photoperiod of 12:12 h (L–D), and were offered 6% glucose solution on which to feed ad libitum. Three to four-days-old females were starved for 12 h and allowed to feed on human arms for a 10-min period on two consecutive evenings at 18:00 h. Unfed mosquitoes were removed from the cage after each blood meal. Fully engorged females were left in the cages until they were gravid and used in oviposition assays on the second night after their last blood meal. Approval for feeding mosquitoes on human subjects was obtained from the Kenya National Ethical Review Board (protocol number KEMRI/RES/7/3/1).

Collection of Anopheline Habitat Water Anopheline habitat water was collected at the start of the assays from natural ground pools around Mbita Point (Minakawa et al. 1999; Sumba et al. 2004a). Presence of anopheline larvae in these pools was confirmed by randomly sampling the water five times with a 350 ml standard dipper and inspecting it for larvae. Collected water was normally turbid with an average pH of 7.4±0.1 and was sieved to remove mosquito larvae or pupae.

Oviposition Response to Conspecific Larvae Oviposition assays were carried out in 25×25×25 cm Plexi®-glass cages under ambient conditions in the screenhouse. A gravid mosquito was placed in each cage and provided with a choice of two artificial oviposition sites, each in a black plastic cup (2 cm deep, 4 cm diameter), placed diagonally at opposite corners of the cage, 30 cm apart. One cup contained test water and the other control water. The test waters were prepared by placing different numbers (0, 1, 5, 10, 20, 30, or 40) of early (first and second) or late (third and fourth) instars in 20 ml of either (1) water taken from freshly collected natural ground pool colonized by anopheline larvae (pool water), or (2) distilled water. Larvae in distilled water were left in the cage for at least 24 h before the assay was started. Control water was either pool water or distilled water without larvae. Mosquitoes were released into the cages at about 17:00 h, and the number of eggs on each cup was counted the following morning. Two or three replicates of each treatment were performed on the same night and the experiment repeated on 10 different nights.

Oviposition Response to Conspecific Eggs Varying number of eggs (0, 1, 5, 10, 20, or 30 eggs) laid the previous night were placed in 20 ml of either a fresh batch of pool water or distilled water in a black plastic cup. Each was paired with another cup with control water (distilled or pool water) without eggs in a Plexi®-glass cage, and a gravid female was introduced into the cage 24 h later, as for the larval assays. The numbers of eggs laid in the two cups were recorded the following morning. Treatments were replicated from 22–27 times.

Nature of Signal(s) Mediating Larval Effects In the 2-choice assays, the greatest increase in oviposition occurred at a concentration of about 10 (early instar) larvae in 20 ml of pool water, and the greatest reduction in oviposition occurred at a concentration of about 40 (late instar) larvae in the same volume. These densities were used in 4-choice experiments carried out in 60×60×60 cm Plexi®-glass cages to determine whether the stimulus was olfactory or otherwise (tactile, contact chemical, or visual) in nature. 'Double cup' oviposition setups (Sumba et al. 2004b), placed at the corners of the cage, were used. Each setup consisted of an outer opaque plastic cup (8 cm deep, 6 cm diameter) containing 20 ml of either pool or distilled water, with or without larvae, and a smaller inner black plastic cup (2 cm deep, 4 cm diameter) containing 20 ml of distilled water that was floating on the water in the outer cup. In a setup designed to restrict exposure of gravid females to volatile chemicals from test substrates, a 6-cm diameter cone of white folded polyester cloth was placed so that it fit the inside of the plastic cup neatly and acted as a barrier against direct tarsal or visual contact of the test water in the outer cup by mosquitoes. This setup allowed olfactory perception of any volatile signal emanating from the test water. Four sets of 4-choice assays were carried out: (1) a choice of distilled water, distilled water with 10 early instars, pool water, and pool water with 10 early instars, with no cone barriers in any set-ups; (2) same as (1) with polyester cones in all setups; (3) same as in (1) but with 40 late instars; and (4) same as (3) with cone barriers. In a given replicate, the four treatments were randomly assigned to a corner of the Plexi®-glass cage. In all experiments, gravid mosquitoes were released into the cages in groups of five at about 17:00 h and the numbers of eggs laid on each treatment (outer and inner cups in treatments without cone barrier and cone in treatments with barrier) were counted the next morning. Fresh gravid mosquitoes and treatments were used on each experimental night. The assays were replicated from 16–22 times (Table 1).

Data Analysis An oviposition index (OI) for each replicate in the dual-choice assays was calculated according to the formula $OI=N_t-N_s/N_t+N_s$ (Kramer and Mulla 1979), with N_t=number of eggs on the test substrate (distilled water or pool water with larvae or eggs) and N_s=number of eggs on the control substrate (distilled water or pool water without larvae or eggs). Thus, OI indices can range from +1 to −1, with positive values indicating that more eggs were laid on the treatment than on the control and negative values the converse. Generally, a substance with an OI of +0.3 or above is considered attractive, whereas one of −0.3 or below is considered a deterrent or repellent (Hwang 1980; Poonam et al. 2002). In the present study, the statistical significance of the OI of each treatment (with larvae or eggs in one of the cup pairs) relative to the control (no larvae or eggs in a cup) was determined by a one-sample *t* test. In the 4-choice experiments, the number of eggs laid on each oviposition site was arcsine transformed (Gomez and Gomez 1984) and the angular values were subjected to ANOVA (SAS Institute Inc. 2003). Means were compared by a Student-Newman-Keuls (SNK) test at a 5% level of significance.

Results

Oviposition Responses to Different Densities of Conspecific Larvae Figure 1 shows OIs of treatments with different densities of early (Fig. 1a,c, and e) or late (Fig. 1b,d, and f) instars, in choices between pool water vs. distilled water control (Fig. 1a,b), pool water vs. pool water control (Fig. 1c,d), and distilled water vs. distilled water control (Fig. 1e, f). Water without larvae, obtained from a natural anopheline pool habitat was significantly more attractive (OI>+0.38; $P<0.05$, $N=27$, *t*-statistics) than the distilled water control (Fig 1a,b), confirming previous results (Sumba et al. 2004a, b). Irrespective of larval age, oviposition responses of gravid mosquitoes in the presence of conspecific larvae in pool

Table 1 Mean numbers of eggs (±SE) laid by gravid *Anopheles gambiae s.s.* given choices of four aqueous substrates: (1) using low density (10) early instars with no cone barriers; (2) same as (1) but with polyester cones preventing contact with aqueous substrate cues; (3) same as (1) but with high density (40) late instars; and (4) same as (3) with polyester cones

	Low larval density		High larval density	
Substrate	(1) (N=16)	(2) (N=22)	(3) (N=19)	(4) (N=22)
Distilled water	23.1±12.9a	36.1±11.5a	72.4±15.5b	27.1±10.8a
Distilled water+larvae	22.8±13.7a	21.4±8.8a	19.3±7.6a	25.4±12.4a
Pool water	96.3±19.8b	93.2±15.1b	232.9±36.7c	79.6±20.1b
Pool water+larvae	197.9±23.9c	198.2±21.7c	37.6±15.7a	169.2±22.9c

Means in the same column sharing a common letter are not significantly different at an alpha of P=0.05 level (SNK test)

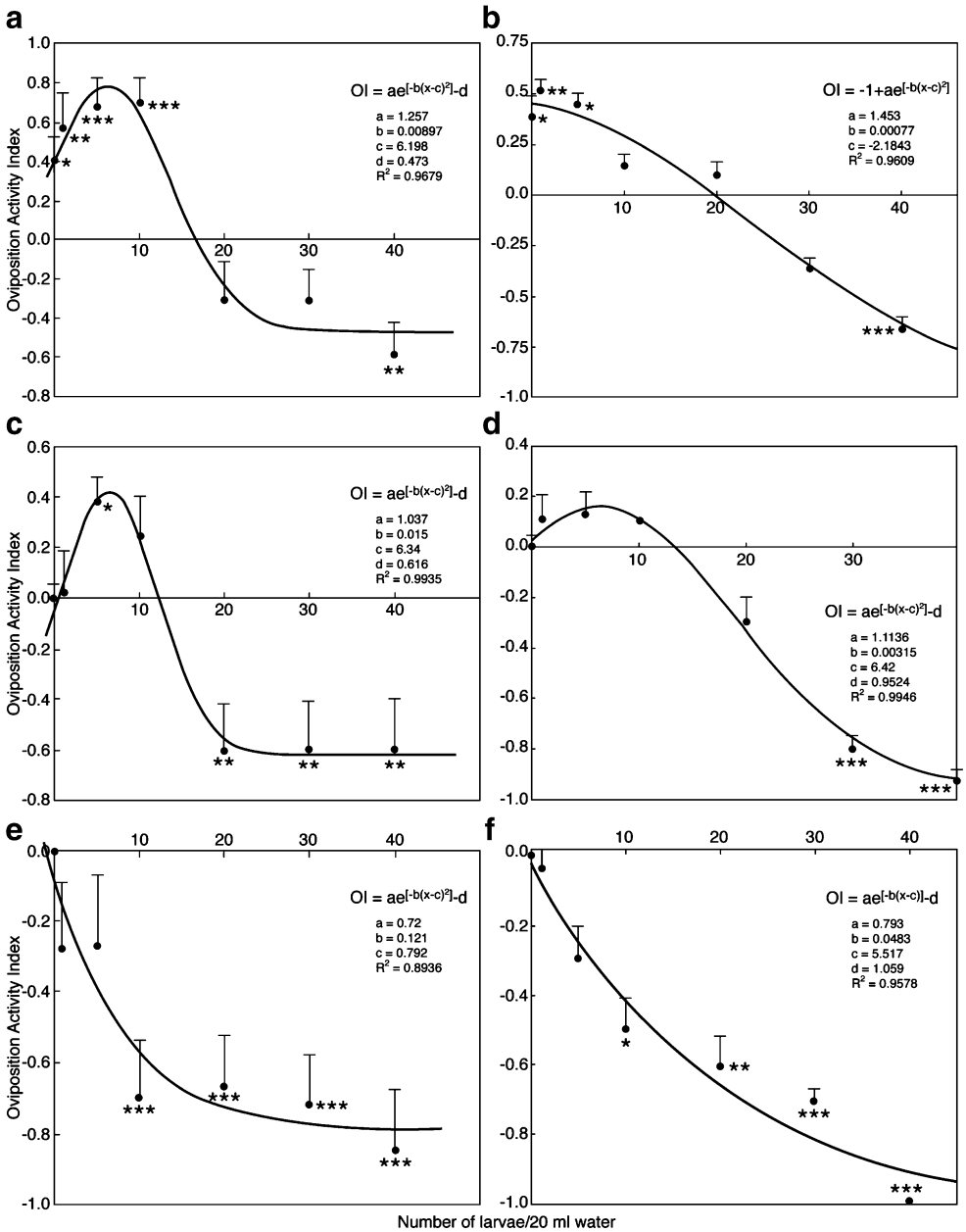

Fig. 1 Oviposition indices (OIs, mean±SE) and regression relationships showing oviposition responses of *Anopheles gambiae s.s.* to different densities of early (**a**, **c**, **e**) and late (**b**, **d**, **f**) conspecific larvae in: (1) pool water with distilled water control (**a** and **b**); (2) pool water with pool water control (**c** and **d**); and (3) distilled water with distilled water control (**e** and **f**). Starred OIs are significantly different from zero (one-sample *t* test) at **P*<0.05, ***P*<0.01 or ****P*<0.001

water were density-dependent, with test substrates with lower densities being preferred. Oviposition indices >+0.3 or <−0.3 were significantly different from zero (one sample *t*-test), indicating the test water was either stimulatory or deterrent, respectively, to females. The presence of larvae, particularly early instars (compare Fig. 1a and c with Fig. 1b and d), at relatively low density in pool water, stimulated oviposition by females (although not all increases were statistically significant). On the other hand, high larval densities, particularly late instars (compare Fig. 1b and d with Fig. 1a and c), deterred oviposition. Interestingly, the presence of larvae in distilled water deterred oviposition by gravid females at most densities (at low densities this was not significant) with late instars appearing particularly effective in this regard (Fig. 1e and f).

Oviposition Responses to Conspecific Eggs In choice assays between distilled water with eggs and a distilled water control, OIs (±SE) were (egg numbers in parentheses): 0.0±0 (0), 0.14±0.20 (1), 0.10±0.20 (5), −0.03±0.20 (10), 0.13±0.10 (20), and −0.12±0.10 (30). In assays involving a batch of pool water with eggs and pool water control, OIs were: 0.0±0 (0), 0.0±0.20 (1), 0.27±0.20 (5), 0.14±0.20 (10), 0.27±0.20 (20), and 0.06±0.10 (30). In assays with pool water with eggs and distilled water control, OIs were: 0.82±0.18 (0), 0.64±0.24 (1), 0.82±0.18 (5), 0.64±0.24 (10), 0.64±0.24 (20), and 0.82±0.18 (30). In all three sets of assays, the presence of 1-day-old eggs had no significant effect (*P*>0.05, *t*-test) on oviposition by *An. gambiae* females at any density tested. The high positive OIs (≥0.64) in the third set of assays between

natural pool and distilled water confirmed the preference for the former by *An. gambiae* females.

Roles of Olfactory and Non-olfactory Cues In all the 4-choice assays, pool water was significantly more attractive than distilled water (Table 1). The presence of larvae at low density (10 early instars in 20 ml) in pool water gave increased oviposition compared to pool water alone (Table 1 (1) and (2)), regardless of whether gravid females had contact with non-olfactory cues or not ($P<0.05$, SNK test). Distilled water showed no effect of low-density larvae (Table 1 (1) and (2)) for both situations. In assays involving the higher density of larvae (40 late instars in 20 ml), in which contact with non-olfactory cues was possible (Table 1 (3)), females laid significantly ($P<0.05$, SNK test) fewer eggs in water with, than in water without, larvae for both pool and distilled water. In contrast, in the situation in which only perception of volatile cues was possible (Table 1 (4)), the number of eggs laid in pool water with larvae was significantly ($P<0.05$, SNK test) greater than in pool water without larvae; there was no such difference observed with distilled water.

Discussion

Immatures of *An. gambiae* occur largely in fresh, sunlit, relatively small, and transient water pools with sparse vegetation (Muirhead-Thomson 1951; Gillies and De Meillon 1968; Service 1993; Minakawa et al. 1999, 2005a, b; Gimnig et al. 2001). Such pools are associated with lower levels of predation compared to large, long-lasting habitats (Service 1977; Washburn 1995; Sunahara et al. 2002). These pools are colonized rapidly within a few days of formation, suggesting that gravid females of this mosquito may actively search for and select such habitats for oviposition (Minakawa et al. 2005a). Laboratory studies have shown that volatile emissions associated with microbial activities in these habitats may partly mediate location of such habitats (Sumba et al. 2004b). Gravid females of two other anopheline species, *Anopheles albimanus* Wiedemann and *Anopheles vesttipennis* Dyar & Knab, are attracted similarly to volatile chemicals from their respective larval habitats (Rejmankova et al. 2005). The effect of the presence of *An. gambiae* larvae in the pools has been explored as an additional mechanism that influences attraction of conspecific females to a preferred oviposition site. Munga et al. (2006) compared oviposition levels of *An. gambiae* in rainwater with and without different numbers of larvae and found that, in all larval densities, the mosquito preferred to lay in clean rainwater over rainwater with larvae. This led the authors to suggest that conspecific larvae play no role in oviposition selection in this mosquito (Munga et al. 2006).

The results of our study demonstrate the role that conspecific larvae play in oviposition by *An. gambiae* and help to clarify some of the mechanisms that underlie the selection and spatial spread of larval habitats of this mosquito. First, the presence of conspecific larvae (but not eggs) influenced oviposition by gravid females. Preferences for ovipositing in water with larvae changed depending on the quality of the water and density of the larvae. In natural anopheline pool water, low densities of larvae (particularly early instars) resulted in increased oviposition, whereas higher densities resulted in decreased oviposition in a dose-dependent manner. Thus, contrary to previous work (Munga et al. 2006), our results show that, depending upon larval density, conspecific larvae of *An. gambiae* may play a dual role of augmenting the inter-specific signal emitted by preferred *An. gambiae* habitats and also of limiting the number of eggs laid in a particular habitat. Thus, conspecific larvae may fine-tune the balance between allowing exploitation of a healthy breeding site and avoiding intra-specific competition and other effects of overcrowding (Gimnig et al. 2002; Spencer et al. 2002; Kiflawi et al. 2003; Koenraadt and Takken 2003; Munga et al. 2006).

The 4-choice assays allowed us to clarify the roles played by inter- and intra-specific signals in oviposition by *An. gambiae*. At the lower larval density, with or without cone barriers, and the higher larval density with cone barriers, more eggs were laid on anopheline pool water substrates with larvae than on those without. This suggests that larvae in a favorable habitat emit a volatile intra-specific signal (pheromone) that augments the attraction to inter-specific volatiles (kairomone) associated with microbial activity in natural anopheline pools (Sumba et al. 2004b; Rejmankova et al. 2005). The presence of larvae, even at low density, in distilled water did not increase oviposition compared to distilled water alone, consistent with the observations made by Munga et al. (2006) in their study of the effects of different densities of larvae in rainwater. This suggests that either the larval pheromone is not stimulatory by itself (i.e., in the absence of the kairomone) or that production of the pheromone by larvae occurs only in *An. gambiae* preferred habitats with suitable organic matter, microbes, and algae (Merritt et al. 1992; Gimnig et al. 2001; Sumba et al. 2004b). The present study then represents the first demonstration of a larval pheromone on the oviposition behavior of *An. gambiae*. Chemical characterization of the pheromone and the kairomone associated with anopheline larval habitats will facilitate further studies on the relative roles of the two semiochemicals and possible manipulation of the oviposition behavior of this mosquito.

Our study of the effects of high larval density in assays with and without cone barriers suggests the presence of

intraspecific non-olfactory cues in addition to the olfactory signal. In the situation (no cone barriers) that allowed contact of females with the substrate, gravid *An. gambiae* females laid significantly fewer eggs in pool water with larvae than in that without larvae, suggesting an inhibitory effect. However, in the situation (with cone barriers) in which only olfactory cues could be perceived, the relative number of eggs laid in pool water with or without larvae resembled that with lower density of larvae. This suggests that gravid *An. gambiae* females are attracted to suitable pools with conspecific larvae, but that the intensity of non-olfactory, close range or contact cues, related to the density of larvae present, influences whether they oviposit or not. The nature of this cue, i.e., whether it is the physical disturbance of the water surface by the feeding larvae, contact with the larvae and/or visual effects, or a contact chemical from larval secretions, remains to be established. However, the work of Munga et al. (2006), who found that rainwater conditioned by different numbers of larvae deterred oviposition by gravid females, suggests the mediation of a chemical (non-volatile pheromone).

Finally, some differences between the effects of early and late instars on oviposition were apparent (Fig. 1). In anopheline pool water, in which gravid females were exposed to both olfactory and non-olfactory cues, comparison of OI values suggests that older instars appeared less effective in increasing oviposition at low density and more effective in deterring oviposition at high density (compare Fig. 1b,d and f with Fig. 1a,c and e). The results may indicate that ovipositing females are less inclined to lay in pools that have late instars compared with those with early instars. Follow-up field and laboratory studies will confirm whether these differences are real or not and, if so, elucidate the underlying factors.

In summary, the present study demonstrates the role that larvae of *An. gambiae* play in attracting and regulating oviposition by conspecific gravid females. Work is needed to elucidate the chemicals involved.

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References

ALLAN, S. A., and KLINE, D. L. 1998. Larval rearing water and pre-existing eggs influence oviposition by *Aedes aegypti* and *Ae. albopictus* (Diptera: Culicidae). *J. Med. Entomol.* 35:943–947.

BENTLEY, M. D., MCDANIEL, T. N., LEE, H.-P., STIEHL, B., and YATAGAI, M. 1976. Studies of *Aedes triseriatus* oviposition attractants producted by larvae of *Aedes triseriatus* and *Aedes atropalpus* (Diptera: Culicidae). *J. Med. Entomol.* 13:112–115.

BENZON, G. L., and APPERSON, C. S. 1988. Re-examination of chemically mediated oviposition behavior in *Aedes aegypti* (L.) (Diptera: Culicidae). *J. Med. Entomol.* 25:158–164.

BLACKWELL, A., MORDUE, A. J., HANSSON, B. S., WADHAMS, L. J., and PICKETT, J. A. 1993. A behavioural and electrophysiological study of oviposition cues for *Culex quinquefasciatus*. *Physiol. Entomol.* 18:343–348.

CHADEE, D. D. 1993. Oviposition response of *Aedes aegypti* (L.) to the presence of conspecific eggs in the field in Trinidad, W.I. J. Fl. *Med. Assoc.* 64:63–66.

CLEMENTS, A. N. 1999. The Biology of Mosquitoes Sensory Reception and Behaviour, 2. p. 756. CABI, UK.

GILLIES, M. T., and DE MEILLON, B. 1968. The Anophelinae of Africa South of the Sahara (Ethiopian zoogeographical region). p. 343. South African Institute for Medical Research, Johannesburg.

GOMEZ, K. A., and GOMEZ, A. A. 1984. Statistical Procedure for Agricultural Research. pp. 3–15. Wiley, New York.

GIMNIG, J. E., OMBOK, M., KAMAU, L., and HAWLEY, W. A. 2001. Characteristics of larval anopheline (Diptera: Culicidae) habitats in Western Kenya. *J. Med. Entomol.* 38:282–288.

GIMNIG, J. E., OMBOK, M., OTIENO, S., KAUFMAN, M., VULULE, J. M., and WALKER, E. D. 2002. Density-dependent development of *Anopheles gambiae* (Diptera: Culicidae) larvae in artificial habitats. *J. Med. Entomol.* 39:162–172.

HWANG, Y. S. 1980. Oviposition attractants and repellents of mosquitoes. Isolation and Identification of oviposition repellents for *Culex* mosquitoes. *J. Chem. Ecol.* 6:71–80.

KALPAGE, K. S. P., and Brust, R. A. 1973. Oviposition attractant produced by immature *Aedes atropalpus*. *Env. Entomol.* 2:729–730.

KIFLAWI, M., BLAUSTEIN, L., and MANGEL, M. 2003. Oviposition habitat selection by the mosquito *Culiseta longiareolata* in response to risk of predation and conspecific larval density. *Ecol. Entomol.* 28:168–173.

KOENRAADT, C. J. M., and TAKKEN, W. 2003. Cannibalism and predation among larvae of *Anopheles gambiae* complex. *Med. Vet. Entomol.* 17:61–66.

KRAMER, W. L., and MULLA, M. S. 1979. Oviposition attractants and repellents of mosquitoes: Oviposition responses of *Culex* mosquitoes to organic infusions. *Env. Entomol.* 8:1111–1117.

LAURENCE, B. R., and PICKETT, J. A. 1985. An oviposition attractant pheromone in *Culex quinquefasciatus* Say (Diptera: Culicidae). *Bull. Entomol. Res.* 75:283–290.

MCCRAE, A. W. 1984. Oviposition by African malaria vector mosquitoes. II. Effects of site tone, water type and conspecific immatures on target selection by freshwater *Anopheles gambiae* Giles, sensu lato. *Ann. Trop. Med. Parasitol.* 78:307–318.

MERRITT, R. W., DADD, R. H., and WALKER, E. D. 1992. Feeding behavior, natural food, and nutritional relationships of larval mosquitoes. *Annu. Rev. Entomol.* 37:349–376.

MINAKAWA, N., MUTERO, C. M., GITHURE, J. C., BEIER, J. C., and YAN, G. 1999. Spatial distribution and habitat characterization of anopheline mosquito larvae in western Kenya. *Ann. Trop. Med. Hyg.* 61:1010–1016.

MINAKAWA, N., SONYE, G., and YAN, G. 2005a. Relationships between occurrence of *Anopheles gambiae* s.l. (diptera:Culicidae) and size and stability of larval habitats. *J. Med. Entomol.* 42:295–300.

MINAKAWA, N., MUNGA, S., ATIELI, E., MUSHINZIMANA, E., ZHOU, Z., GITHEKO, A. K., and YAN, G. 2005b. Spatial distribution of anopheline larval habitats in western Kenya highlands: effects of land cover types and topography. *Am. J. Trop. Med. Hyg.* 73:157–165.

MUIRHEAD-THOMSON, R. C. 1945. Studies on the breeding places and control of *Anopheles gambiae* and *Anopheles gambiae* Var. *melas*

in the coastal district of Sierra Leone. *Bull. Entomol. Res.* 36:185–252.

MUNGA, S., MINAKAWA, M., ZHOSU, G., BARRACK, J. O-O., GITHEKO, A. K., and YAN, G. 2006. Effects of larval competitors and predators on oviposition site selection of *Anopheles gambiae* Sensu Stricto. *J. Med. Entomol.* 43:221–224.

OSGOOD, C. E. 1971. An oviposition pheromone associated with the egg rafts of *Culex tarsalis*. *J. Econ. Entomol.* 64:1038–1041.

POONAM, S., PAILY, K. P., and BALARAMAN, K. 2002. Oviposition attractancy of bacterial culture filtrates-Response of *Culex quinquefasciatus*. *Mem. Inst. Oswaldo Cruz* 97:359–362.

REISEN, W. K., and SIDDIQUI, T. F. 1978. The influence of conspecific immatures on the oviposition preferences of the mosquitoes *Anopheles stephensi* and *Culex tritaeniorhynchus*. *Pak. J. Zool.* 10:31–41.

REJMANKOVA, E. R., HIGASHI, R., GRIECO, J., ACHEE, N., and ROBERTS, D. 2005. Volatile substances from larval habitats mediate species-specific oviposition in *Anopheles* mosquitoes. *J. Med. Entomol.* 42:95–103.

SAS INSTITUTE INC. 2003. Proceedings of the Twenty-Eight Annual SAS Users Group International Conference. Cary, NC: SAS Institute Inc.

SERVICE, M. W. 1977. Mortalities of the immature stages of species of the *Anopheles gambiae* complex in Kenya: comparison between rice fields and temporary pools, identification of predators, and effects of insecticidal spraying. *J. Med. Entomol.* 13:535–545.

SERVICE, M. W. 1993. Mosquito Ecology: Field Sampling Methods. p. 988. Chapman and Hall, London.

SEYOUM, A., PÅLSSON, K., KUNG'A, S., KABIRU, E. W., LWANDE, W., KILLEEN, G. F., HASSANALI, A., and KNOLS, B. G. J. 2002. Traditional use of mosquito-repellent plants in western Kenya and their evaluation in semi-field experimental huts against *Anopheles gambiae*: ethnobotanical studies and application by thermal expulsion and direct burning. *Trans. Royal Soc. Trop. Med. Hygiene* 96:225–231.

SPENCER, M. L., BLAUSTEIN, L., and COHEN, J. E. 2002. Oviposition habitat selection by mosquitoes (*Culiseta longiareolata*) and consequences for population size. *Ecology* 83:669–679.

SUMBA, L. A., OKOTH, K., DENG, A. L., GITHURE, J., KNOLS, B. G. J., BEIER, J. C., and HASSANALI, A. 2004a. Daily oviposition patterns of the African malaria mosquito *Anopheles gambiae* Giles (Diptera: Culicidae) on different types of aqueous substrates. *J. Circadian Rhythms* 2:6.

SUMBA, L. A., GUDA, T. O., DENG, A. L., HASSANALI, A., BEIER, J. C., and KNOLS, B. G. J. 2004b. Mediation of oviposition site selection in the African malaria mosquito *Anopheles gambiae* (Diptera: Culicidae) by semiochemicals of microbial origin. *Int. J. Trop. Insec. Sci.* 24:260–265.

SUNAHARA, T., ISHIZAKA, K., and MOGI, M. 2002. Habitat size: a factor determining the opportunity for encounters between mosquito larvae and aquatic predators. *J. Vector Ecol.* 27:8–20.

TRIMBLE, R. M., and WELLINGTON, W. G. 1980. Oviposition stimulant associated with fourth-instar larvae of *Aedes togoi* (Dipetera: Culicidae). *J. Med. Entomol.* 17:509–514.

WASHBURN, J. O. 1995. Regulatory factors affecting larval mosquito populations in container and pool habitats: implications for biological control. *J. Am. Mosq. Contol Assoc.* 11:279–283.

ZAHIRI, N., and RAU, M. E. 1998. Oviposition attraction and repellency of *Aedes aegypti* (Diptera: Culicidae) to waters from conspecific larvae subjected to crowding, confinement, starvation, or infection. *J. Med. Entomol.* 35:782–787.

ZAHIRI, N., RAU, M. E., and LEWIS, D. J. 1997. Oviposition responses of *Aedes aegypti* and *Ae. atropalpus* (Diptera: Culicidae) females to waters from conspecific and heterospecific normal larvae and from larvae infected with Plagiorchis elegans (Trematoda: Plagiorchiidae). *J. Med. Entomol.* 34:565–568.

species secrete a sex pheromone that attracts males in a manner similar to many other nocturnal moths. Information about the sex pheromones of the diurnal arctiid species is limited, whereas pheromones of about 20 nocturnal species in the Arctiinae, another subfamily of Arctiidae, have been reported (Ando 2008). In the family Sesiidae, which also includes many diurnal moth species, 3,13-and 2,13-octadecadienyl compounds have been identified as sex pheromones of about 20 species and have been reported as sex attractants of more than 80 species (Naka et al. 2006; Ando 2008; El-Sayed 2008). Most of the known arctiid pheromones are composed of polyunsaturated hydrocarbons and their epoxy derivatives with a C_{17}–C_{23} straight chain. These compounds without a terminal functional group, which are also produced by females in the Geometridae, Noctuidae, and Lymantriidae, comprise a class of sex pheromones (Type II pheromones) that are differentiated from the compounds with a terminal functional group (Type I pheromones), such as the pheromones produced by the Sesiidae (Ando et al. 2004). For physiological and taxonomic reasons, we examined the pheromone components of *S. imaon* and revealed that the mating communication of the wasp moth is accomplished with a C_{21} 3,6,9-triene and 1,3,6,9-tetraene (i.e., a Type II pheromone).

In addition to characterizing the chemistry of Type II pheromone components, we studied their biosynthesis, mainly in the Japanese giant looper, *Ascotis selenaria cretacea* Butler (Geometridae). The female moths produce a C_{19} 3,6,9-triene and its epoxy derivative, as do females of the European strain, *Ascotis* (*Boarmia*) *selenaria* Denis & Schiffermüller (Becker et al. 1990). Both components are expected to be biosynthesized from dietary linolenic acid (C_{18} 9,12,15-trienoic acid) after its chain elongation to a C_{20} acid and decarboxylation. It has been confirmed that the epoxy pheromone was derived from the triene by monoepoxidation carried out in the pheromone gland (Miyamoto et al. 1999; Fujii et al. 2007). The triene is supplied to the pheromone gland via the hemolymph with the help of lipophorin after production outside of the pheromone gland, presumably in oenocytes and/or the fat body (Wei et. al. 2003; Matsuoka et al. 2006; Ando et al. 2008). However, the elongation and decarboxylation have not been demonstrated experimentally and the C_{20} acyl intermediate has not been identified. If the decarboxylation proceeds rapidly after the formation of the intermediate, trace amounts may be difficult to detect among an abundance of many common fatty acids. Since identification of the intermediate is an important essential step in a biosynthetic study, we purified and identified the hypothetical C_{20} acid intermediate from *A. s. cretacea* by high performance liquid chromatography (HPLC). In order to confirm this experiment with *A. s. cretacea*, we also analyzed fatty acids in *S. imaon* and identified not only C_{20} but also C_{22} trienoic acids. These acids have double bonds at the same positions as linolenic acid, relative to the terminal methyl group, i.e., they bear structures of intermediates of Type II pheromones. In addition to reporting the identification of the sex pheromone components secreted by *S. imaon* females, this paper deals with analyses of the fatty acids in abdominal integument tissues of *S. imaon* and *A. s. cretacea* to confirm the biosynthetic pathways of the pheromones.

Methods and Materials

Analytical Instruments The electroantennogram (EAG) activity of natural pheromonal components of *S. imaon* was measured with a gas chromatograph equipped with an electroantennographic detector (GC–EAD; Struble and Arn 1984). An HP-5890 Series II gas chromatograph (Hewlett-Packard, Wilmington, DE, USA) was equipped with a DB-23 column (0.25 mm i.d., 30 m length, 0.25 μm film thickness, J & W Scientific, Folsom, CA, USA). The column temperature was programmed for 50°C for 2 min, 10°C/min to 160°C, and 4°C/min to 220°C. The effluent from the column was split into two lines, which led to a flame ionization detector (FID) and EAD at a ratio of 1:1. An antenna was excised at the base from the male moth and a few distal segments were cut off. Each end of the antenna was attached to a droplet of a saline solution on an electrode of the EAD device such that the sensilla faced the airflow from the GC. Electron impact GC–mass spectrometry (GC–MS) was achieved by using an HP 5973 mass spectrometer system (quadrupole type, Hewlett Packard) equipped with the same DB-23 column operated at the same temperatures as the GC–EAD except for the analysis of the dimethyl disulfide (DMDS) derivatives, which were analyzed with an HP-5 column (0.25 mm i.d., 30 m length, 0.25 μm film thickness, Hewlett-Packard) and another program of 50°C for 2 min and 10°C/min to 320°C. For GC–MS analysis of fatty acid methyl esters (FAMEs) with the DB-23 column, the temperature program was 80°C for 1 min and 8°C/min to 220°C. The ionization voltage was 70 eV, and the flow rate of the carrier gas (He) was 1.0 ml/min. HPLC involved a Jasco PU-980 liquid chromatograph equipped with an integrator (System Instrument Chromatocorder 21J), a UV spectrometric detector (Jasco UV-970, 210 nm), and a reversed-phase column (Inertsil ODS, 4.6 mm i.d., 25 cm length; GL Sciences Inc., Tokyo, Japan). As the eluent, 5% water in methanol was used at a flow rate of 1.0 ml/min.

Insects and Pheromone Extraction Adults of *S. imaon* and larvae of *A. s. cretacea* were collected in a coppice in the Yonaguni-jima Islands and a tea garden in Mie Prefecture in

Japan, respectively. They were continuously reared on a semisynthetic diet for insects (Insecta LF(S), Nippon-Nosan-Kogyo Co., Yokohama, Japan) and sexed at the pupal stage. All insects were maintained under a 16:8 h, L–D cycle at 25°C. Since *S. imaon* virgin females showed a calling position during photophase, their abdominal ends were excised 4 h after the beginning of photophase to extract pheromone components. Each gland was separately immersed in hexane (100 μl) for 10 min to yield a crude extract.

Diimide Reduction and DMDS Derivatization Solvent of a crude pheromone extract of *S. imaon* (20 FE) was removed under a N_2 gas stream and the residue was successively treated with a N_2H_4 solution (0.3 ml of N_2H_4 in 10 ml of ethanol, 0.1 ml) and a H_2O_2 solution (0.04 ml of 30% H_2O_2 in 10 ml of ethanol, 0.1 ml) (Yamaoka et al. 1976). After warming at 65°C for 8 h, the reaction mixture was acidified with 1 N HCl and extracted with hexane (0.2 ml ×3). The hexane extract was passed through a short column with powdered Na_2SO_4 (0.1 g) and analyzed by GC–MS. The reduced products were subjected to DMDS derivatization (Buser et al. 1983) without any purification. After removing the solvent, residue was dissolved in DMDS (20 μl) that included a catalytic amount of iodine and kept at 40°C for 2 h. The DMDS adducts were extracted with hexane and analyzed by GC–MS. In the same manner, synthetic (*Z,Z,Z*)-1,3,6,9-henicosatetraene (1,*Z*3,*Z*6,*Z*9-21:H, 50 μg) also was treated with N_2H_4 and DMDS and the products of each reaction were analyzed by GC–MS to compare the results with those of the natural pheromone.

Field Test The synthetic lures for *S. imaon* were tested in 2007 in the Yonaguni-jima Islands. Rubber septa (white rubber, 8 mm o.d., Sigma-Aldrich, St. Louis, MO, USA) were used as dispensers, and synthetic compounds dissolved in hexane (100 μl) were applied to them. Each lure was placed at the center of a sticky board trap (30×27 cm bottom plate with a roof, Takeda Chem. Co., Tokyo, Japan). Two traps were used for each lure, and two other traps including the septa treated with only hexane (100 μl) were tested as control. The traps were placed at least 10 m apart at about 1.5 m above the ground in a coppice dominated by *Leucaena* trees, which are not the host plant of *S. imaon*, but were a convenient scaffold from which to hang the traps.

Chemicals (*Z,Z,Z*)-3,6,9-Trienes, which had been synthesized in our laboratory (Ando et al. 1993), were used as authentic standards for GC–MS analysis of a pheromone extract and as lures for a field test. (*Z,Z,Z*)-1,3,6,9-Tetraenes were newly synthesized by utilizing a one-pot double-Wittig approach (Pohnert and Boland 2000). The procedure and chemical data for the synthetic 1,*Z*3,*Z*6,*Z*9-21:H and other related polyenes have been reported (Yamamoto et al. 2008). The chemical structures of fatty acids and the methyl esters are abbreviated with the numbers before the colon, indicating the carbon number of a straight chain, and after the colon, indicating the degree of unsaturation.

Extraction and Methanolysis of Lipids in Integuments Abdomens from ten 0-day-old virgin females of *S. imaon* were isolated after elimination of their tips. Abdomens were incised along the midline, and their insides, including the ovaries, ovary-associated fat bodies, and digestive systems, were removed to yield integument tissues associated with oenocytes and peripheral fat bodies. Tissues were washed with cold saline (a 10 mM phosphate buffer, 150 mM NaCl, pH 6.5)×3, and then lipids included in the tissues were extracted with a mixed solvent (0.5 ml/tissue) of methanol and chloroform (Arima et al. 1991). Free acids, which were derived from the lipids by basic methanolysis, were converted further to methyl esters (FAMEs) by a reaction with diazomethane, and analyzed by GC–MS or subjected to HPLC fractionation. The quantities of common FAMEs were estimated by GC–MS analysis based on the peak areas in total ion chromatograms (TIC) of authentic standards (Sigma-Aldrich). The same experiment was carried out with ten virgin females of *A. s. cretacea* and the semisynthetic diet for insects (4.0 g) to yield their FAMEs.

Purification and Identification of Trace FAMEs The crude FAME mixture (1 FE) of each species was injected into an ODS column, and fractions were then collected every 1 min for 9–23 min and every 2 min for 23–61 min. This HPLC fractionation was repeated four more times, and fractions with the same fraction number were combined. After evaporation of methanol, the residues of each fraction were mixed with water (2 ml) and extracted with hexane (2 ml ×3). The extract was concentrated by evaporation and analyzed by GC–MS to identify the trace FAMEs included. Quantities were estimated by comparing peak areas with those of the authentic standards with a similar structure. Furthermore, two HPLC fractions (14–15 and 20–21 min) were separately collected from the FAME mixtures of *S. imaon* (10 FE) in order to confirm the structures of the esters with a trienyl moiety by micro chemical reactions, diimide reduction and DMDS derivatization, which was used to determine the double-bond positions of the *S. imaon* pheromone components.

Results

Identification of the S. imaon Pheromone In GC–EAD analysis with a pheromone gland extract from the *S. imaon* female, male antennae were constantly stimulated by two

components, Comp. I [retention time (Rt) 16.8 min] and Comp. II (Rt 17.9 min) (Fig. 1a). The structures of the two components were determined by GC–MS analysis of another crude pheromone extract. The mass spectrum of Comp. I (Rt 16.09 min) showed M^+ at *m/z* 290 (relative intensity, 2%), and characteristic fragment ions at *m/z* 234 (14%), 108 (63%), and 79 (100%) indicating a C_{21} 3,6,9-triene (Fig. 1b). The spectrum and Rt were closely coincident with those of synthetic (*Z,Z,Z*)-3,6,9-henicosatriene (*Z*3,*Z*6, *Z*9-21:H) (Ando et al. 1993). The mass spectrum of Comp. II (Rt 17.43 min) showed M^+ at *m/z* 288 (2%) and characteristic ions at *m/z* 234 (11%), 106 (33%), 91 (92%), and 79 (100%), indicating a C_{21} 1,3,6,9-tetraene (Fig. 1c). The spectrum and Rt were closely coincident with those of synthetic 1,*Z*3,*Z*6,*Z*9-21:H (Yamamoto et al. 2008). Moreover, the GC–MS analysis revealed a trace amount of the C_{20} triene and tetraene, (*Z,Z,Z*)-3,6,9-icosatriene (*Z*3,*Z*6, *Z*9-20:H, Rt 14.97 min, Comp. III) and (*Z,Z,Z*)-1,3,6,9-icosatetraene (1,*Z*3,*Z*6,*Z*9-20:H, Rt 16.28 min, Comp. IV), respectively. The C_{20} triene showed the spectrum with M^+ at *m/z* 276 (3%) and characteristic ions at *m/z* 220 (15%), 108 (65%), and 79 (100%), and the C_{20} tetraene showed the spectrum with M^+ at *m/z* 274 (2%) and characteristic ions at *m/z* 220 (11%), 106 (30%), 91 (87%), and 79 (100%). The average ratio of these four components (I–IV) analyzed with the extracts of seven individual females was 32:67:0.6:0.7. The content of the most abundant component (Comp. II, 1,*Z*3,*Z*6,*Z*9-21:H) was 1.5±0.2 μg/female.

Determination of Unsaturated Positions of the Tetraenyl Pheromone Component After the diimide reduction, the crude pheromone extract of *S. imaon* showed peaks of the following eight compounds (a–h) in a ratio of 10:7:7:5:4:4:4:1: a=icosane [Rt 14.85 min, *m/z* 296 (M^+, 2%) and 57 (100%)]; b=C_{21} monoene [Rt 15.00 min, *m/z* 294 (M^+, 13%) and 55 (100%)]; c=C_{21} monoene [Rt 15.14 min, *m/z* 294 (M^+, 13%) and 55 (100%)]; d=C_{21} monoene [Rt 15.35 min, *m/z* 294 (M^+, 11%) and 55 (100%)]; e=C_{21} diene [Rt 15.44 min, *m/z* 292 (M^+, 15%) and 67 (100%)]; f=C_{21} diene [Rt 15.55 min, *m/z* 292 (M^+, 2%) and 82 (100%)]; g=C_{21} diene [Rt 15.74 min, *m/z* 292 (M^+, 11%) and 82 (100%)]; and h=*Z*3,*Z*6,*Z*9-21:H [Rt 16.04 min, *m/z* 290 (M^+, 2%) and 79 (100%)]. Further treatment of the reduced products with DMDS yielded the following derivatives from the three monoenes: a DMDS adduct of 9-icosene (Rt 24.27 min, M^+ at *m/z* 388, diagnostic ions at *m/z* 215 and 173); a DMDS adduct of 6-icosene (Rt 25.37 min, M^+ at *m/z* 388, diagnostic ions at *m/z* 257 and 131); and a DMDS adduct of 3-icosene (Rt 24.88 min, M^+ at *m/z* 388, diagnostic ions at *m/z* 299 and 89). This result confirmed that the tetraenyl pheromone component (Comp. II) had three double bonds at the same positions as the trienyl component (Comp. I), i.e., the 3-, 6-, and 9-positions. The additional double bond in the tetraenes was estimated to be located at a terminal, which might be more easily attacked by N_2H_4 than the other bisubstituted double bonds. The experiment with synthetic, pure 1,*Z*3,*Z*6, *Z*9-21:H showed only the same products, indicating the selectivity of the diimide reduction.

Field Evaluation of the Synthetic S. imaon Pheromone Traps baited with a lure including a mixture of 1,*Z*3,*Z*6,*Z*9-21:H (1.0 mg/septum) and *Z*3,*Z*6,*Z*9-21:H (0.5 mg/septum) captured 31 male *S. imaon* from 28 June to 5 July, 2007, whereas no males were attracted by single component lures with each C_{21} polyene (1.0 mg/septum) and by the control traps.

GC–MS Analyses of Crude FAMEs In some lepidopteran insects, it has been reported that cuticular and pheromonal hydrocarbons are produced in the oenocytes located around the abdominal integument (Romer 1991; Jurenka et al. 2003). Therefore, the abdominal integument was dissected from female moths of *S. imaon* and *A. s. cretacea* to find

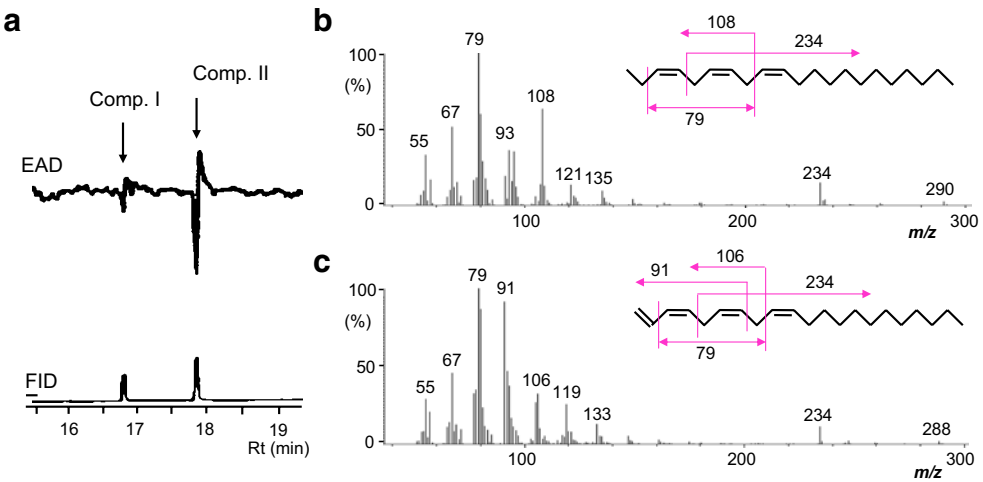

Fig. 1 Analyses of the sex pheromone components of *S. imaon* females by GC–EAD with the male antenna and GC–MS equipped with a DB-23 column. (**a**) Components I and II in a pheromone extract (0.5 FE) detected by a flame ionization detector (FID) and an electro-antennographic detector (EAD). (**b**) Mass spectrum of Comp. I (*Z*3,*Z*6,*Z*9-21:H) from the extract (1 FE). (**c**) Mass spectrum of Comp. II (1,*Z*3,*Z*6,*Z*9-21:H) from the extract (1 FE)

acyl intermediates involved in the pheromone biosynthesis. FAMEs prepared from extracts of one integument were dissolved in hexane (1 ml), and an aliquot (1 μl) was subjected to GC–MS analysis to reveal six common fatty acids (16:0, 16:1, 18:0, 18:1, 18:2, and 18:3) in *S. imaon* (Fig. 2a) and *A. s. cretacea* (Fig. 2b). The profiles of the FAMEs differed in the relative amounts of the two major components (peaks **1** and **4**). The 16:0 and 18:1 esters represented 40 and 25% of the total FAMEs in *S. imaon*, respectively, but 31 and 44% in *A. s. cretacea*. In these analyses, however, the proposed acyl intermediates of the type II pheromone components, such as 20:3, 22:3, 20:4, and 22:4 esters, could not be detected beyond a common 18:3 ester (peak **6**), which offered a mass spectrum with a base peak at *m/z* 79 (Fig. 2c). Even by using selected-ion monitoring, chromatograms at *m/z* 320 (M^+ of 20:3), 348 (M^+ of 22:3), 318 (M^+ of 20:4), 346 (M^+ of 22:4), and 79 showed no peaks around the Rts expected for the long-chain esters.

Analyses of the FAMEs after HPLC Fractionation From an ODS column, fatty alcohols and their derivatives with a shorter chain and a higher degree of unsaturation elute faster than the corresponding compounds with a longer chain and lower degree of unsaturation (Ando et al. 1986). Similar elution relationships hold for FAMEs of a series of authentic standards (C_{16} to C_{22}) (Fig. 3a). HPLC analyses of the esters from lipids of *S. imaon* (Fig. 3b) and *A. s. cretacea* (Fig. 3c) showed six peaks (**1**–**6**). With reference to the Rts of the standards, the chromatographic behaviors of the six FAMEs indicated that their chemical structures had a C_{16} or C_{18} chain, similar to those identified by GC–MS analysis (Fig. 2). On the other hand, GC–MS analyses of HPLC-fractionated elutants revealed the occurrence of FAMEs derived from other minor fatty acids with a C_{17}, C_{19}, C_{20}, C_{21}, or C_{22} chain. The chain length and degree of unsaturation of each ester were determined by M^+. While a 20:3 ester was recovered from the 14–15 min HPLC fraction of the FAMEs in both species, a 22:3 ester was detected only in the 20–21 min HPLC fraction of the *S. imaon* FAMEs. Together with the Rts of the authentic standards, the approximate Rts of trace FAMEs showed elution patterns that one might expect based on the chain length and degree of unsaturation (Fig. 3a). This analysis predicted that 20:4 and 22:4 esters might be recovered from the HPLC fractions of 9–12 and 16–19 min, respectively, but the GC–MS analysis of these fractions did not confirm the occurrence of these compounds.

Structure Determination of C_{20} and C_{22} Trienoates Trienoates were partially purified from an FAME mixture (1 FE) by using HPLC and were analyzed by GC–MS fractionation (Fig. 4) The FAMEs of *S. imaon* and *A. s. cretacea* included the same 20:3 ester (Rt 18.8 min), which showed a mass spectrum with M^+ at *m/z* 320 (6%) and fragment ions at *m/z* 289 (M-31, 4%), 277 (M-43, 2%), and 264 (M-56, 5%) (Fig. 4b). Each ion was larger than that of linolenate (18:3 ester, Fig. 2c) by 28 mass units (C_2H_4). Furthermore, the ion fragments lower than *m/z* 150 in the spectrum of the 20:3 ester were almost identical to that of 18:3 ester,

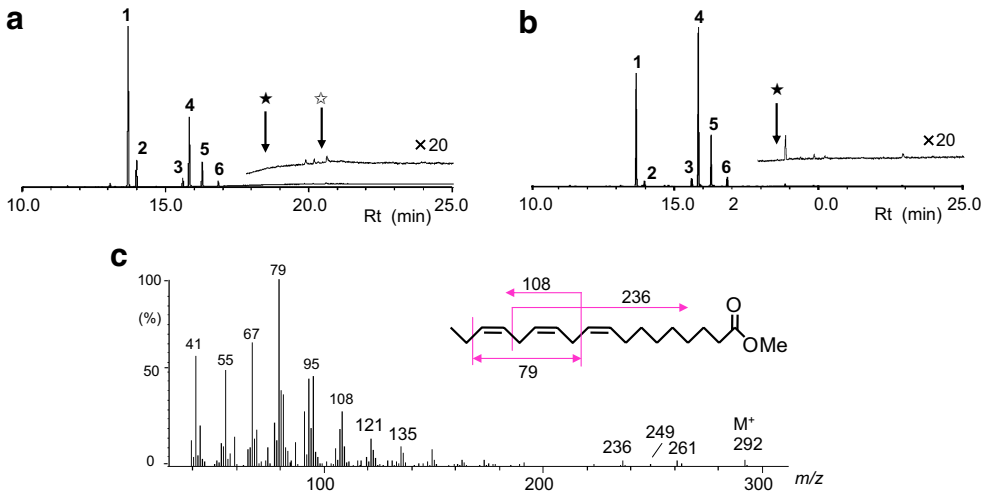

Fig. 2 Analyses of crude mixtures of fatty acid methyl esters (FAMEs), which were derived from the lipid extracts of abdominal integuments, by GC–MS equipped with a DB-23 column. (**a**) TIC (total ion chromatogram) of FAMEs from the *S. imaon* female (0.001 FE). (**b**) TIC of FAMEs from the *A. s. cretacea* female (0.001 FE). (**c**) Mass spectrum of the 18:3 ester of the *S. imaon* female (linolenate, peak **6**). Other esters: 16:0 (palmitate, peak **1**), 16:1 (peak **2**), 18:0 (stearate, peak **3**), 18:1 (oleate, peak **4**), and 18:2 (linoleate, peak **5**). The *arrows* in the amplified chromatograms show the expected Rts of 20:3 (*closed star*) and 22:3 (*open star*) esters, which could not be detected in these analyses

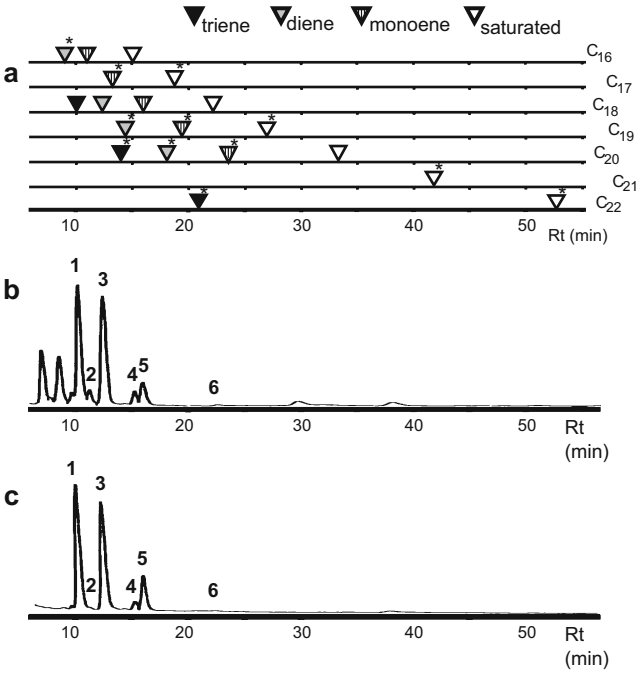

Fig. 3 Analyses of FAMEs, authentic standards and mixtures derived from the female abdominal integuments, by HPLC equipped with an ODS column. (**a**) Retention times (Rts) of saturated, monoenyl, dienyl, and trienyl FAMEs with a straight chain (C_{16}–C_{22}). The Rts of trace FAMEs with an *asterisk* were estimated by GC–MS analysis of the HPLC elutants, which were fractionated every one or 2 min after injection of insect FAMEs. (**b**) Chromatogram of FAMEs from the *S. imaon* female (1 FE). (**c**) Chromatogram of FAMEs from the *A. s. cretacea* female (1 FE). Upon injection of insect FAMEs, the following esters eluted with 5% water in methanol (flow rate, 1.0 ml/min; UV, 210 nm): 18:3 (linolenate, peak **1**), 16:1 (peak **2**), 18:2 (linoleate, peak **3**), 16:0 (palmitate, peak **4**), 18:1 (oleate, peak **5**), and 18:0 (stearate, peak **6**)

indicating the same trienoic structure. This characteristic spectrum suggests that the 20:3 ester is methyl (*Z,Z,Z*)-11,14,17-icosatrienoate derived from the predicted biosynthetic intermediate. This identification was confirmed by its partial reduction of the 20:3 ester, which yielded a mixture of three 20:1 esters. These monoenyl esters were treated with DMDS, and the GC–MS analysis of the reaction mixture revealed the production of the following three compounds: a DMDS adduct of 11-icosenoate (Rt 25.88 min, M^+ at *m/z* 418, diagnostic ions at *m/z* 245, 213, and 173); a DMDS adduct of 14-icosenoate (Rt 25.98 min, M^+ at *m/z* 418, diagnostic ions at *m/z* 287, 255, and 131); and a DMDS adduct of 17-icosenoate (Rt 26.45 min, M^+ at *m/z* 418, diagnostic ions at *m/z* 329, 297, and 89).

The 22:3 ester (Rt 20.5 min), which was detected only in the FAMEs of *S. imaon*, showed a mass spectrum with M^+ at *m/z* 348 (9%) and characteristic fragment ions at *m/z* 317 (M-31, 6%), 305 (M-43, 2%), and 292 (M-56, 5%) (Fig. 4d). Each ion was larger than that of linolenate by 56 mass units (C_4H_8). In the portion of the spectrum that was lower than *m/z* 150, the spectrum of the 22:3 ester was also identical to that of the 18:3 ester, indicating that the ester was methyl (*Z,Z,Z*)-13,16,19-docosatrienoate. Since the amount of the 22:3 ester purified from the FAME mixture was very low, the double-bond positions were not confirmed by the DMDS derivatization of monoenyl esters produced by its diimide reduction.

Relative Contents of Trace Fatty Acids In addition to 20:3 and 22:3 esters, the FAME mixtures of two species included many esters of the following trace fatty acids: 16:2, 17:0, 17:1, 19:0, 19:1, 19:2, 20:0, 20:1, 20:2, 21:0, and 22:0. While about 0.3 and 0.4 mg/female of the 16:0 ester were present in the crude FAME mixtures of *S. imaon* and *A. s. cretacea*, respectively, these trace esters were found at an ng range. Their relative contents based on the 16:0 ester were under 1% as shown in Table 1. Particularly, the amount of the 22:3 ester was quite low. In this study, 22:1 and 22:2 esters were not detected. If these esters are present in the mixtures, their contents are estimated to be

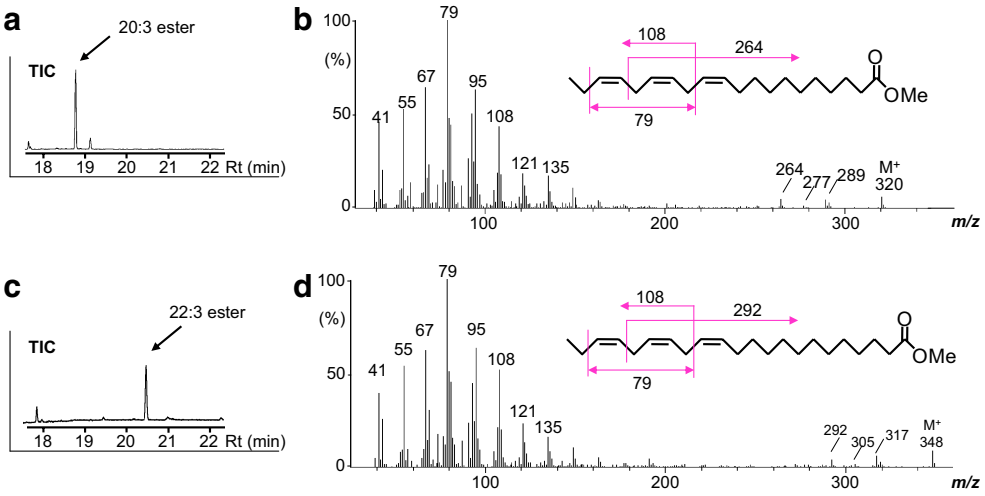

Fig. 4 GC–MS analyses of C_{20} and C_{22} trienoates, which were purified by HPLC fractionation of the FAMEs derived from the lipids in integuments of the *S. imaon* females. (**a**) TIC of the HPLC-elutants in a fraction of 14–15 min. (**b**) Mass spectrum of the 20:3 ester [methyl (*Z,Z,Z*)-11,14,17-icosatrienoate, Rt 18.8 min]. (**c**) TIC of the HPLC elutants in a fraction of 20–21 min. (**d**) Mass spectrum of the 22:3 ester [methyl (*Z,Z,Z*)-13,16,19-docosatrienoate, Rt 20.5 min]

Table 1 Relative contents of FAMEs with a C_{16}–C_{22} chain, which were derived from the lipids in abdominal integuments from female *Syntomoides imaon* and *Ascotis selenaria cretacea*

Ester	GC–MS[a]	Relative content (%±SEM)[b]	
	Rt (min)	*S. imaon*	*A. s. cretacea*
16:0	13.8	100	100
16:1	14.0	22±1	5.7±0.2
16:2	14.4	0.20±0.03	0.15±0.04
17:0	14.7	0.76±0.04	0.80±0.10
17:1	14.9	0.38±0.01	0.22±0.04
18:0	15.7	8.3±0.2	8.2±1.0
18:1	15.9	61±3	150±0
18:2	16.4	35±6	56±2
18:3	16.9	11±1	17±4
19:0	16.5	0.06±0.02	0.19±0.05
19:1	16.7	0.05±0.02	0.05±0.02
19:2	17.1	0.02±0.01	0.03±0.01
20:0	17.4	0.33±0.02	0.92±0.20
20:1	17.7	0.09±0.01	0.16±0.07
20:2	18.2	0.05±0.01	0.09±0.02
20:3	18.8	0.08±0.04	0.16±0.02
21:0	18.2	0.02±0.02	0.06±0.03
22:0	19.1	0.41±0.09	0.29±0.16
22:3	20.5	0.03±0.01	ND

[a] Analysis on a DB-23 capillary column
[b] The contents were measured by TIC of the GC–MS and expressed as a relative percentage of the amount of 16:0 ester (100 %)

under 0.01%. In order to examine a possibility that the 20:3 and 22:3 acids were derived from the artificial diet, which was provided for larvae of both species, the FAMEs of the diet were also analyzed by the same procedure as insect integuments. The trienoates were not detected in the artificial diet.

Discussion

A pheromone extract of the *S. imaon* females included four poly-unsaturated hydrocarbons, *Z*3,*Z*6,*Z*9-21:H, 1,*Z*3,*Z*6,*Z*9-21:H, *Z*3,*Z*6,*Z*9-20:H, and 1,*Z*3,*Z*6,*Z*9-20:H, at a ratio of 32:67:0.6:0.7. The former two C_{21} components showed strong EAG activities (Fig. 1), while the latter C_{20} components were not detected on GC–EAD analysis of the pheromone extract because of their low contents. In a coppice in the Yonaguni-jima Islands, we confirmed that the traps baited with a lure that included a 1:2 mixture of *Z*3,*Z*6,*Z*9-21:H and 1,*Z*3,*Z*6,*Z*9-21:H could capture males. After a preliminary field test in 2007, violent typhoons hit the islands and caused serious damage everywhere. Since the larvae and adults of *S. imaon* were not observed on the islands after the storm, the optimum mixing ratio and the roles of two minor components in the synthetic lures were not evaluated. However, the trapping revealed that the pheromone is the most significant cue in the searching behavior of the male moths around the females of this diurnal species. On the other hand, visual cues may play a role in final recognition of the partner because the adults have colorful species-specific striped bodies. By using a wind tunnel, we are examining exactly how the shape of the *S. imaon* female is recognized by the males attracted by the synthetic pheromone.

To date, sex pheromones have been reported for two other species in the Syntominae, *Empyreuma mucro* and *Syntomeida epilais* (Descoins et al. 1989). Although the identified components have not been evaluated in the field, the pheromones were composed of Type II pheromone components, *Z*3,*Z*6,*Z*9-21:H and the epoxy derivative. Our study with *S. imaon* confirmed that females of diurnal arctiid species secrete Type II pheromones, as the nocturnal arctiid species do. Species in the Syntominae evolved in the tropics, and only three other species are distributed throughout Japan. We also identified the Type II pheromone from another wasp moth, *Amata fortunei fortunei* (Kondo et al., unpublished). Sex pheromones of species in another subfamily, Lithosiinae, have not been reported, with the exception of female *Lyclene dharma dharma* (Ando 2008). We recently identified novel EAG-active compounds (methyl-branched 2-ketones) from this species, and the compounds were not classified into Types I or II (Yamamoto et al. 2007). The sex pheromones of arctiid moths are taxonomically interesting, and these results will be reported elsewhere.

To add to the above two Syntominae species, *Z*3,*Z*6,*Z*9-21:H has been identified as a pheromone component from more than 20 species in Geometridae, Noctuidae, Lymantriidae, and Arctiidae (Ando 2008). 1,*Z*3,*Z*6,*Z*9-21:H has been identified from the following three species: *Epirrita autumnata* (Geometridae) (Zhu et al. 1995), *Arctia villica* (Arctiidae) (Einhorn et al. 1984), and *Utetheisa ornatrix* (Arctiidae) (Jain et al. 1983; Choi et al. 2007). Similar to *S. imaon*, the latter two arctiid species produced a mixture of *Z*3,*Z*6,*Z*9-21:H and 1,*Z*3,*Z*6,*Z*9-21:H, but field tests of the synthetic mixture have not been reported for them. The preliminary field data from our study with *S. imaon* is the first case where the mixture identified from females was attractive to conspecific males.

In addition to the pheromone components, the lipid extracted from abdominal integuments, which were associated with oenocytes and peripheral fat bodies, was examined. A direct GC–MS analysis of FAMEs derived from the lipid of the *S. imaon* females was fruitless (Fig. 2). However, after HPLC fractionation of the FAMEs (Fig. 3, Table 1), methyl (*Z,Z,Z*)-11,14,17-icosatrienoate and (*Z,Z,Z*)-13,16,19-docosatrienoate were identified by GC–MS analysis (Fig. 4). These novel 20:3 and 22:3 acid moieties

might be biosynthesized by chain elongation of linolenic (18:3) acid, and are presumed to be biosynthetic precursors of the trienyl component (*Z*3,*Z*6,*Z*9-21:H) of the *S. imaon* pheromone. The C_{21} triene can be produced by decarboxylation of the 22:3 acid, which has the double bonds at the same positions counting from the terminal methyl group. On the other hand, only the 20:3 ester was found in FAMEs of *A. s. cretacea* females (Table 1), which secreted C_{19} pheromone components, (*Z,Z,Z*)-3,6,9-nonadecatriene and *cis*-3,4-epoxy-(*Z,Z*)-6,9-nonadecadiene. The difference of acid composition in these two species reflects the different chain lengths of the pheromones, indicating that the biosyntheses of Type II pheromones are strictly regulated by a species-specific system of the chain elongation. This result concurred with biosynthetic experiments of cuticle hydrocarbon pheromones in cockroaches and houseflies (Vaz et al. 1988; Tillman-Wall et al. 1992; Chertemps et al. 2007). It has been reported that the chain lengths of the pheromones are regulated by the step of fatty acyl-CoA elongation.

Studies on the biosynthetic pathways of Type II pheromones began from research with females of two arctiid species, *Estigmene acrea* and *Phragmatobia fuliginosa*, which secreted (*Z,Z*)-*cis*-9,10-epoxy-3,6-henicosadiene (Rule and Roelofs 1989). Since the females injected with ^{14}C-labeled 18:3, 20:3, or 22:3 acids produced the radioactivity-incorporated pheromones, the pathway via chain elongation of 18:3 acid was proposed. The 20:3 and 22:3 acids, however, could not be detected in the females. Moreover, no unsaturated fatty acids with a C_{20} or longer chain have been chemically determined from any insects, including the species that produce unsaturated hydrocarbons with a long chain (Blomquist et al.1987; Jurenka et al.1987). Our identification of the acyl intermediates in Type II pheromone biosynthesis is the first example that confirms the occurrence of long-chain unsaturated fatty acids in insects, to the best of our knowledge.

It is well known that cuticle hydrocarbons are produced in oenocytes associated with either epidermal tissue or fat body cells in insects (Romer 1991; Fan et al. 2003). In the case of insects that secrete a Type II pheromone, polyunsaturated hydrocarbons characterized as a pheromone component or biosynthetic precursor are also expected to be biosynthesized there and transported to the pheromone gland via the hemolymph after association with lipophorin (Matsuoka et al. 2006). From the *S. imaon* females, the triene and tetraene were detected in the hemolymph (data not shown), as other polyunsaturated hydrocarbons of *A. s. cretacea*, *Hemerophila artilineata* (Geometridae), and *Spilosoma imparilis* (Arctiidae) were (Wei et al. 2003). Although the contents of the 20:3 and 22:3 acids in *S. imaon* and *A. s. cretacea* were very low (Table 1), they were detected only in the lipids derived from the abdominal integuments and not from any other regions, such as the hemolymph and a pheromone gland (data not shown). This confirmed the site for the production and decarboxylation of 20:3 and 22:3 acids.

Recently, the biosynthetic pathway of the 1,*Z*3,*Z*6,*Z*9-21:H was examined with *U. ornatrix* (Choi et al. 2007). Deuterated *Z*3,*Z*6,*Z*9-21:H injected into female pupae was not incorporated into 1,*Z*3,*Z*6,*Z*9-21:H, indicating that the triene was not a biosynthetic precursor of the tetraene and that the terminal double bond was introduced at an early step in the biosynthetic pathway. With female *S. imaon*, we tried to identify the tetraenyl acids corresponding to the tetraene, but none of the hypothetical tetraenyl acids, i.e., 18:4, 20:4, and 22:4, was detected in FAMEs. If the tetraene is biosynthesized via desaturation of the acyl intermediate starting from dietary linolenic (18:3) acid, we can speculate that the desaturation step is linked effectively with decarboxylation and the tetraenyl acid does not accumulate. Further studies are necessary to determine the biosynthetic pathway of the tetraene. While conversion of deuterated 19:3 acid into a C_{18} trienyl pheromone was reported recently in *Erannis bajaria* (Goller et al. 2007), the biosynthetic steps that produce polyenyl hydrocarbons with an odd-numbered chain (not only tetraenes, but also trienes and dienes) have not been confirmed experimentally with labeled compounds. The *S. imaon* female seems to be a desirable insect for biosynthetic experiments, since the pheromone content is higher than 1 μg/female. Based on the structures determined in this study, we are synthesizing labeled candidates of precursors of the *S. imaon* pheromone to confirm the biosynthesis.

References

ANDO, T. 2008. Internet database: http://www.tuat.ac.jp/~antetsu/LepiPheroList.htm.

ANDO, T., HASEGAWA, Y., and UCHIYAMA, M. 1986. Separation of lepidopterous sex pheromones by reversed-phase thin layer chromatography and high performance liquid chromatography. *Agric. Biol. Chem.* 50:2935–2937.

ANDO, T., OHSAWA, H., UENO, T., KISHI, H., OKAMURA, Y., and HASHIMOTO, S. 1993. Hydrocarbons with a homoconjugated polyene system and their monoepoxy derivatives: Sex attractants of geometrid and noctuid moths distributed in Japan. *J. Chem. Ecol.* 19:787–798.

ANDO, T., INOMATA, S., and YAMAMOTO, M. 2004. Lepidopteran sex pheromones. *Topics Current Chem.* 239:51–96.

ANDO, T., KAWAI, T., and MATSUOKA, K. 2008. Epoxyalkenyl sex pheromones produced by female moths in highly evolved groups: Biosynthesis and its endocrine regulation. *J. Pestic. Sci.* 33:17–20.

ARIMA, R., TAKAHARA, K., KADOSHIMA, T., NUMAZAKI, F., ANDO, T., UCHIYAMA, M., NAGASAWA, H., and SUZUKI, A. 1991. Hormonal regulation of pheromone biosynthesis in the silkworm moth, *Bombyx mori*. *Appl. Entomol. Zool.* 26:137–147.

BECKER, D., CYJON, R., COSSÉ, A., MOORE, I., KIMMEL, T., and WYSOKI, M. 1990. Identification and enantioselective synthesis

of *(Z,Z)*-6,9-*cis*-3*S*,4*R*-epoxynonadecadiene, the major sex pheromone component of *Boarmia selenaria*. *Tetrahedron Lett.* 31:4923–4926.

BLOMQUIST, G. J., NELSON, D. R., and dE RENOBALES, M. 1987. Chemistry, biochemistry, and physiology of insect cuticular lipids. *Arch. Insect Biochem. Physiol.* 6:227–265.

BUSER, H.-R., ARN, H., GUERIN, P., and RAUSCHER, S. 1983. Determination of double bond position in mono-unsaturated acetates by mass spectrometry of dimethyl disulfide adducts. *Anal. Chem.* 55:818–822.

CHERTEMPS, T., DUPORTETS, L., LABEUR, C., UEDA, R., TAKAHASHI, K., SAIGO, K., and WICKER-THOMAS, C. 2007. A female-biased expressed elongase involved in long-chain hydrocarbon biosynthesis and courtship behavior in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. U S A* 104:4273–4278.

CHOI, M.-Y., LIM, H., PARK, K. C., ADLOF, R., WANG, S., ZHANG, A., and JURENKA, R. 2007. Identification and biosynthetic studies of the hydrocarbon sex pheromone in *Utetheisa ornatrix*. *J. Chem. Ecol.* 33:1336–1345.

DESCOINS, C., LALANNE-CASSOU, B., FRÉROT, B., MALOSSE, C., and RENOU, M. 1989. Comparative study of pheromonal secretions produced by arctiid and ctenuchid female moths (insects, Lepidoptera) from the neotropical area. *C. R. Acad. Sci., Ser. 3* 309:577–581.

EINHORN, J., BONIFACE, B., RENOU, M., and MILAT, M.-L. 1984. Study on the sex pheromone of *Arctia villica* L. (Lepidoptera, Arctiidae). *C. R. Acad. Sci., Ser. 3* 298:573–576.

EL-SAYED, A. M. 2008. Internet database: http://www.pherobase.com/.

FAN, Y., ZUREK, L., DYKSTRA, M. J., and SCHAL, C. 2003. Hydrocarbon synthesis by enzymatically dissociated oenocytes of the abdominal integument of the German cockroach, *Blattella germanica*. *Naturwissenschaften* 90:121–126.

FUJII, T., SUZUKI M. G., KAWAI, T., TSUNEIZUMI, K., OHNISHI, A., KURIHARA, M., MATSUMOTO, S., and ANDO, T. 2007. Determination of the pheromone producing region that has epoxidation activity in the abdominal tip of the Japanese giant looper, *Ascotis selenaria cretacea* (Lepidoptera: Geometridae). *J. Insect Physiol.* 53:312–318.

GOLLER, S., SZÖCS, G., FRANCKE, W., and SCHULZ, S. 2007. Biosynthesis of (3*Z*,6*Z*,9*Z*)-3,6,9-octadecatriene: The main component of the pheromone blend of *Erannis bajaria*. *J. Chem. Ecol.* 33:1336–1345.

JAIN, S. C., DUSSOURD, D. E., CONNER, W. E., EISNER, T., GUERRERO, A., and MEINWALD, J. 1983. Polyene pheromone components from an arctiid moth (*Utetheisa ornatrix*): characterization and synthesis. *J. Org. Chem.* 48:2266–2270.

JURENKA, R. A., dE RENOBALES, M., and BLOMQUIST, G. J. 1987. De novo biosynthesis of polyunsaturated fatty acid in the cockroach *Periplaneta americana*. *Arch. Biochem. Biophys.* 225:184–193.

JURENKA, R. A., SUBCHEV, M., ABAD, J.-L., CHOI, M.-Y., and FABRIAS, G. 2003. Sex pheromone biosynthetic pathway for disparlure in the gypsy moth, *Lymantria dispar*. *Proc. Natl. Acad. Sci. U S A* 100:809–914.

MATSUOKA, K., TABUNOKI, H., KAWAI, T., ISHIKAWA, S., YAMAMOTO, M., SATO, R., and ANDO, T. 2006. Transport of a hydrophobic biosynthetic precursor by lipophorin in the hemolymph of geometrid female moth which secretes an epoxyalkenyl sex pheromone. *Insect Biochem. Mol. Biol.* 36:576–583.

MIYAMOTO, T., YAMAMOTO, M., ONO, A., OHTANI, K., and ANDO, T. 1999. Substrate specificity of the epoxidation reaction in sex pheromone biosynthesis of the Japanese giant looper (Lepidoptera: Geometridae). *Insect Biochem. Mol. Biol.* 29:63–69.

NAKA, H., NAKAZAWA, T., SUGIE, M., YAMAMOTO, M., HORIE, Y., WAKASUGI, R., ARITA, Y., and ANDO, T. 2006. Synthesis and characterization of 3,13-and 2,13-octadecadienyl compounds for identification of the sex pheromone secreted by a clearwing moth, *Nokona pernix*. *Biosci. Biotechnol. Biochem.* 70:508–516.

POHNERT, G. and BOLAND,W. 2000. High efficient one-pot double-Wittig approach to unsymmetrical (1*Z*,4*Z*,7*Z*)-homoconjugated trienes. *Eur. J. Org. Chem.* 1821–1826.

ROMER, F. 1991. The oenocytes of insects: Differentiation, changes during molting, and heir involvement in the secretion of molting hormone, pp. 532–567, in A.P. Gupta (ed.). Morphogenetic Hormones of Arthropods: Roles in Histogenesis, Organogenesis, and Morphogenesis Rutgers University Press, New Brunswick, NJ.

RULE, G. S., and ROELOFS, W. L. 1989. Biosynthesis of sex pheromone components from linolenic acid in arctiid moths. *Arch. Insect Biochem. Physiol.* 12:89–97.

STRUBLE, D. L., and ARN, H. 1984. Combined gas chromatography and electroantennogram recording of insect olfactory responses, pp. 161–178, in H.E. Hummel, and T.A. Miller (eds.). Techniques in Pheromone Research Springer-Verlag, New York, NY.

TILLMAN-WALL, J. A., VANDERWEL, D., KUENZLI, M. E., REITZ, R. C., and BLOMQUIST, G. J. 1992. Regulation of sex pheromone biosynthesis in the housefly, *Musca domestica*: Relative contribution of the elongation and reductive steps. *Arch. Biochem. Biophys.* 299:92–99.

VAZ, A. H., JURENKA, R. A., BLOMQUIST, G. J., and REITZ, R. C. 1988. Tissue and chain length specificity of the fatty acyl-CoA elongation system in the American cockroach. *Arch. Biochem. Biophys.* 267:551–557.

WEI, W., MIYAMOTO, T., ENDO, M., MURAKAWA, T., PU, G.-Q., and ANDO, T. 2003. Polyunsaturated hydrocarbons in the hemolymph: Biosynthetic precursors of epoxy pheromones of geometrid and arctiid moths. *Insect Biochem. Mol. Biol.* 33:397–405.

YAMAMOTO, M., KAMATA, T., DO, N. D., ADACHI, Y., KINJO, M., and ANDO, T. 2007. A novel lepidopteran sex pheromone produced by females of a Lithosiinae species, *Lyclene dharma dharma*, in the family of Arctiidae. *Biosci. Biotechnol. Biochem.* 71:2860–2863.

YAMAMOTO, M., YAMAKAWA, R., OGA, T., TAKEI, Y., KINJO, M., and ANDO, T. 2008. Synthesis and chemical characterization of hydrocarbons with a 6,9,11-, 3,6,9,11-, or 1,3,6,9-polyene system, pheromone candidates in Lepidoptera. *J. Chem. Ecol.* 34:1057–1064.

YAMAOKA, R., FUKAMI, H., and ISHII, S. 1976. Isolation and identification of the female sex pheromone of the potato tuberworm moth, *Phthorimaea operculella* (Zeller). *Agric. Biol. Chem.* 40:1971–1977.

ZHU, J.-W., LÖFSTEDT, C., PHILIPP, P., FRANCKE, W., TAMMARU, T., and HAUKIOJA, E. 1995. A sex pheromone component to the Geometridae identified from *Epirrita autumnata*. *Entomol. Exp. Appl.* 75:159–164.

Fig. 1 Crown-like arrangement boring dust (frass) surrounding the entrance hole constructed by male *M. mutatus* in *P. canadensis*. Volatiles emitted from the frass and entrance hole attract individuals of the opposite sex

Santoro (1963) reported that *M. mutatus* emerge primarily at sunrise. Observations (Gatti Liguori, unpublished results) suggested that newly emerged *M. mutatus* are susceptible to dehydration once they are outside their gallery system in the xylem. Therefore, it would be reasonable to expect that, immediately after emergence, female flight is orientated to locate males *via* the volatile plume emitted early in the morning. The response is necessary to initiate courtship and copulation.

Here, we report an analysis of the frequency profile of diurnal and nocturnal female emergence. Furthermore, volatile emission was analyzed during the morning hours when female emergence and courtship and mating occur at high frequency. Finally, the behavioral activities of candidate compounds from the volatile blend were evaluated by using the female response in a walking behavioral assay.

Methods and Materials

Biological Material Insects were collected from our Institute's plantation of *Populus canadensis* Moench and *Quercus palustris* Muenchh. located in Villa Martelli, Province of Buenos Aires, Argentina (34°22′ S, 58°30′W). Beetles were collected during emergence by using plastic traps that were specially designed to avoid antagonistic interactions (Gatti Liguori et al. 2007) and immediately sexed. Only females that emerged within a period of 3 h or less were used in laboratory experiments.

Temporal Pattern of Female Emergence The number of emerged females was recorded every 2 h from 7–23 h, between 1 and 20 October, 2006. A relative frequency chart was made based on the data collected. The times of day with the highest frequency of emergence were used to select the time to collect and analyze male volatiles.

Collection and Analysis of Male Volatiles Live *Populus alba* L., *Q. palustris* Munchh, and *Casuarina stricta* L. trees that were at least 10 years old and had a diameter at breast height (dbh) of 20 cm were artificially infested with 59, 15, and 94 virgin males, respectively. Each insect was confined in a transparent plastic jar (60×30 mm) to ensure its interaction with the tree bark surface. The date and time at which tunneling activity began was recorded.

At times during maximal female emergence, males were collected from the frass crown arrangement with entomological forceps and immediately placed in a 20-ml glass vial (Scientific Specialties Service, Inc., Baltimore, MD, and Reno, NV, USA) with a teflon-coated cap (teflon septum with glass-reinforced polypropylene resin open cap). The physical integrity of each male was checked, and the volatiles from the headspace were collected at 30°C for 30 min by using a Solid Phase Microextraction (SPME) holder and fiber covered with a Carbowax®/divinylbenzene polar phase (Supelco Inc., Bellefonte, PA, USA). This coating is specific for low-molecular weight alcohols and ketones. Samples were analyzed immediately by GC-MS (see below), yielding a volatile emission profile for each insect.

Synthetic Chemicals Sulcatone (6-methyl-5-hepten-2-one) and 3-pentanol were analytical grade (Sigma-Aldrich Co., St. Louis, MO, USA); (+)-sulcatol [(+)-6-methyl-5-hepten-2-ol] 99% chemical purity, was purchased from Pherotech Inc., Delta, B.C., Canada.

Linked Gas Chromatography–Mass Spectrometry (GC-MS) Analyses SPME fibers were analyzed by GC-MS with a Shimadzu QP 5050A instrument equipped with a non-polar, fused silica HP-1 capillary column with crossed linked methyl silicone (50 m length, 0.32 mm diameter, and 0.52 μm thickness) (Hewlett Packard, Santa Clara, CA, USA). Samples were injected in the splitless mode. Volatiles from the SPME fibers were desorbed in the injection port at 190°C for 1.5 min. The GC column was held at 60°C for 1 min, after which the temperature was programmed to increase 3°C/min up to 105°C, and then 40°C/min to 250°C where it was maintained for 5 min. The carrier gas was helium with a head pressure of 30 kPa. The MS detector was set on electron impact mode at 70 eV.

Behavioral Bioassays Walking behavior of female *M. mutatus* was evaluated in an experimental arena with a video-tracking technique (Alzogaray et al. 2000). The floor of the test arena was covered with a round piece of Whatman No. 1 filter paper (125 mm diameter; Whatman Ltd., Maidstone, UK), and a glass cover (20×20 mm) was placed in the center of the paper. Next, the filter paper and glass cover were both covered with a rectangular piece of wire mesh (100×100 mm; 1 mm mesh size). A colorless

glass ring (100 mm diameter; 50 mm high) was used to confine the insects. The glass ring and wire mesh were washed previously with 96% ethanol and kept at 250-300°C for at least 1 week. A new glass cover and filter paper were used in each replicate.

A closed-circuit video camera that provided black and white images (VC 1910; Sanyo Electrical Co., Tokyo, Japan) was suspended 22 cm over the center of the test arena. A circular fluorescent tube (22 W, OSRAM, Buenos Aires, AR) was placed 64 cm above the video camera.

An image analyzer (Videomex V, Columbus, OH, USA) received input from the video camera, converting the analog signal into digital data. The resolution was 256×192 pixels, and the acquisition and processing speed was 30 fps. The video signal colors were inverted in the monitor, therefore white objects appeared black and vice versa. The presence of insects in the arena was determined by visual contrast between the individuals (white) and the arena background (dark), and scored as the number of "ON" pixels. The area occupied by the insects was recorded by using the Multiple Zone Motion Monitor for Videomex software.

The arena image was divided into a central square (4 cm^2, 5% of the total area) and a circular outer area. The center of the glass cover was located in the center of the virtual central square. A female *M. mutatus* was placed on the wire mesh and allowed to acclimatize for 5 min before starting the bioassay. During this time, the insect moved all around the arena. Insect movement was recorded for 60 min. During the first 30 min, 20 µl of carbon dioxide-free distilled water were placed on the glass cover. Then, 20 µl of the test solution were placed on the cover by using carbon dioxide-free distilled water as carrier. Temperature varied between 25 and 30°C. Water took about 30 min to evaporate within this temperature range. The first 30 min of each test was the control, and the remaining 30 min was the experimental treatment. Thus, the occupation level of the central circle during the first 30 min (control) was compared to the occupation level during second 30 min (following the introduction of the test substance).

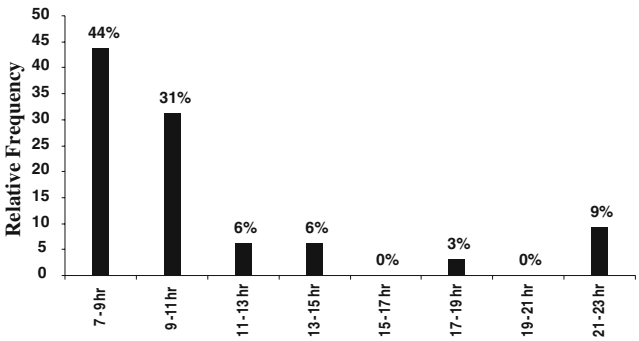

Fig. 2 Relative frequencies, expressed as percentages, of emerging female *M. mutatus* during each 2-h time interval, from 7–23 h. Samples were taken between 1 and 20, October, 2006. Total number of females was 31

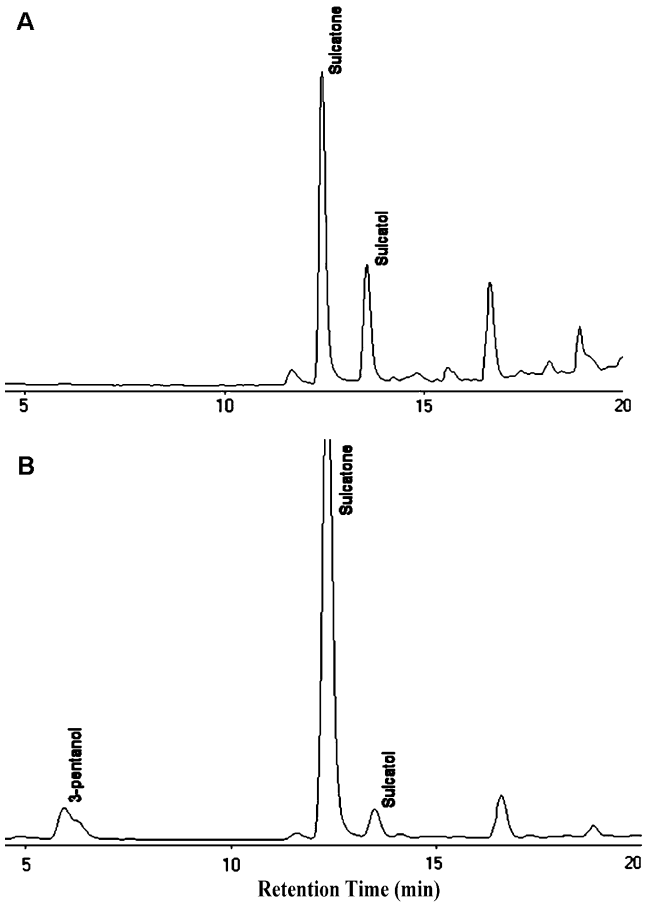

Fig. 3 GC trace of volatiles collected by SPME above a male *M. mutatus* boring in *C. stricta*. **a** GC trace representative of 91.7% of samples. **b** GC trace representative of 8.3% of the samples showing the presence of 3-pentanol in the volatile emission

For preparation of test solutions, sulcatol, sulcatone, and 3-pentanol were weighed and added to carbon dioxide free distilled water (100 ml total volume). Concentrations of stock solutions were chosen in order to obtain the required amounts of test substance in the 20 or 40 µl test aliquots. Solutions were sonicated and stored at 5°C until 1 h before they were used in the behavioral assays. At least four different concentrations of each compound were assayed. Carbon dioxide-free distilled water alone was used to evaluate the behavior of the control group. Each experiment was repeated at least eight times.

To assay the comparative responses to (+)-sulcatol and sulcatone (binary mixture) versus (+)-sulcatol, sulcatone, and 3-pentanol (ternary mixture), 40 µl of solution containing 22 µg of each component were tested in the same conditions. Each experiment was replicated al least 16 times.

We used the Central Area Occupation (CAO) parameter, defined as the total number of "ON" pixels in the central circle (where the test compound is placed) during a

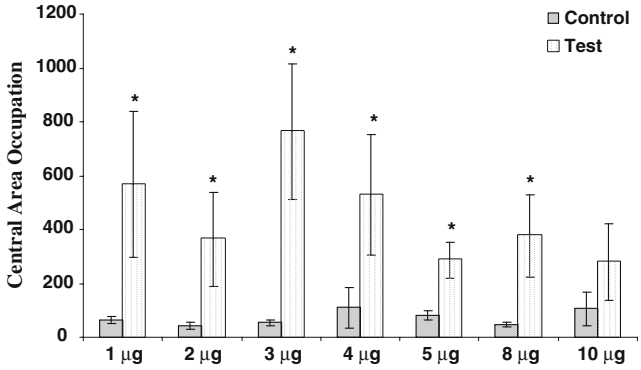

Fig. 4 Response of female *M. mutatus* (mean±SE) measured as the Central Area Occupation (=number of *on* pixels) for each dose of 3-pentanol compared to its respective control. Each test was replicated at least eight times. *Indicates significant difference compared to control values ($P \leq 0.05$)

replicate (Fontán et al. 2002), to quantify insect behavior. A mean CAO value was obtained for each treatment and compared to its respective control.

Statistical Analysis Data from the behavioral assay were analyzed by non-parametric Kruskal–Wallis ANOVA (STATISTICA'99 1999). Edition software by StatSoft, Inc (Kernel Release 5.5 A ©1984–1999).

Results

Temporal Pattern of Female Emergence Analyses of female emergence with time of day showed that 75% of the females left the gallery between 7 and 11 h (Fig. 2). Based on these results, we performed our analysis of male volatiles by using males collected during the first hours of this interval, hypothesizing that pheromone emission would be in progress at this time.

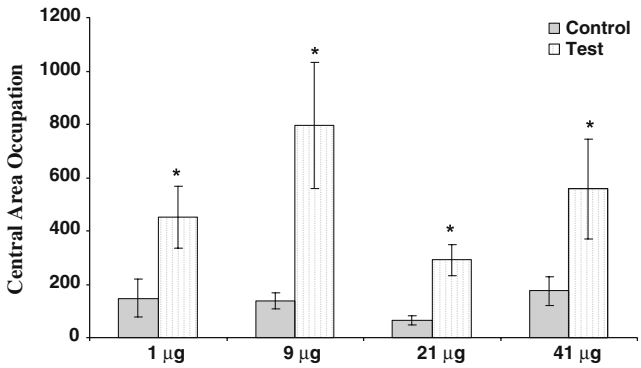

Fig. 5 Response of female *M. mutatus* (mean ± SE) measured as the Central Area Occupation (=number of *on* pixels) for each dose of (+)-sulcatol compared to its respective control. Each test was replicated at least eight times. *Indicates significant difference compared to control values ($P \leq 0.05$)

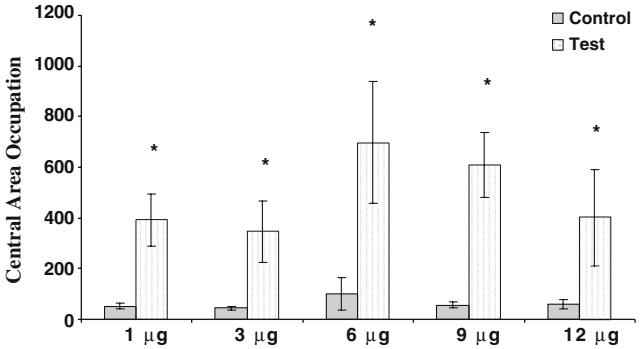

Fig. 6 Response of female *M. mutatus* (mean±SE) measured as the Central Area Occupation (=number of *on* pixels) for each dose of sulcatone compared to its respective control. Each test was replicated at least eight times. *Indicates significant difference compared to control values ($P \leq 0.05$)

Collection and Analysis of Male Volatiles GC-traces showed that (+)-sulcatol and sulcatone were found in 91.7% of the male samples collected on *P. alba*, *Q. palustris*, and *C. stricta* trees between 7 and 11 AM. However, an additional peak was detected in 8.3% of the samples (Fig. 3). This peak was identified as 3-pentanol by comparison of its retention time and mass spectrum with an authentic standard. Although there was considerable variability among insects, we estimated a mean relative amount of 3-pentanol as 13.9±6.4%, whereas sulcatone and sulcatol were 34.9±9.3% and 51.2±10.7%, respectively. 3-Pentanol was not found in any of the system blanks.

Behavioral Bioassays Results were analyzed based on the Central Area Occupation (CAO) parameter. Significant occupation of the central area can be interpreted as an effective attraction to the source followed by an arrestment in the area (Fontán et al. 2002).

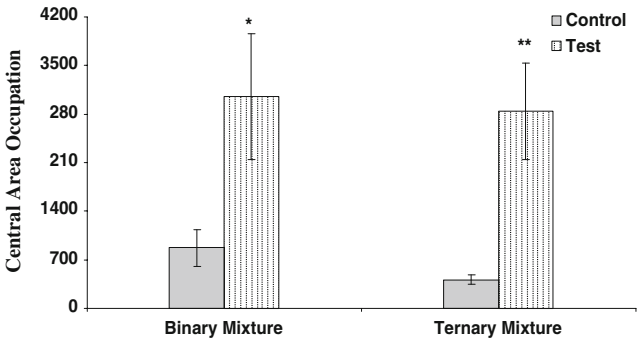

Fig. 7 Response of female *M. mutatus* (mean±SE) measured as the Central Area Occupation (=number of *on* pixels) for the binary mixture and the ternary mixture compared to their respective controls. The binary mixture contained 22 µg each of (+)-sulcatol and sulcatone. The ternary mixture contained 22 µg each of (+)-sulcatol, sulcatone, and 3-pentanol. *Indicates significant difference compared to control values ($P \leq 0.05$). **Indicates significant difference compared to control values ($P \leq 0.001$). Controls were not significantly different

CAO values of female *M. mutatus* exposed to different concentrations of 3-pentanol revealed that doses between 1 and 8 μg elicited a significant behavioral response ($P \leq 0.05$) when compared with their respective controls (Fig. 4). Thus, females were attracted to the stimulus source. Only the highest concentration (10 μg) failed to elicit any significant response from females. In this case, the value of CAO was not significant differently from its control ($P = 0.07$). This can be interpreted as a repellence or saturation phenomenon that attracts the insects to the plume, but then repels them when they get closer.

The values of CAO for (+)-sulcatol were different from their respective controls for all doses tested ($P \leq 0.05$). Therefore, a significant attraction to the source was observed whenever the insects were exposed to (+)-sulcatol (Fig. 5). There were no differences among the different concentrations ($P > 0.05$).

The values of CAO for sulcatone were different from their respective controls ($P \leq 0.05$) at all doses tested. Thus, the insects were significantly attracted to the source in all cases of exposure to sulcatone (Fig. 6). No differences were observed among the different concentrations ($P > 0.05$).

In the assay to evaluate the role of 3-pentanol in the pheromone blend, the values for CAO for the binary mixture [(+)-sulcatol+sulcatone] were not significantly different from those of the ternary mixture [(+)-sulcatol+ sulcatone+3-pentanol] (Fig. 7).

Discussion

Our results showed that during the assay period (1 to 20 October), female *M. mutatus* emerge preferably between 7 and 11 AM. This preference could be due to this insect's particular susceptibility to dehydration (Gatti Liguori, unpublished results).

Chemical analyses of volatiles emitted by male *M. mutatus* between 7 and 11 AM showed that the extracts were composed primarily of sulcatone and (+)-sulcatol. However, an additional compound, 3-pentanol, was identified in a small percentage of samples. Walking behavioral bioassays with video image analysis showed that at the doses tested, 3-pentanol, (+)-sulcatol, and sulcatone each elicited an attractive response from female *M. mutatus*. As no significant differences in response were observed among the different concentrations, we cannot conclude whether one of the concentrations was more attractive than the others under the current the experimental conditions. The assay to compare the attractiveness of the two vs. three component mixtures did not show significant differences in their behavioral activity. So, although we have demonstrated that 3-pentanol is attractive to *M. mutatus* females, we were not able to address its role in the sex pheromone blend.

3-Pentanol has been described as a minor pheromonal component in West Indian sugarcane weevils, *Metamasius hemipterus sericeus* (Oliv.) (Perez et al. 1994), an insect pest of bananas, pineapples, palms, and sugarcane in Central and South America, the Caribbean, and Africa (Vaurie 1966). It has also been described in volatile emissions from the metasternal glands of *Triatoma infestans* (Klug), the etiological agent of Chagas disease (Manrique et al. 2006). For *M. mutatus*, we hypothesize based on the superior response to the ternary mixture with respect to its control ($P < 0.001$ vs. $P = 0.01$ for the binary mixture with the same test), that 3-pentanol could diminish the uncertainty in the process of location of the pheromone source (Witzgall and Arn 1990) and/or arrest the *M. mutatus* female at the point of origin of the plume (i.e., the male in the natural system).

Our efforts are now aimed at testing the role of 3-pentanol combined with (+)-sulcatol and sulcatone in the attraction of female *M. mutatus* in the field. We are also investigating whether the male proctodeum is the site of pheromone storage and/or production. We are evaluating the attractant activity of this male tissue compared to a blend of synthetic pheromone components, and are conducting an exhaustive chemical analysis of this tissue for minor pheromonal components.

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References

ACHINELLI, F. G., LILJERSTHRÖM, G., APARICIO, A., DELGADO, M., JOUANNY, M., and MASTRANDREA, C. 2005. Daños por taladrillo (*Megaplatypus mutatus* (= *Platypus sulcatus*)) en plantaciones de álamo (*Populus* spp.) de Alberti, Buenos Aires: análisis preliminar de la magnitud y distribución de fustes quebrados. *Rev. Asoc. Ftal. Arg*. 59:8–11. (In Spanish).

ALFARO, R., HUMBLE, L. M., GONZALEZ AUDINO, P., VILLAVERDE, R., and ALLEGRO, G. 2007. The threat of the ambrosia beetle *Megaplatypus mutatus* (Chapuis) [=*Platypus mutatus* Chapuis] to world poplar resources. *Forestry* 80:471–479.

ALZOGARAY, R. A., FONTÁN, A., and ZERBA, E. 2000. Repellency of deet to nymphs of *Triatoma infestans*. *Med. Vet. Entomol.* 14:6–10.

FONTÁN, A., GONZÁLEZ AUDINO, P., MARTÍNEZ, A., ALZOGARAY, R. A., ZERBA, E. N., CAMPS, F., and CORK, A. 2002. Attractant volatiles released by female and male *Triatoma infestans* (Hemiptera: Reduviidae), a vector of Chagas disease: chemical analysis and behavioral bioassays. *J. Med. Entomol.* 39:191–197.

GATTI LIGUORI, P., ZERBA, E., and GONZALEZ AUDINO, P. 2007. New trap for emergent *Megaplatypus mutatus*. *Can. Entomol.* 139:894–896.

GONZALEZ AUDINO, P., VILLAVERDE, R., ALFARO, R., and ZERBA, E. 2005. Identification of volatile emissions from *Platypus mutatus* (=*sulcatus*) (Coleoptera: Platypodidae) and their behavioral activity. *J. Econ. Entomol*. 98:1506–1509.

MANRIQUE, G., VITTA, A., FERREIRA, R., ZANI, C., RIKARD, UNELIUS, C., LAZZARI, C., DIOTAIUTI, L., and LORENZO, M. 2006. Chemical communication in Chagas disease vectors. Source, identity, and potential function of volatiles released by the metasternal and Brindley's glands of *Triatoma infestans* adults. *J. Chem. Ecol.* 32:2035–2052.

MILLIGAN, R. H., and YTSMA, G. 1988. Pheromone dissemination by male *Platypus apicalis* White and *P. gracilis* Broun (Col., Platypodidae). *J. Appl. Ent.* 106:113–118.

PEREZ, A. L., CAMPOS-PIEDRA, Y., CHINCHILLA, C. M., OEHLSCHLAGER, A. C., GRIES, G., GRIES, R., CASTRILLO, G., GIBLIN-DAVIS, R. M., PEÑA, J. E., DUNCAN, R. E., GONZALEZ, L. M., PIERCE, Jr, H. D., MCDONALD, R., and ANDRADE, R. 1995. Aggregation pheromones and host kairomones of the West Indian sugarcane weevil, *Metamasius hemipterus sericeus* (Oliv.) (Coleoptera: Curculionidae). *J. Chem. Ecol.* 23:869–888.

SANTORO, H. F 1963. Bioecología de *Platypus sulcatus* Chapuis (Coleoptera: Platypodidae). *Revista de Investigaciones Forestales* 4:47–79. (In Spanish).

STATISTICA'99, 1999. StatSoft, Inc., Kernel Release 5.5 A ©1984–1999. Tulsa, OK (USA).

VAURIE, P. 1966. A revision of the Neotropical genus *Metamasius* (Coleoptera: Curculionidae, Rhynchophorinae). Species groups I and II. *Bull. Am. Mus. Nat. Hist.* 131:213–337.

WITZGALL, P., and ARN, H. 1990. Direct measurement of the flight behavior of male moths to calling females and synthetic pheromones. *Zeitschrift für Naturforschung* 45c:1067–1069.

WOOD, S. L. 1993. Revision of the genera of Platypodidae (Coleoptera). *Great Basin Nat.* 53:259–281.

WOOD, S. L. 2007. Bark and Ambrosia Beetles of South America (Coleoptera, Scolytidae). Brigham Young University, M.L. Bean Life Science Museum, Provo, Utah, 900 pp.

Nelson et al. 2001; Copren et al. 2005). Finally, it is difficult to distinguish *Reticulitermes* taxa solely on the basis of morphology, particularly if only workers are available.

Given this problem, numerous investigators have employed chemical characters, such as cuticular hydrocarbons (CHCs) and soldier defense secretions (SDSs), for distinguishing taxa of *Reticulitermes* (Howard et al. 1978, 1982; Parton et al. 1981; Zalkow et al. 1981; Baker et al. 1982; Clément et al. 1985, 1986, 1988; Bagnères et al. 1988, 1990, 1991; Lemaire et al. 1990; Haverty et al. 1996a, 1999b; Haverty and Nelson 1997; Clément and Bagnères 1998; Takematsu 1999; Takematsu and Yamaoka 1999; Nelson et al. 2001; Page et al. 2002; Quintana et al. 2003). Studies that correlate mitochondrial DNA sequences with CHC phenotypes of *Reticulitermes* give additional credibility to the value of CHCs as taxonomic characters (Jenkins et al. 2000; Clément et al. 2001; Copren et al. 2005).

We have successfully used CHCs and SDSs to separate taxa in studies on the foraging ecology of *Reticulitermes* in northern California (Getty et al. 1999a; Haverty et al. 1999c, 2000) and to evaluate baiting technology for control of *Reticulitermes* in structures (Getty et al. 1999b, 2000b). This chemotaxonomic information has also been critical for studies of behavior of *Reticulitermes* (Haverty et al. 1999a, 2003; Getty et al. 2000a; Delphia et al. 2003; Copren 2004; Copren et al. 2005).

Recently, we expanded our studies to locations in southern California to fill in gaps in the biogeography of *Reticulitermes* and to facilitate evaluation of bait technology. The study reported here includes more extensive collections and a complete description of the phenotypic differences first reported in Haverty and Nelson (1997), Nelson et al. (2001), and Copren et al. (2005). We also characterized SDSs for the CHC phenotypes found in southern California, and we discuss the state of current taxonomic research on *Reticulitermes*.

Methods and Materials

Insects Termites were collected from monitoring stations or naturally infested wood at locations in Los Angeles, Orange, Riverside, San Bernardino, San Diego, and Santa Barbara counties in southern California (Table 1). In San Bernardino County, we collected at the type locality of *R. hesperus*, Little Bear Lake, San Bernardino Mountains, CA (Snyder 1949). This area is now known as Lake Arrowhead (Robinson 1989). In addition, we include information from collections used in our study of the CHCs of *Reticulitermes* from northern California (Table 1) (Haverty and Nelson 1997).

Once separated from wood and debris, samples of up to 200 termite workers were placed in 20-ml scintillation vials and kept at −15°C until extraction of CHCs. Termite soldiers (1–10) were placed in *n*-pentane (ca 200–600 µl) in 4-ml vials and stored at −15°C until analysis of SDSs. Voucher specimens for each colony were placed in 70% ethanol and are maintained by the authors.

Cuticular Hydrocarbons Frozen termite workers were thawed, dried at ca 60°C, and immersed in 10 ml of *n*-hexane for 10 min. After extraction, hydrocarbons were separated from other compounds by pipetting the extract through 4 cm of activated silica gel (70–230 mesh), in Pasteur pipet mini-columns, followed by elution with 5 ml of *n*-hexane. The solvent was removed under a stream of nitrogen, and the residue was redissolved in 60 µl of *n*-hexane for gas chromatography-mass spectrometry (GC-MS) analysis.

GC-MS analyses were performed on a Hewlett-Packard (HP) 5890 gas chromatograph interfaced with an HP 5970B Mass Selective Detector. The GC-MS was equipped with an HP-1 fused silica capillary column (25 m×0.2 mm ID). Split injection (split ratio of 8:1) and a temperature program of 200–320°C at 3°C min^{-1} were used in the analysis. Electron impact (EI) mass spectra were obtained at 70 eV. Samples collected in 2001 and 2002, numbered SC-182-244 (Table 1), were analyzed under the same conditions with an Agilent 6890 GC coupled with a 5973 MSD.

n-Alkanes and methyl-branched alkanes were identified by mass spectral fragmentation patterns (Blomquist et al. 1987; Nelson 1993; Page et al. 1997). Alkenes, alkadienes, and alkatrienes were identified by mass spectra, but double bond positions were not determined (see Haverty and Nelson 1997). Equivalent chain lengths (ECL) were calculated for some of the unsaturated compounds, so as to distinguish isomers.

In the text and tables, we use shorthand nomenclature to identify individual hydrocarbons or mixtures of hydrocarbons. This shorthand uses a descriptor for the total number of carbons (CXX) in the hydrocarbon component, excluding the methyl branch(es), the location of methyl groups (X-me), and the number of double bonds following a colon (CXX:Y). Thus, normal pentacosane is *n*-C25; 5-methylpentacosane is 5-meC25; 5,17-dimethylheptacosane is 5,17-dimeC27; and pentacosatriene is C25:3. Integration of the total ion chromatogram was performed by using HP Chemstation data analysis software. GC-MS peak areas were converted to percentages of the total hydrocarbon fraction. A summary of the relative amounts of each peak is presented in table form using all samples of each phenotype.

CHC phenotypes are identified by a prefix that indicates geographic region from where specimens were collected, and a suffix indicating the distinct phenotype for that geographic region. In Haverty and Nelson (1997), northern California phenotypes were labeled with a single letter to

Table 1 Collection localities for *Reticulitermes* samples

Sample	County	Locality	Date collected	Latitude (°N)	Longitude (°W)	Elevation (m)
Phenotype SC-A						
SC-104	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-106	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-107	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-108	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-109	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-110	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-114	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-184	Santa Barbara	Goleta	9/24/2001	34.443945	119.843309	18
SC-186	Santa Barbara	Goleta	10/31/2001	34.443945	119.843309	18
SC-207	Santa Barbara	Goleta	1/28/2002	34.443945	119.843309	18
SC-221	Santa Barbara	Goleta	5/22/2001	34.443945	119.843309	18
SC-222	Santa Barbara	Goleta	5/22/2001	34.443945	119.843309	18
SC-236	Santa Barbara	Goleta	3/25/2002	34.443945	119.843309	18
SC-111 (SCA2)[a]	Santa Barbara	Goleta	7/20/2000	34.450375	119.831185	24
SC-112	Santa Barbara	Goleta	7/20/2000	34.450375	119.831185	24
SC-113	Santa Barbara	Goleta	7/20/2000	34.450375	119.831185	24
SC-182	Los Angeles	Baldwin Park	9/27/2001	34.086216	117.978931	98
SC-208	Los Angeles	Baldwin Park	1/29/2002	34.086216	117.978931	98
SC-209	Los Angeles	Burbank	3/7/2002	34.174138	118.322956	169
SC-210	Los Angeles	Burbank	3/7/2002	34.174138	118.322956	169
SC-211	Los Angeles	Burbank	3/7/2002	34.174138	118.322956	169
SC-204	Los Angeles	Burbank	1/15/2002	34.168448	118.334144	171
SC-213	Los Angeles	Burbank	3/7/2002	34.168448	118.334144	171
SC-242	Los Angeles	Chatsworth	3/27/2002	34.257484	118.614576	291
SC-241	Los Angeles	Los Angeles	3/15/2002	34.091573	118.196865	200
SC-124 (SCA5)[a]	Los Angeles	Los Angeles	8/22/2000	34.093894	118.198291	222
SC-185	Orange	Irvine	10/9/2001	33.678302	117.757289	63
SC-205	Orange	Irvine	1/15/2002	33.678302	117.757289	63
SC-228	Orange	Trabuco Canyon	5/22/2001	33.663889	117.589444	317
SC-ALP2	San Diego	Alpine	4/29/1998	32.835000	116.765556	562
SC-ALP3	San Diego	Alpine	4/29/1998	32.835000	116.765556	562
Phenotype SC-B						
SC-105 (SCB2)[a]	Santa Barbara	Goleta	7/20/2000	34.443945	119.843309	18
SC-231	Santa Barbara	Goleta	3/25/2002	34.443945	119.843309	18
SC-232	Santa Barbara	Goleta	3/25/2002	34.443945	119.843309	18
SC-101	Santa Barbara	Carpinteria	7/20/2000	34.395631	119.517370	8
SC-206	Santa Barbara	Carpinteria	1/28/2002	34.395631	119.517370	8
SC-225	Santa Barbara	Carpinteria	5/22/2001	34.395631	119.517370	8
SC-239	Santa Barbara	Carpinteria	3/25/2002	34.395631	119.517370	8
SC-103	Santa Barbara	Carpinteria	7/20/2000	34.395780	119.517548	8
SC-227	Santa Barbara	Carpinteria	5/22/2001	34.395780	119.517548	8
SC-212	Los Angeles	Burbank	3/7/2002	34.174138	118.322956	169
SC-219	Los Angeles	Chatsworth	3/10/2002	34.257484	118.614576	291
SC-119	Los Angeles	El Monte	8/3/2000	34.078531	118.024463	91
SC-120	Los Angeles	El Monte	8/3/2000	34.078531	118.024463	91
SC-121	Los Angeles	El Monte	8/3/2000	34.078531	118.024463	91
SC-123 (SCB4)[a]	Los Angeles	Los Angeles	8/22/2000	34.093894	118.198291	222
SC-200	Los Angeles	Los Angeles	12/7/2001	34.093894	118.198291	222
SC-244	Los Angeles	Los Angeles	8/18/2002	34.092844	118.199101	202
SC-229	Los Angeles	Los Angeles	5/17/2001	34.089372	118.199912	194
SC-215	Los Angeles	Los Angeles	3/19/2002	34.089382	118.199431	202
SC-218	Los Angeles	Los Angeles	3/18/2002	34.087747	118.204024	176
SC-202	Los Angeles	Malibu	1/2/2002	34.104218	118.858461	549
SC-203	Los Angeles	Malibu	1/2/2002	34.070140	118.894261	351
SC-189	San Bernardino	Rialto	11/14/2001	34.092112	117.382250	361

Table 1 (continued)

Sample	County	Locality	Date collected	Latitude (°N)	Longitude (°W)	Elevation (m)
SC-190	San Bernardino	Rialto	11/14/2001	34.092112	117.382250	361
SC-196	San Bernardino	Rialto	12/4/2001	34.092112	117.382250	361
SC-197	San Bernardino	Rialto	12/4/2001	34.092112	117.382250	361
SC-198	San Bernardino	Rialto	12/4/2001	34.092112	117.382250	361
SC-SF1	San Diego	Scissors Crossing	4/29/1998	33.095350	116.475420	700
SC-SF2	San Diego	Scissors Crossing	4/29/1998	33.095350	116.475420	700
SC-TP	San Diego	Torrey Pines	4/30/1998	32.920736	117.254518	91
Phenotype SC-B'						
SC-MR2	Riverside	Motte Rimrock Reserve	4/30/1998	33.803356	117.261084	552
SC-MR3	Riverside	Motte Rimrock Reserve	4/30/1998	33.803356	117.261084	552
SC-MR4	Riverside	Motte Rimrock Reserve	4/30/1998	33.803356	117.261084	552
SC-PTF1	Riverside	UC Riverside Campus	4/30/1998	33.975478	117.331206	315
SC-PTF2	Riverside	UC Riverside Campus	4/30/1998	33.975478	117.331206	315
Phenotype CA-A/A'[b,c]						
SC-115 (LBL1)[a]	San Bernardino	Lake Arrowhead	8/2/2000	34.258360	117.168260	1587
SC-116	San Bernardino	Lake Arrowhead	8/2/2000	34.258360	117.168260	1587
SC-117 (LBL2)[a]	San Bernardino	Lake Arrowhead	8/2/2000	34.258360	117.168260	1587
SC-118	San Bernardino	Lake Arrowhead	8/2/2000	34.258360	117.168260	1587
CA-PPE	El Dorado	Placerville	8/14/1994	38.742110	120.744060	828
CA-Wc7	El Dorado	Placerville	9/16/1993	38.742110	120.744060	828
CA-Yq31	El Dorado	Placerville	9/16/1993	38.742110	120.744060	828
CA-Wk64	El Dorado	Placerville	10/24/1994	38.742110	120.744060	828
CA-Zp8	El Dorado	Placerville	6/20/1994	38.742110	120.744060	828
CA-Xi21	El Dorado	Placerville	8/14/1995	38.742110	120.744060	828
CA-L34	Marin	Larkspur	7/6/1995	37.928127	122.536179	59
CA-SAC	Sacramento	Sacramento	2/5/1995	38.582848	121.489305	7
CA-L55	Marin	Larkspur	6/15/1992	37.928127	122.536179	59
CA-St25	Marin	Novato	6/7/1995	38.108880	122.572440	7
CA-St63	Marin	Novato	4/16/1996	38.108880	122.572440	7
CA-St87 (CAAP1)[a]	Marin	Novato	6/6/1995	38.108880	122.572440	7
CA-F167	Marin	Novato	8/2/1995	38.091654	122.595103	44
CA-HAS	Monterey	Hastings Reserve	8/15/1990	36.408161	121.590645	600
CA-BM	Lassen	Blacks Mountain	8/27/1996	40.777460	121.192220	2182
CA-HC	Shasta	Hat Creek	8/29/1996	40.744640	121.488820	1117
Phenotype CA-B[b,c]						
CA-Xm14	El Dorado	Placerville	9/16/1993	38.742110	120.744060	828
CA-Wb36 (CAB5)[a]	El Dorado	Placerville	9/16/1993	38.742110	120.744060	828
CA-Yv34 (CAB1)[a]	El Dorado	Placerville	9/16/1993	38.742110	120.744060	828
CA-Zv31	El Dorado	Placerville	8/14/1995	38.742110	120.744060	828
CA-Wc10	El Dorado	Placerville	8/14/1995	38.742110	120.744060	828
Phenotype CA-C[b,c]						
CA-Zn11 (CAC2)[a]	El Dorado	Placerville	6/20/1994	38.742110	120.744060	828
CA-PV	El Dorado	Placerville	6/20/1994	38.742110	120.744060	828
CA-Wi74 (CAC1)[a]	El Dorado	Placerville	12/21/1994	38.742110	120.744060	828
CA-Yt2 (CAC3)[a]	El Dorado	Placerville	7/12/1995	38.742110	120.744060	828
Phenotype CA-D[b,c]						
CA-UCB	Alameda	UC Berkeley Campus	6/15/1994	37.872355	122.262462	76
CA-L5	Marin	Larkspur	7/6/1995	37.928127	122.536179	59
CA-St21	Marin	Novato	7/6/1995	38.108880	122.572440	7
CA-St116	Marin	Novato	7/6/1995	38.108880	122.572440	7
CA-St253	Marin	Novato	7/6/1995	38.108880	122.572440	7
CA-St314	Marin	Novato	7/6/1995	38.108880	122.572440	7

[a] Sequences of the cytochrome oxidase II region of mtDNA for these collections were reported in Copren et al. 2005.
[b] The cuticular hydrocarbon phenotypes for these samples are reported in Haverty and Nelson 1997, excluding samples SC-115–118.
[c] The soldier defense secretion phenotypes for these samples are reported in Nelson et al. 2001, excluding samples SC-115–118.

indicate the phenotype (e.g., A, A′, B, C, D). However, in subsequent publications, we added the prefix CA- to distinguish these phenotypes from those characterized from other regions, such as Arizona (AZ-) and Georgia (GA-). Here, we use the prefix SC- for southern California collections.

Soldier Defense Secretions Monoterpenes, sesquiterpenes, and diterpenes were identified and quantified by GC-MS (Nelson et al. 2001). GC-MS analysis was carried out with splitless injection, a 0.25 mm ID×30 m 5% phenyl–95% methylpolysiloxane capillary column, helium as carrier gas, and a temperature program of 35°C (held for 0.7 min) to 280°C at 6°C min^{-1}. Most compounds were identified by retention time and MS comparison with authentic material or components of well-characterized essential oils. In some cases, identification was by comparison with published GC-MS data only and is considered tentative (compound name in parentheses). Terpenoids in bold characters in the tables are novel reports for termite soldiers. Unknowns are designated by apparent molecular weight and an identifying suffix letter. The suffix designations for the unknowns correspond to the same unknowns reported in Nelson et al. (2001).

Quantities of each terpenoid are calculated as a percentage of total peak area from the total ion chromatogram without correction for response factors. Germacrene A, which gradually rearranges to β-elemene under GC conditions, appeared as a broad hump following the β-elemene peak. Its quantity was determined as the sum of the areas of the β-elemene peak plus the hump, minus the areas of the other peaks superimposed on the hump. Germacrene B behaved similarly (rearranging to γ-elemene) and was quantified by the same approach.

(+)-γ-Cadinene from pooled samples of various collections was purified by silica gel LC and preparative GC as described elsewhere (Kim et al. 1994) and positively identified by diffuse-reflectance Fourier-transform infrared spectroscopy by using ca 50 μg of the neat material on powdered KBr in a microcup. Its optical rotation (α_D) was measured in hexane at 20°C.

Enantiomeric identity of a number of the monoterpenes and sesquiterpenes was determined by enantioselective GC (EGC). Conditions were: 10% permethylated β-cyclodextrin-90% OV-1701 capillary column, 0.25 mm ID×30 m; temperature program of 60–80°C for monoterpenes, 90–140°C for sesquiterpenes, with a 1-°C min^{-1} temperature increase; splitless injection (0.7 min); flame-ionization detection; internal standards of *n*-undecane for monoterpenes, and *n*-tetradecane or *n*-hexadecane for sesquiterpenes. EGC analysis was done on one soldier collection from the CHC phenotype that was richest in the relevant terpene(s). Before analysis, oxygenated components were removed by passing the pentane extract through activated alumina. Enantiomer identification was by retention time comparison with authentic materials. In the case of (−)-germacrene A, pooled samples were purified as for γ-cadinene, although under preparative GC conditions only β-elemene was recovered. This was then analyzed by EGC and compared to authentic (−)-β-elemene. For brevity, enantiomeric designations are not specified in the text, although they are given in the tables.

Cluster Analysis of CHC Mixtures From Termites The percentage of each hydrocarbon peak was used as the response variable. The presence of co-eluting compounds precluded exact quantification of many individual hydrocarbons. Several samples of each of the five northern California phenotypes described by Haverty and Nelson (1997) were included in the cluster analysis (Table 1).

Cluster analysis was first performed with all the samples listed in Table 1. There was a definite division between samples that lacked 5-methylalkanes and 5,17-dimethylalkanes and those that contained these compounds. Thus, in order to simplify the analysis, samples were divided into two groups based on this distinction, which corresponds to the lineage designations of Page et al. (2002). The first group included collections that lacked 5-methylalkanes and 5,17-dimethylalkanes. The predominant compounds in these samples were internally branched monomethyl- and dimethylalkanes, which place this group in lineage I. Phenotypes in lineage I were CA-A/A′ and SC-A. The second group, lineage II, was composed of collections with abundant 5-methylalkanes and 5,17-dimethylalkanes in the CHCs. Phenotypes in this lineage were CA-B, CA-D, and SC-B/B′. We also included four samples designated as CA-C from Placerville, CA (Haverty and Nelson 1997); although this phenotype did not neatly fit into lineage I or II, these samples were grouped with the former because of the absence of 5-methylalkanes and 5,17-dimethylalkanes.

The standardized Euclidean distances were calculated separately for the samples in lineages I and II by using all hydrocarbon peaks (Takematsu and Yamaoka 1999; Haverty et al. 2005; Baker and Haverty 2007; Haverty and Nelson 2007) using R Statistical Language (R Development Core Team 2004). Results are displayed as dendrograms. Euclidean distances of 15.0 were used to define clusters that we infer represent discrete taxa within each of the two lineages.

Results

Cuticular Hydrocarbons As reported in Copren et al. (2005), the two CHC phenotypes found in southern California were designated SC-A and SC-B. These pheno-

types belong to lineages I and II, respectively (Page et al. 2002). Samples representing each phenotype often were found at the same sites, indicating sympatry. For example, at study sites in Goleta (Santa Barbara Co.) and Burbank (Los Angeles Co.), we collected samples of SC-A and SC-B from nearby monitoring stations on the same day (Table 1: SC-231 and SC-236; SC-209 and SC-212).

Figure 1 shows the dendrogram based on cluster analysis of Californian *Reticulitermes* samples belonging to lineage I. There were three main clusters. One, SC-A, is composed of 18 samples from southern California ranging from Goleta to Alpine in San Diego County (Fig. 1; Tables 1 and 2). A second cluster is composed of CA-A and CA-A′ samples from northern California, as well as the two *R. hesperus* topotype samples from Lake Arrowhead (SC-115 and SC-117), and one sample from Los Angeles (SC-241; Fig. 1; Tables 1 and 2). Three samples (CA-Yt2, CA-Zn11, and CA-Wi74), previously designated as phenotype CA-C (Haverty and Nelson 1997), clustered together (Fig. 1; Tables 1 and 2), and a fourth sample (CA-PV), also previously included in CA-C, fell out by itself in the cluster analysis, despite the fact that the predominant compounds present (C27:2; 11,15-dimeC27) were typical of phenotype CA-C. For this reason, this sample is considered phenotype CA-C.

Phenotype SC-A closely resembles phenotypes CA-A and CA-A′ from northern California (Table 2), with a few quantitative and qualitative differences. All three of these phenotypes were dominated by internally branched monomethylalkanes and dimethylalkanes, with the methyl branches usually on carbons 11 or 13. SC-A produced several monomethylene-interrupted dimethylalkanes, 9,11-dimeCXX, in small amounts; these were not seen in CA-A, CA-A′, nor in the *R. hesperus* topotype samples from Lake Arrowhead (Fig. 2; Table 2).

SC-A profiles contained the same pair of pentacosatrienes (C25:3, ECL 25.98 and 26.07) as CA-A′, although the relative proportions differed (Table 2). Most samples from southern California produced more of the later eluting isomer (ca 1:7; Fig. 2a), whereas CA-A′ produced approximately equal amounts of both (Fig. 2b). Topotype samples from Lake Arrowhead had more of the earlier eluting isomer (ca 4:1; Fig. 2c). CA-A lacked these two compounds (Fig. 2d).

The one sample (SC-241) from Los Angeles that clustered with the northern California phenotypes had very small amounts of 9,11-dimethylalkanes, which may have caused it to cluster as it did (Fig. 1). However, we designate this sample as SC-A based on the presence of these compounds and the proportion of pentacosatrienes.

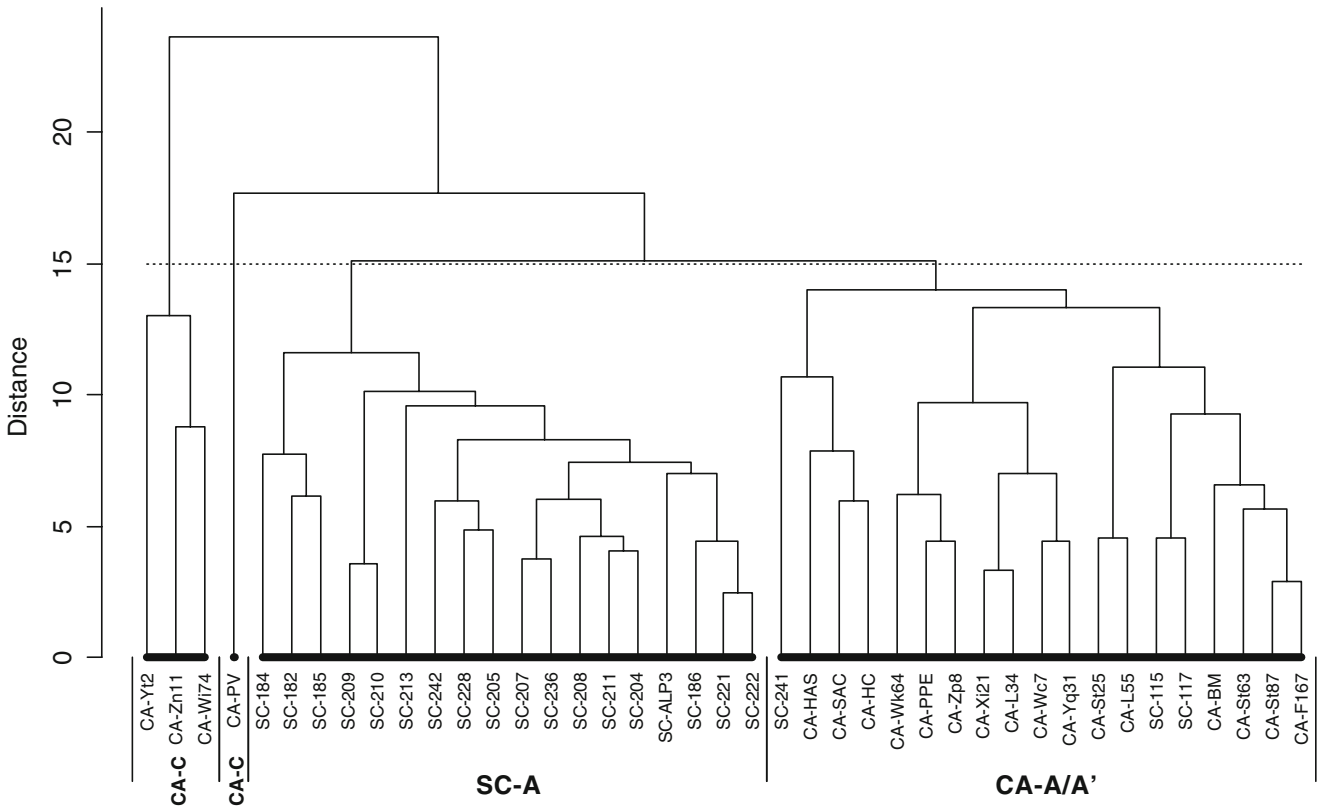

Fig. 1 Dendrograms from cluster analyses based on Euclidean distance of cuticular hydrocarbons extracted from samples of *Reticulitermes* workers from California. These samples lack 5-methylalkanes and 5,X-dimethylalkanes. See Tables 1 and 2 for data pertaining to individuals and phenotypes

Table 2 Mean percent composition (and SD) of cuticular hydrocarbons from all clusters

Hydrocarbon[a]	SC-A *N*=18	CA-A/A' *N*=16	CA-C *N*=4	SC-B *N*=23	SC-B' *N*=4	CA-B *N*=5	CA-D *N*=6
C21			0.05 (0.06)				
C22		0.08 (0.11)	0.21 (0.19)				
2-; 3-meC22		0.03 (0.06)	0.11 (0.07)				
C23:1 (ECL=22.70)		0.16 (0.27)					0.22 (0.04)
C23	2.67 (0.97)	3.85 (1.14)	1.11 (0.28)	0.86 (0.49)	0.54 (0.03)	0.23 (0.05)	3.59 (0.75)
11-; 9-meC23	0.91 (0.34)	2.79 (0.70)	0.13 (0.09)				1.66 (0.20)
9,11-dimeC23	**0.97 (0.38)**	**0.03 (0.13)**					
7-meC23				0.40 (0.38)			0.18 (0.02)
5-meC23				0.02 (0.02)			0.42 (0.10)
2-meC23	0.53 (0.17)	1.67 (0.46)	1.28 (0.19)	0.07 (0.05)	0.48 (0.27)		0.48 (0.06)
3-meC23; 9,13-dimeC23[b]	1.02 (0.36)	1.54 (0.37)	0.82 (0.21)	0.05 (0.04)	0.19 (0.23)		0.55 (0.08)
C24	0.87 (0.37)	1.83 (0.68)	1.68 (0.85)	0.71 (0.39)	1.05 (0.15)	0.67 (0.16)	1.26 (0.26)
12-; 11-; 10-; 9-meC24	1.39 (0.32)	2.31 (0.53)	0.46 (0.08)		0.06 (0.11)	0.15 (0.07)	1.94 (0.22)
9,11-dimeC24	0.20 (0.07)						
8-meC24				0.25 (0.28)			
6-meC24				0.16 (0.15)			0.54 (0.10)
5-meC24				0.23 (0.22)	1.22 (0.23)	0.26 (0.10)	0.54 (0.10)
2-meC24; C25:2; C25:1; 9,13-dimeC24; 3-meC24[c]	6.87 (1.33)	7.85 (1.60)	8.82 (1.49)	3.14 (1.57)	4.84 (0.89)	1.00 (0.28)	3.75 (0.71)
6,16-dimeC24				0.13 (0.11)			
C25:1 (ECL=24.70)	0.13 (0.11)	0.02 (0.08)		0.07 (0.06)			
C25:1 (ECL=24.80)	0.18 (0.15)	0.03 (0.12)		0.05 (0.09)			
C25	5.86 (1.83)	7.81 (2.49)	10.55 (7.16)	6.29 (2.13)	7.76 (1.26)	4.96 (1.22)	5.81 (1.04)
13-; 11-; 9-meC25; C25:2[d]	28.56 (4.87)	24.94 (5.39)	8.75 (1.07)	4.75 (2.95)	11.91 (1.65)	4.10 (1.13)	21.17 (1.33)
7-meC25; 11,13-dimeC25[e]			0.83 (0.22)	2.48 (1.74)	1.77 (2.07)	0.17 (0.05)	1.59 (0.10)
C25:2 (ECL=25.50)	0.80 (0.23)	1.17 (0.92)					
5-meC25				11.30 (2.78)	11.22 (2.66)	7.78 (0.51)	7.65 (1.79)
9,11-dimeC25; C25:2	**1.26 (0.84)**	**0.02 (0.09)**					
9,11,13-trimeC25	**0.52 (0.33)**						
11,15-; 9,13-dimeC25; 2-meC25[f]	12.03 (3.52)	8.86 (2.20)	4.14 (2.38)	0.97 (0.31)	2.19 (0.42)	1.03 (0.13)	2.71 (0.61)
3-meC25	4.16 (0.74)	4.49 (1.11)	4.84 (3.32)	2.02 (0.69)	1.82 (0.43)	1.81 (0.18)	2.46 (0.26)
7,17-dimeC25				0.89 (0.51)			
5,15-; 5,9-dimeC25					**3.22 (0.20)**	**1.96 (0.19)**	
5,17-dimeC25				**7.68 (5.55)**	**1.00 (0.19)**	**1.10 (0.18)**	**12.54 (2.71)**
C26:1 (ECL=25.70)		0.08 (0.10)					
C25:3 (ECL=25.98)[g]	**1.36 (0.43)**	**2.75 (2.71)**		**1.44 (2.96)**	**1.00 (0.18)**		
C26		0.25 (0.34)	1.48 (0.68)		0.66 (0.25)	1.36 (0.31)	
3,7-dimeC25		0.11 (0.15)		0.41 (0.19)			0.08 (0.20)
C25:3 (ECL=26.07)[g]	**9.47 (3.60)**	**3.42 (3.94)**		**0.13 (0.22)**	**0.61 (0.05)**		**2.66 (1.55)**
5,9,17-trimeC25				0.57 (0.28)	0.88 (0.14)		0.39 (0.18)
C27:1 (ECL=26.30)	0.14 (0.09)			0.02 (0.03)			
13-; 12-; 11-meC26; C26:2	0.50 (0.08)	0.50 (0.18)	1.98 (0.73)	0.14 (0.13)	0.68 (0.11)	1.22 (0.16)	1.09 (0.05)
C25:3 (ECL=26.47)	0.33 (0.31)	0.20 (0.23)		0.12 (0.33)			
6-meC26				1.09 (0.26)	1.57 (0.19)	1.79 (0.14)	0.99 (0.23)
5-meC26				0.12 (0.08)	0.25 (0.17)	0.37 (0.05)	0.10 (0.02)
4-meC26				0.15 (0.05)	0.19 (0.01)	0.26 (0.05)	0.14 (0.02)
9,13-dimeC26; 2-meC26; C27:2; C27:1[h]	0.33 (0.17)	0.39 (0.21)	11.23 (1.18)	0.47 (0.21)	1.27 (0.08)	1.50 (0.27)	0.31 (0.05)
C27:2 (ECL=26.70)				0.05 (0.05)			
3-meC26		0.04 (0.06)				0.15 (0.01)	
6,18-;5,17-dimeC26				1.35 (0.28)	1.01 (0.28)	1.79 (0.18)	0.87 (0.18)
4,16-; 4,18-dimeC26				0.27 (0.08)	0.27 (0.21)	0.43 (0.05)	0.20 (0.03)
C26:3 (ECL=26.81)		0.06 (0.08)					
C27	0.21 (0.19)	0.42 (0.27)	3.22 (1.24)	0.33 (0.17)	1.11 (0.33)	2.49 (0.51)	0.17 (0.03)

Table 2 (continued)

Hydrocarbon[a]	SC-A *N*=18	CA-A/A' *N*=16	CA-C *N*=4	SC-B *N*=23	SC-B' *N*=4	CA-B *N*=5	CA-D *N*=6
C26:3 (ECL=27.15)	0.05 (0.09)	0.06 (0.08)					
4,8,16-trimeC26				0.12 (0.08)			
13-; 11-meC27; C27:2; 11,13-dimeC27[i]	**0.17 (0.19)**	**0.74 (0.38)**	**12.89 (2.60)**	**0.66 (0.36)**	**2.22 (0.27)**	**7.93 (1.67)**	**0.71 (0.10)**
7-meC27				0.62 (0.23)	1.09 (0.08)	1.37 (0.19)	0.17 (0.02)
C27:2 (ECL=27.50)	0.04 (0.08)	0.06 (0.10)	0.62 (0.22)				
5-meC27				**2.34 (0.88)**	**3.46 (0.47)**	**4.67 (0.85)**	**0.65 (0.07)**
11,15-dimeC27; 2-meC27[j]		**0.10 (0.09)**	**6.66 (2.24)**			**0.52 (0.15)**	
3-meC27		0.10 (0.09)	0.39 (0.06)				
5,17-dimeC27				**27.32 (10.74)**	**9.84 (1.41)**	**18.45 (2.00)**	**1.51 (0.40)**
C28		0.11 (0.23)	0.40 (0.21)			0.53 (0.33)	
C27:3 (ECL=28.03)	0.41 (0.21)	0.10 (0.13)	0.27 (0.22)				0.63 (0.60)
3,7-dimeC27						0.30 (0.05)	
C27:3 (ECL=28.24)	0.43 (0.26)	0.11 (0.15)	0.59 (0.48)				0.21 (0.12)
5,9,17-trimeC27				0.71 (0.45)	0.34 (0.10)	0.85 (0.11)	0.18 (0.06)
14-; 13-; 12-;10-meC28			0.77 (0.15)			0.31 (0.06)	
11,15-dimeC28			0.35 (0.31)				
6-meC28						0.29 (0.06)	
3-meC28; C29:2; C29:1			0.28 (0.19)				
6,18-dimeC28						0.83 (0.16)	
C29:3 (ECL=28.65)	0.02 (0.05)						
C29			0.18 (0.17)			0.43 (0.20)	
15-; 13-; 11-meC29; C29:2[k]			0.44 (0.30)			0.23 (0.04)	
C29:2 (ECL=29.50)			0.22 (0.15)				
5-meC29						0.27 (0.06)	
5,17-dimeC29						0.48 (0.06)	
C30			0.12 (0.09)			0.24 (0.11)	
C31			0.06 (0.07)			0.19 (0.09)	
C32			0.04 (0.05)			0.13 (0.05)	
5,17-dimeC32				0.14 (0.14)			
C33			0.05 (0.06)			0.13 (0.04)	
13-; 11-meC33					0.08 (0.17)	1.17 (0.27)	
13,17-dimeC33					0.14 (0.29)		
5,19-; 5,17-dimeC33[l]				**4.55 (3.32)**	**4.60 (0.71)**	**2.47 (0.47)**	
14-meC34					0.13 (0.16)	0.22 (0.04)	
6,18; 5,17-dimeC34				0.55 (0.40)	1.62 (2.43)	0.43 (0.06)	
15-; 13-; 11-meC35	**1.37 (0.49)**	**2.59 (1.04)**	**1.91 (0.42)**		**2.68 (0.37)**	**1.14 (0.20)**	**0.35 (0.09)**
13,17-; 11,15-dimeC35	**0.55 (0.34)**	**3.32 (1.23)**	**0.46 (0.32)**		**1.07 (0.35)**		**0.01 (0.03)**
7-meC35							0.08 (0.03)
5-meC35							0.09 (0.03)
5,17-dimeC35				**5.16 (2.72)**	**3.94 (0.57)**	**3.61 (0.63)**	**1.68 (0.44)**
5,9,17-trimeC35				0.03 (0.15)	0.07 (0.14)	0.84 (0.16)	0.15 (0.08)
12-; 11-meC36	0.76 (0.20)	0.39 (0.12)	0.31 (0.21)	0.02 (0.08)	0.46 (0.07)	0.30 (0.04)	0.30 (0.03)
12,16-; 11,15-dimeC36	0.15 (0.14)	0.59 (0.27)					0.05 (0.04)
6,18-; 5,17-dimeC36				0.41 (0.27)	0.19 (0.18)	0.55 (0.09)	0.48 (0.14)
15-; 13-; 11-meC37	**5.07 (0.97)**	**4.15 (0.75)**	**2.18 (0.39)**	**0.03 (0.12)**	**1.63 (0.30)**	**2.54 (0.60)**	**2.91 (0.58)**
13,17-; 11,15-dimeC37	**3.84 (1.62)**	**5.10 (1.32)**	**1.21 (0.42)**		**0.72 (0.27)**		**0.78 (0.65)**
5,17-dimeC37				**2.45 (1.25)**	**1.60 (0.30)**	**3.37 (0.48)**	**2.78 (0.58)**
5,9,17-trimeC37				0.02 (0.07)	0.07 (0.13)	0.79 (0.19)	0.28 (0.05)
12-; 11-meC38	0.15 (0.12)	0.20 (0.14)	0.16 (0.11)				0.26 (0.04)
12,16-; 11,15-dimeC38	0.52 (0.12)	0.28 (0.18)	0.19 (0.13)				0.05 (0.07)
6,18-; 5,17-dimeC38				0.40 (0.35)		0.35 (0.10)	0.45 (0.12)
15-; 13-; 11-meC39	0.51 (0.21)	0.98 (0.43)	1.15 (0.19)	0.02 (0.05)	0.14 (0.16)	0.71 (0.18)	0.89 (0.17)
13,17-dimeC39	**3.36 (0.88)**	**2.04 (0.84)**	**2.16 (0.48)**		**0.26 (0.18)**		**0.47 (0.14)**
5,17-dimeC39				2.65 (1.49)	1.17 (0.19)	2.18 (0.48)	1.39 (0.27)

Table 2 (continued)

Hydrocarbon[a]	SC-A *N*=18	CA-A/A' *N*=16	CA-C *N*=4	SC-B *N*=23	SC-B' *N*=4	CA-B *N*=5	CA-D *N*=6
5,9,17-trimeC39						0.16 (0.06)	
12-; 11-meC40	0.01 (0.03)	0.04 (0.05)	0.09 (0.06)				
12,16-dimeC40	0.01 (0.03)	0.07 (0.10)	0.17 (0.12)				
5,17-dimeC40						0.16 (0.06)	0.37 (0.24)
15-; 13-; 11-meC41	0.21 (0.19)	0.26 (0.19)	0.89 (0.14)				0.49 (0.19)
13,17-dimeC41	0.39 (0.18)	0.74 (0.40)	1.88 (0.35)		0.07 (0.14)		0.72 (0.24)
5,17-dimeC41				1.00 (0.81)	0.61 (0.10)	1.92 (0.32)	3.26 (0.54)
12-; 11-meC42		0.01 (0.03)					
12,16-; 11,15-dimeC42		0.01 (0.04)					
15-; 13-; 11-meC43						0.17 (0.10)	
15-; 13-; 11-meC43	0.03 (0.06)	0.03 (0.07)	0.33 (0.22)		0.37 (0.25)		
13,17-dimeC43	0.71 (0.32)	0.10 (0.15)	0.87 (0.35)				0.43 (0.22)
5,17-dimeC43				1.59 (0.95)	2.73 (0.39)	2.20 (0.76)	1.25 (0.31)

[a] Presented in order of elution. Co-eluting compounds are listed together. *ECL* equivalent chain length (approximate). **Bold type** indicates diagnostic peaks.
[b] 9,13-dimeC23 is only present in SC-A and CA-D.
[c] 9,13-dimeC24 is present only in SC-A, CA-A/A', CA-D; only 2-meC24 is present in CA-B.
[d] C25:2 is absent in CA-C and CA-B.
[e] 11,13-dimeC25 is present only in CA-C.
[f] Only 2-meC25 is present in CA-C, SC-B and CA-B.
[g] These trienes are not found in CA-A; their absence is diagnostic for this phenotype.
[h] 9,13-dimeC26 is present only in SC-A and CA-A/A'; C27:2 and C27:1 are present only in CA-C.
[i] C27:2 is present only in CA-A/A', CA-C, and CA-D; 11,13-dimeC27 is present only in CA-C.
[j] 11,15-dimeC27 is present only in CA-A/A' and CA-C.
[k] C29:2 is present only in CA-C.
[l] 5,19-dimeC33 is present with 5,17-dimeC33 only in CA-B.

Phenotype CA-C is distinguished from phenotypes CA-A/A′ and SC-A by the presence of large quantities of C27:1, C27:2, 11-; 13-meC27, and 11,15-dimeC27 (Fig. 2e; Table 2). A phylogenetic analysis based on the COII mtDNA gene also separated this phenotype from all others reported in this paper (Copren et al. 2005). Thus far, this phenotype appears to be rare and has been found only at our site in Placerville, CA.

Cluster analysis of the lineage II *Reticulitermes* resulted in four clusters (Fig. 3), two with all the southern California samples and two with all the northern California samples. Twenty-three samples from southern California comprise phenotype SC-B, while the smaller cluster is composed of four samples we designated as SC-B′ (Table 2). The two remaining clusters included one with five samples from northern California that had been previously designated as CA-B, and one with six samples that had been previously designated as CA-D (Table 2) (Haverty and Nelson 1997).

CHC phenotype SC-B is similar to phenotypes CA-B and CA-D from northern California (Fig. 4a-c; Table 2). The most abundant compound in the majority of phenotype SC-B samples was 5,17-dimeC27, and there was a homologous series of similar compounds, with carbon chain length from 33 to 43. This pattern is comparable to that of CA-B and CA-D. However, several monomethylalkanes present in CA-B and CA-D, such as 11-meC35 and 11-; 13-meC37, were absent in most samples of SC-B (Fig. 4a–c; Table 2). CA-D produced less 5,17-dimeC27 than SC-B or CA-B, and more internally branched monomethylalkanes, such as 11- and 13-meC25 and 11- and 13-meC37 (Fig. 4a-c; Table 2).

Two variants of SC-B were seen in San Diego County. In one variant, which included samples SC-SF1 and SC-SF2, 5,17-dimeC27 was less abundant, and 5,17-dimeC33 was absent (Fig. 4d; Table 2). These two samples grouped together with another, SC-TP, within the main SC-B cluster. Closer examination of the profile of SC-TP indicated that it is more similar to the other SC-B samples in the main cluster than to the two SC-SF samples, as it contained a large amount of 5,17-dimeC27 (19.2%), and 5,17-dimeC33 was present (3.4%).

The other variant (samples SC-MR2, -3, -4, and SC-PTF) had significant quantities of internally branched mono- and dimethylalkanes with chain lengths of 35 to 39 carbons, and smaller amounts of 5,17-dimeC27 (Fig. 4e; Table 2). Because the four samples formed a separate cluster and had other distinguishing features in common, they were designated as SC-B′ and are possibly a separate taxon.

We indicate in bold type the diagnostic CHCs that distinguish the phenotype clusters in Table 2. It is noted

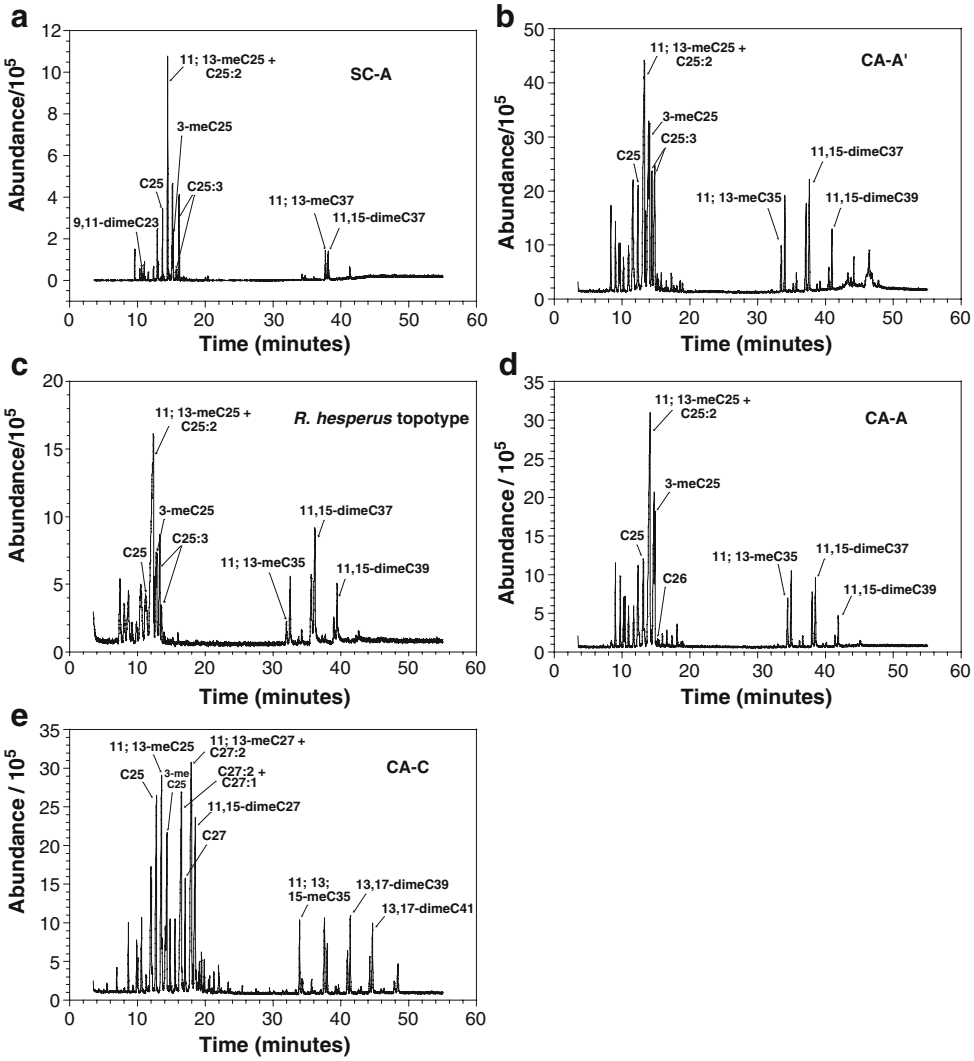

Fig. 2 Total ion chromatograms of cuticular hydrocarbons from *Reticulitermes* workers collected from **a** Goleta, Santa Barbara Co., CA (SC-A); **b** Novato, Marin Co., CA (CA-A′); **c** Lake Arrowhead, San Bernardino Co., CA (*R. hesperus* topotype); **d** Placerville, El Dorado Co., CA (CA-A); **e** Placerville, El Dorado Co., CA (CA-C)

that since phenotypes CA-A and CA-A′ clustered together, their hydrocarbon data were combined in Table 2. However, they can be distinguished easily from each other by the absence of the two isomers of C25:3 in CA-A, which is not apparent in the table, but can be seen in Fig. 2b and d. Haverty et al. (1991) proposed that if CHCs are to be reliable as taxonomic characters, they should be abundant components (at least 1%, but preferably 5% or more of the total hydrocarbon mixture). They should also be unique or present in only a few of the species, or conversely, they should be common in most of the species yet completely absent or in insignificant quantities in one or a few species. These are the criteria that we used to select the diagnostic CHCs listed in bold in Table 2.

Soldier Defense Secretions Soldiers of CHC phenotypes from southern California generally produced substantial amounts of germacrene A in the SDS (Table 3). The defense secretions of soldiers taken from colonies with CHC phenotype SC-A consisted almost entirely of germacrene A (>95%), except for one sample (SC-111) that had 79.0% germacrene A and greater amounts of γ-cadinene (15.0%) than the rest. Soldiers from the Lake Arrowhead collections (SC-115, -116, -117, and -118) produced mainly γ-cadinene (>78%) in their SDS, similar to the samples of CA-A and CA-A′ (Nelson et al. 2001). Other compounds, such as 3-octanol, *Z,E*-germacrene A, and δ-amorphene, were present in the Lake Arrowhead samples in significant amounts (0.5–9.9%, 5.0–11.5%, 1.2–2.9%, respectively), but were either absent or present in trace quantities in CA-A and CA-A′. The latter two compounds were previously identified in the SDS of CA-B soldiers (Table 3; Nelson et al. 2001).

The SDS of phenotype SC-B also contained significant amounts of germacrene A; however, there was variation in the relative quantities, ranging from 16.2% to 97.2%. Most of the samples had >62% germacrene A, along with moderate amounts of γ-cadinene (Table 3). Two collections

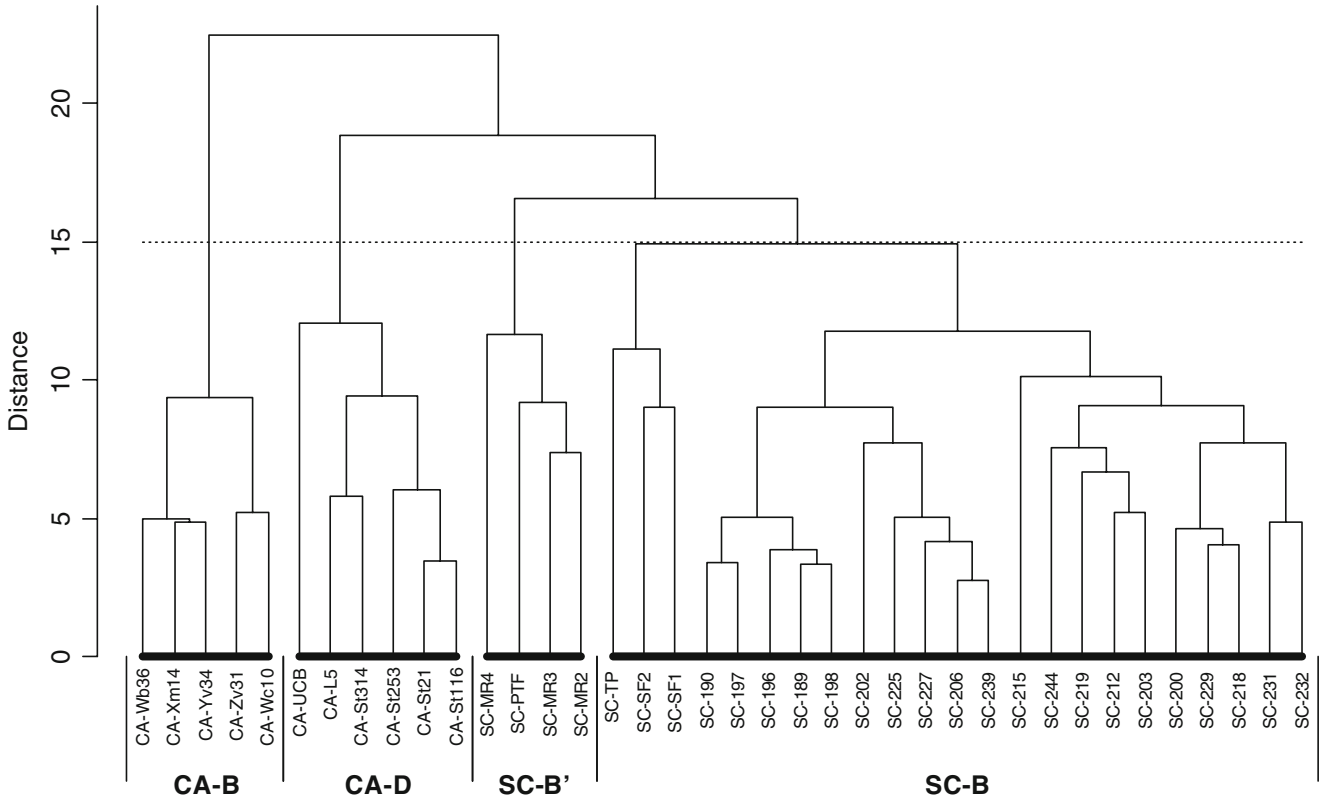

Fig. 3 Dendrograms from cluster analyses based on Euclidean distance of cuticular hydrocarbons extracted from samples of *Reticulitermes* workers from California. These samples contain 5-methylalkanes and 5,X-dimethylalkanes. See Tables 1 and 2 for data pertaining to individuals and phenotypes

from San Diego County (SC-SF1 and SC-SF2) contained much less germacrene A and substantially more geranyl linalool (Table 3). These same samples had notable differences in their hydrocarbon mixture when compared with other SC-B samples (Fig. 4a,d).

Collections from Riverside County, which were designated as hydrocarbon phenotype SC-B′, contained predominantly germacrene A (56.7–98.7%), and γ-cadinene (0.8–16.6%; Table 3). Samples MR2, MR4, and PTF1 contained modest amounts of dauca-8,11-diene and dauca-4(11),8-diene (1.4 to 5.8% and 0.3 to 2.2%, respectively). These compounds have not been reported before from *Reticulitermes* soldiers. A small amount of dauca-8,11-diene (0.6%) was observed also in SC-SF1, and dauca-4(11),8-diene was found in SC-SF1 and SC-TP. Several other components (daucene, β-santalene, zingiberene, β-sesquiphellandrene) were found in these samples as well. Small amounts of unknowns designated as 204f and 204g were seen in SC-SF1, SC-MR2, SC-MR4, SC-PTF1, and 204g was also found in SC-TP from San Diego Co. (Table 3).

Zingiberene was reported in trace amounts in *Reticulitermes* phenotype AZ-C(I) from Arizona (Nelson et al. 2001). Daucene, 3-octanol, β-santalene, (*Z*,*E*)-α-farnesene, and β-sesquiphellandrene also have not been reported previously from *Reticulitermes* soldiers, although 3-octanol was reported in pentane extracts from workers of four European *Reticulitermes* species (Reinhard et al. 2003). Nerolidol was found in one sample of SC-A (0.4%) and two samples of SC-B (<0.03%). This compound was reported previously, by Quintana et al. (2003), in soldiers of *R. lucifugus* and *R. lucifugus corsicus*.

Discussion

Before addressing the particular results of the current study, it is helpful to review the current state of taxonomic research on *Reticulitermes*.

We are troubled by several things in the literature on termite taxonomy: exclusive reliance on morphological determination of species, with use (or misuse) of inadequate keys; use of samples from the GenBank sequence database that may or may not be correctly identified to species; and misrepresentation of the value of chemical characters in delimiting species in termites. We also agree with Uva et al. (2004) who, in a study of *Reticulitermes* from southern Europe, warned against the following assumptions: (1) the classification of termites is definitive, and (2) that identification of termites can be made based on geographic origin alone. Unfortunately, these cautions, and other difficulties

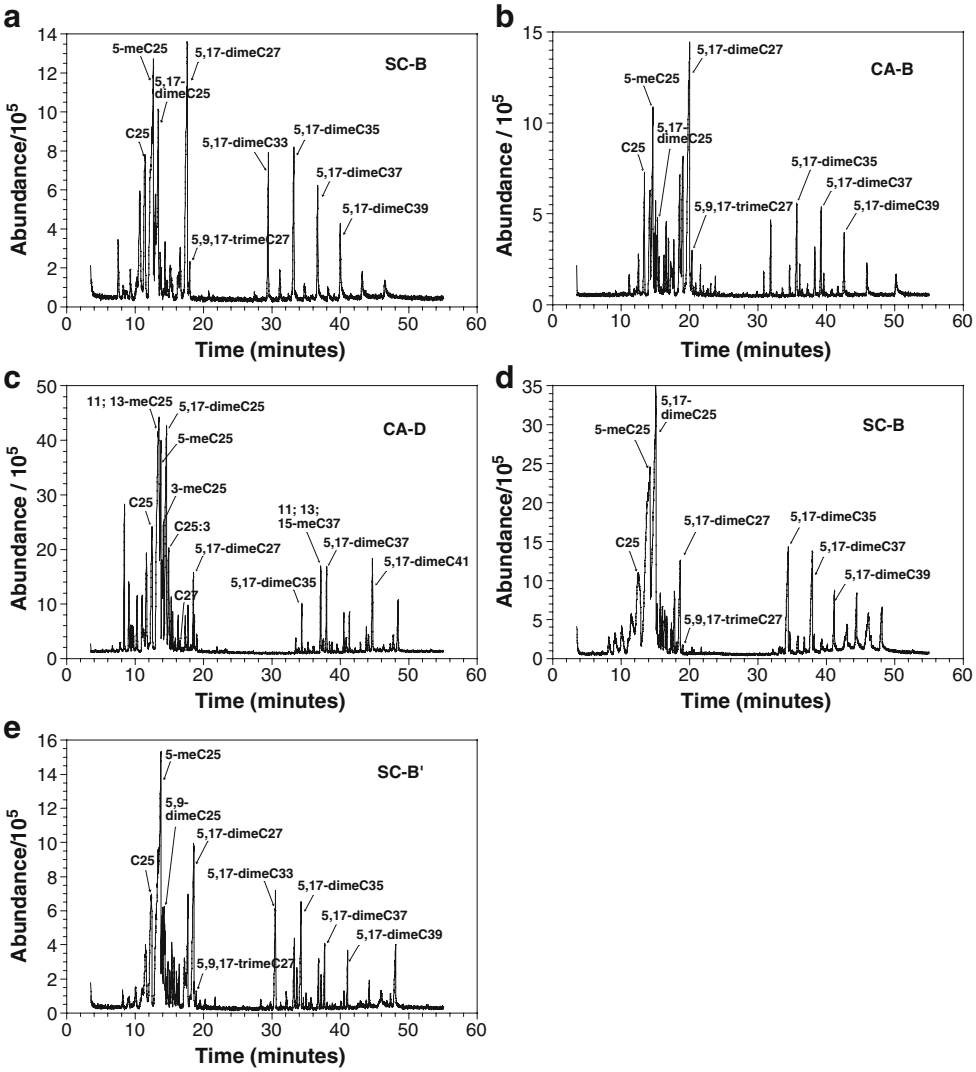

Fig. 4 Total ion chromatograms of cuticular hydrocarbons from *Reticulitermes* workers collected from **a** Carpinteria, Santa Barbara Co., CA (SC-B); **b** Placerville, El Dorado Co., CA (CA-B); **c** Novato, Marin Co., CA (CA-D); **d** Scissors Crossing, San Diego Co., CA (SC-B); **e** Motte Rimrock Reserve, Riverside Co., CA (SC-B′)

inherent in the taxonomic study of *Reticulitermes*, often have been overlooked.

Taxonomy of North American Reticulitermes Much of the biogeographical and taxonomic information on *Reticulitermes* was developed in the first half of the past century (Banks and Snyder 1920; Light 1934; Pickens 1934a, b; Banks 1946; Miller 1949; Snyder 1954). Banks and Snyder (1920) provided the first "descriptions" of species within this genus, and Snyder (1949) established the number of species of *Reticulitermes* in North America at six: *R. arenincola* Goellner, *R. flavipes* (Kollar), *R. hageni* Banks, *R. hesperus* Banks, *R. tibialis* Banks, and *R. virginicus* (Banks). Snyder (1954) reiterated that there are only six species of *Reticulitermes* in North America, and this has been affirmed by Weesner (1965, 1970) and Nutting (1990) and assumed by most termite biologists (Haverty et al. 1999b). However, the growing body of literature on the diversity of *Reticulitermes* suggests that there are more species than the six currently recognized. Our own chemotaxonomic analyses (Haverty et al. 1996a, 1999b; Haverty and Nelson 1997, 2007; Jenkins et al. 2000; Nelson et al. 2001; Copren et al. 2005) and ethological studies (Haverty et al. 1999a, 2003; Delphia et al. 2003) indicated that undescribed species of *Reticulitermes* occur in the United States. Su et al. (2006) reported two undescribed species from California, based on mtDNA sequences, as did Tripodi et al. (2006). Szalanski et al. (2006) named a new species, *R. okanaganensis*, from the western United States and Canada, based on the mtDNA 16S gene. Austin et al. (2007) provided genetic and other evidence to confirm the existence of *R. malletei*, first described by Clément et al. (1986), in Georgia and several other states. Correspondence of genetic data with those from CHC, biometric, and ethological analyses support resurrecting this species name, which had never been formally accepted. Taken together, studies like these underscore the outdated and incomplete nature of North

American *Reticulitermes* taxonomy. As Weesner (1970) put it, "...this genus is woefully in need of a critical taxonomic study..."

Identification of Reticulitermes *Specimens* Assigning insect specimens to the correct species has been done traditionally by using dichotomous keys and/or surmised by the geographic location of the collection. In practice, there are serious problems and pitfalls with both methods.

The morphological keys most commonly used and cited for identification of *Reticulitermes* species are those by Snyder (1954), Weesner (1965), and Nutting (1990). These keys are based on the original keys in Banks and Snyder (1920), with additional characters added and the acceptance of the synonomies presented by Snyder (1949). The keys developed by Scheffrahn and Su (1994) and Hostettler et al. (1995) were intended for identifying termites only from Florida, but have been cited in numerous papers as useful for identifying specimens of *Reticulitermes* from Arkansas, Louisiana, Missouri, and Virginia (Szalanski et al. 2003), Texas (Austin et al. 2004b; Foster et al. 2004), Oklahoma (Austin et al. 2004c), California (Tripodi et al. 2006), Oregon (McKern et al. 2006, 2007), and Delaware (King et al. 2007). In their genetic analysis of *Reticulitermes*, including samples from Europe, Asia, and North America, Austin et al. (2002) stated that they identified species in their study by using keys by Krishna and Weesner (1969), Scheffrahn and Su (1994), and Donovan et al. (2000). There are no keys for identification of species in Krishna and Weesner (1969), nor in Donovan et al. (2000). Furthermore, Scheffrahn and Su (1994) only have keys to three species: *R. flavipes*, *R. virginicus*, and *R. hageni*, and they restrict their focus to Florida specimens. It is unclear how specimens from China, Israel, Japan, South Korea, Arizona, or California can be identified by using these references.

In their phylogenetic analyses of the family Rhinotermitidae, Austin et al. (2004a) claimed to have identified termites collected from North America and the Caribbean by applying keys by Goellner et al. [sic] (1931), Scheffrahn and Su (1994), and Hostettler et al. (1995). Again, the last two references include termite species known to occur only in Florida. It appears that all but three samples [*R. arenicola* (sic), *Coptotermes formosanus* Shiraki, and *Heterotermes cardini*] were gathered from GenBank and not collected by the authors. It is questionable how the *H. cardini* sample was identified by using any of these keys.

In their study of the phylogeography of *Reticulitermes* in California, Tripodi et al. (2006) claim that "*R. flavipes* and *R. tibialis* were morphologically identified when alates were available by using the keys of Krishna and Weesner (1969), Banks and Snyder (1920), and Hostettler et al. (1995)." As mentioned earlier, there is no key in Krishna and Weesner (1969). The key in Banks and Snyder (1920) is timeworn, and if it is agreed that the genus needs revision, reliance on such a key is inherently problematic. The keys to *Reticulitermes* of Florida in Hostettler et al. (1995) do not include *R. tibialis*. We have serious reservations about morphological identifications of *Reticulitermes* species by using geographically inappropriate keys, i.e., that do not include all the species. Equally suspect are identifications that claim to use sources (such as Krishna and Weesner 1969 or Donovan et al. 2000) that do not contain keys to species (Austin et al. 2002, 2004b, c, 2005a; Szalanski et al. 2003; Tripodi et al. 2006, McKern et al. 2007).

A major problem with existing morphological keys to *Reticulitermes* species is the complication of the caste used for identification. If only workers of *Reticulitermes* are collected, identification to species is all but impossible, as there are no morphological keys to workers available. For imagoes (alates) and soldiers, the most common approach is to have separate keys (see Snyder 1954; Weesner 1965; Nutting 1990; Scheffrahn and Su 1994). However, the great majority of *Reticulitermes* collections involve either foraging groups, which rarely contain imagoes, or flights of imagoes (alates) without the associated soldiers. Weesner (1965) lacks a key to the soldiers for *Reticulitermes* from the Pacific Coastal region, and thus makes species identification of *Reticulitermes* foraging groups impossible. The problem is exacerbated when both castes are present in the same sample, and keys to alates indicate a species different from that indicated by the keys to soldiers (Haverty et al. 1999b; King et al. 2007).

While ignoring geography can lead to dubious identifications of specimens, uncritical reliance on the location of a collection in its identification is also hazardous. In their discussion of phylogeography of *Reticulitermes* in California, Tripodi et al. (2006) stated that their "... experience with this group shows that it consistently has been misidentified because of prejudices or assumptions about their respective distributions (Austin et al. 2002, 2005b)." Weesner (1970) gave detailed geographic information on the distribution of *Reticulitermes* species that could lead to erroneous conclusions. For example, if one collected a *Reticulitermes* sample anywhere in Arizona, USA, and referred to Weesner (1970) or Nutting (1990), the conclusion would be that it could only be *R. tibialis*. Now, we know that there are at least four CHC phenotypes of *Reticulitermes* in Arizona, that we believe represent four different species, only one (or none) of which could actually be *R. tibialis* (Haverty and Nelson 2007). Identification of *Reticulitermes* in California has proven difficult (Haverty and Nelson 1997; Tripodi et al. 2006), especially considering the possible occurrence of exotic species, such as *R. flavipes* (Kollar), or undescribed species (Haverty and Nelson 1997; Su et al. 2006; Tripodi et al. 2006). The assumed species distributions in extant keys for California would allow only two choices, *R. hesperus* or *R. tibialis*.

Table 3 Terpenoid percent composition of soldier defense secretions from *Reticulitermes* from southern and northern California[a]

Terpenoid[b]	SC-104 SC-A	SC-106 SC-A	SC-107 SC-A	SC-108 SC-A	SC-109 SC-A	SC-110 SC-A	SC-111 SC-A	SC-112 SC-A	SC-113 SC-A	SC-114 SC-A	SC-124 SC-A	SC-ALP2 SC-A
(—)-α-Pinene												
(—)-β-Pinene												
Myrcene							0.6		0.0	0.1	0.1	
Daucene												
(—)-Limonene	0.0	0.0				0.0	0.1		0.0	0.0	0.0	
(3-Octanol)	0.0	0.1	0.1	0.1	0.3	0.2	0.8	0.2	0.2	0.1	0.1	
(Z)-Ocimene	0.0	0.1				0.0	0.2		0.0	0.0	0.0	
(E)-Ocimene	0.0	0.1				0.0	0.2		0.0	0.0	0.0	
Unk. 204b												
δ−Elemene	0.0	0.1	0.1		0.1	0.1	0.4	0.1	0.1	0.1	0.1	
(+)-α-Himachalene												
(*E*)-β-Farnesene	0.1	0.4	0.2	0.1	0.2	0.1	1.2	0.2	0.2	0.2	0.7	
β−Santalene												
Unk. 204f												
Unk. 204g												
α−Humulene												
cis-Muurola-4(14),5-diene												
Unk. 204c												
γ−Himachalene					0.0							
γ−Humulene	0.0	0.1	0.1	0.0	0.1	0.1	0.8	0.2	0.1	0.0	0.3	
(—)-β-Selinene												
(—)-α-Selinene												
β−Himachalene												
(Zingiberene)												
***(Z,E)*-α-Farnesene**	0.1	0.4				0.1	1.4	0.3	0.2	0.2	0.6	
dauca-8,11-diene												
β−Bisabolene												
((*Z,E*)-Germacrene A)												
(—)-Germacrene A	98.3	95.7	98.9	98.5	96.7	97.5	79.0	95.5	96.1	98.1	90.9	99.6
δ−Amorphene												
(+)-γ-Cadinene	1.3	2.3	0.3	0.9	1.5	1.3	14.7	3.2	2.6	0.9	6.9	0.3
δ−Cadinene												
β−Sesquiphellandrene												
dauca-4(11),8-diene												
Unk. 204e												
α−Cadinene		0.1			0.0	0.0	0.2	0.1	0.1	0.0	0.3	
Nerolidol					0.4							
Germacrene B												0.0
((*E*)-γ-Bisabolene)												
(+)-γ-Cadinenal												
Unk. 272a												
Unk. 238a												
Unk. 290a												
Geranyl linalool	0.1	0.5	0.4	0.3	0.7	0.5	0.5	0.3	0.3	0.2		

[a] Values of 0.0 indicate the compound was detected, but at a level <0.05% of the total SDS.
[b] Terpenoids are listed in GC elution order. Terpenoids in **bold type** have not previously been reported for termite soldiers. Compound name in parentheses or brackets indicates identification is tentative. Unknowns are designated by an apparent molecular weight and an identifying suffix letter.
[c] The terpene phenotypes for these samples are reported in Nelson et al. 2001.

All these difficulties have led us, as well as other researchers, to investigate chemical or molecular characters as a better means of distinguishing termite taxa based on soldiers or workers (pseudergates; (Haverty and Nelson 1997; Takematsu 1999; Takematsu and Yamaoka 1999; Jenkins et al. 2000, 2001; Nelson et al. 2001; Austin et al. 2002, 2004a, b, c, 2005a, b, 2007; Szalanski et al. 2003, 2006; Foster et al. 2004; Uva et al. 2004; Ye et al. 2004; Copren et al. 2005; Su et al. 2006; Tripodi et al. 2006; King et al. 2007).

Chemical Taxonomy of Reticulitermes Howard and Blomquist pioneered the use of CHCs as taxonomic characters for delimiting species or taxa of *Reticulitermes* (Howard et al.

SC-ALP3 SC-A	SC-101 SC-B	SC-103 SC-B	SC-105 SC-B	SC-119 SC-B	SC-120 SC-B	SC-121 SC-B	SC-123 SC-B	SC-TP SC-B	SC-SF1 SC-B	SC-SF2 SC-B	SC-MR2 SC-B'	SC-MR3 SC-B'	SC-MR4 SC-B'
									0.1				0.0
0.0										0.0	0.1		
											0.4		2.5
	0.1	0.8	0.5	0.1	0.4		0.7						
	0.1		0.1	0.0	0.0		0.0						
0.3	1.6	0.3	0.6	0.1	0.1	0.1	0.1	0.8	0.2	0.2	0.2	0.1	0.5
											0.2		1.0
									0.3		0.5		1.8
								0.2	0.3		0.3		1.0
										0.1			
	0.7												
0.3	0.7	0.7	0.4	0.9	0.7	0.8	0.6	0.4	0.8	1.1	0.6		0.5
										0.1			
											0.8		2.8
	1.4	0.1	0.8	0.1									
									0.6		1.4		5.8
	1.7												
98.2	71.3	76.4	84.6	66.3	66.3	62.4	76.8	82.5	22.7	16.2	74.3	97.2	56.7
1.1	20.3	20.9	11.7	27.8	21.1	23.3	18.5	12.1	18.2	31.6	16.6	2.0	16.1
									0.3		0.5		2.2
								0.1	0.2		1.3		6.4
0.0	0.8	0.4	0.3	0.7	0.6	0.7	0.6	0.4	0.6	0.9	0.5	0.1	0.7
	0.0		0.0										
0.0											0.0	0.0	0.0
									4.1	0.1			
				2.3	10.2	7.2	1.4	3.3		10.9	1.6	0.4	0.6
	1.3	0.4	1.1	1.7	0.5	5.6	1.3	0.2	51.4	38.7	0.6	0.1	1.5

1978, 1982; Howard and Blomquist 1982). With a few exceptions, most termites have species-specific mixtures of CHCs (Kaib et al. 1991; Howard 1993; Page et al. 2002). CHCs are homologous characters; they represent hierarchical distributions of shared characters and are independent characters with discrete states (Page et al. 2002). Termites synthesize most, if not all, of their complement of CHCs de novo (Blomquist and Dillwith 1985). As such, hydrocarbon composition is an expression of a taxon's genotype, and therefore, we can assume there is a genetic basis for different hydrocarbon composition among species (Page et al. 2002).

Use of CHCs to sort specimens has led to identification of new species and has resulted in morphological descriptions supplemented by chemical data (Takematsu 1999; Takematsu

Table 3 (continued)

Terpenoid[b]	SC-PTF1 SC-B'	SC-PTF2 SC-B'	SC-115 CA-A'	SC-117 CA-A'	SC-118 CA-A'	CA-A (N=16) Mean[c]	CA-A' (N=10) Mean[c]	CA-B (N=6) Mean[c]	CA-C (N=6) Mean[c]	CA-D (N=10) Mean[c]
(—)-α-Pinene						0.1	0.0	0.0		0.0
(—)-β-Pinene	0.0					0.1	0.1	0.0	0.0	0.0
Myrcene	0.0					0.1		0.0	0.0	0.0
Daucene	1.2									
(—)-Limonene						0.1	0.0		0.0	0.0
(3-Octanol)			9.9	4.8	8.3					
(Z)-Ocimene										
(E)-Ocimene										
Unk. 204b								0.7		
δ−Elemene										
(+)-α-Himachalene								5.0		
(*E*)-β-Farnesene	0.2	0.1							0.2	0.2
β−Santalene	0.5									
Unk. 204f	1.3									
Unk. 204g	0.6									
α−Humulene			0.3	0.1	0.2			0.4		
cis-Muurola-4(14),5-diene						0.0	0.1	0.0		0.0
Unk. 204c						0.0				0.0
γ−Himachalene			0.3	0.3	0.3			13.4		
γ−Humulene	0.2		0.4	0.1		1.6	1.8	1.2	0.0	0.7
(—)-β-Selinene									0.2	0.9
(—)-α-Selinene									2.0	9.1
β−Himachalene								4.9		
(Zingiberene)	1.7									
***(Z,E)*-α-Farnesene**										
dauca-8,11-diene	3.3									
β−Bisabolene						1.1	0.9			
((*Z,E*)-Germacrene A)			5.0	11.5	9.1	0.1	0.2	6.8		
(—)-Germacrene A	76.8	98.7				1.3	1.0		92.8	33.0
δ−Amorphene			1.2	2.9	2.3			8.5		
(+)-γ-Cadinene	8.5	0.8	81.6	79.8	78.2	90.0	94.3	39.6	3.7	17.4
δ−Cadinene						0.2		0.7		
β−Sesquiphellandrene	1.4									
dauca-4(11),8-diene	3.6									
Unk. 204e								1.4		
α−Cadinene	0.4		1.0	0.6		1.6	0.8	0.8	0.0	0.3
Nerolidol										
Germacrene B										
((*E*)-γ-Bisabolene)						0.3				
(+)-γ-Cadinenal	0.2									
Unk. 272a		0.3						0.0		
Unk. 238a						0.2		0.0	0.1	
Unk. 290a										0.3
Geranyl linalool	0.0	0.1	0.4		1.7	3.1	0.8	16.7	0.7	38.0

and Yamaoka 1999). These studies examined the CHCs of six species of *Reticulitermes* from Japan, Korea, and Taiwan, observed nine different hydrocarbon phenotypes, and recommended that four subspecies be elevated to species status. They concluded that the nine species had unique compounds that can be used to separate species and that hydrocarbons are of value for classification of *Reticulitermes* species. These studies demonstrated that *R. speratus* is a complex of three species, and the investigators found new morphological characters for separating species. Similarly, important phenotypic differences in CHC profiles (Bagnères et al. 1988, 1991) and SDS composition (Parton et al. 1981; Bagnères et al. 1990; Quintana et al. 2003) were found between the different species of *Reticulitermes* in Europe.

Two recent studies (Szalanski et al. 2006; Tripodi et al. 2006)[1] have, in our opinion, misrepresented the value of CHCs for taxonomy, even though two of the authors used CHCs (data from *P. Uva*) in an earlier study to validate the species status of *R. lucifugus* from Turkey (Austin et al. 2002) and to confirm the identity of samples of *R. malletei* in the eastern United States (Austin et al. 2007). Szalanski et al. (2006) stated "Recently, Copren et al. (2005) found evidence for as many as seven new species of *Reticulitermes* from the western United States based upon cuticular hydrocarbon phenotypes, but resolved to designate them as putative new species after attempting to corroborate their relationship with molecular phylogenetics and reproductive flight dates. These discrepancies likely are attributable to the environmental plasticity of cuticular hydrocarbons and stresses (sic) the need for fixed character states for species identification such as mtDNA sequences." Tripodi et al. (2006) echoed this line of thought. Actually, there were no discrepancies; this is a misinterpretation of Copren et al. (2005) as she and her colleagues corroborated the species groups based on CHCs and COII mtDNA sequences. Thus, taxa determined by CHC analyses agreed with taxa determined by mtDNA sequences. The "environmental plasticity" caveat has been carefully considered, and the references provided by Szalanski et al. (2006) and Tripodi et al. (2006) are not relevant to this analysis. Our phenotype designations are based on numerous, repeatable qualitatively different profiles. There can be minor quantitative variation among collections of the same phenotype, in which case, we do not distinguish them as separate phenotypes (Haverty et al. 1991, 1996b).

Tripodi et al. (2006) state "The application of cuticular hydrocarbons for chemotaxonomy requires fixed patterns of hydrocarbons within taxa (Kaib et al. 1991). This approach is inherently problematic because of the plastic nature of hydrocarbon composition. Although hydrocarbon compositions are assumed to be species-specific (Kaib et al. 1991), variation between groups may be more greatly attributable to environmental differences and available food sources (Liang and Silverman 2000)." The study by Liang and Silverman (2000) involved colonies, not of termites but of the Argentine ant, *Linepithema humile* (Mayr), which were feeding on and mixing with various species of cockroaches. It is clear that predatory insects feeding on waxy organisms, such as laboratory-reared cockroaches, will acquire foreign hydrocarbons that could easily affect kin recognition. This study, however, has no relevance with regard to the alleged "plastic nature" of termite CHC mixtures.

In their discussion on the effects of diet on aggression in *Coptotermes formosanus* Shiraki, Tripodi et al. (2006) claim that "differences in diet can influence hydrocarbon composition and intercolonial aggression (Florane et al. 2004). Therefore, although cuticular hydrocarbons probably play a key role in nestmate recognition among colonies of termites, considerable variation of hydrocarbons across small spatial distances within an apparently single morphological species may occur and should alert taxonomists to interpret cuticular hydrocarbon patterns with care." Florane et al. (2004) do not show effects of diet on CHC composition, but rather on volatile compounds (collected by headspace solid-phase microextraction of workers) that possibly affected kin recognition and intercolonial aggression.

In summary, we find no valid objections to the use of CHC profiles in *Reticulitermes* taxonomy. The extensive body of knowledge demonstrating the taxonomic value of termite CHCs allows us to accept distinct, repeatable CHC profiles as descriptive of distinct taxa.

Reticulitermes soldiers, like soldiers from other termite genera, synthesize their defense secretions *de novo* (Prestwich 1979, 1983). It follows that these SDS mixtures, like CHCs, could be useful as taxonomic characters. Several studies have characterized and compared SDSs from *Reticulitermes* species. Parton et al. (1981) compared SDS mixtures from populations of *R. lucifugus* and *R. santonensis* in Europe, finding geographic variation in the *lucifugus* complex, while *R. santonensis* was homogeneous. In the southeastern United States, Clément et al. (1985) identified two SDS phenotypes in *R. flavipes*, one in *R. virginicus* and another in *R. malletei*. Bagnères et al. (1990) examined chemical characters from *R. flavipes* in the southeastern United States and *R. santonensis* in Europe, and reported six SDS phenotypes in *R. flavipes*, while *R. santonensis* was, again, homogeneous. In a study of the same characters from *Reticulitermes* samples from Georgia, Arizona, and northern California, Nelson et al. (2001) reported that some SDS phenotypes pair with more than one CHC phenotype; however, with two exceptions, each hydrocarbon phenotype is associated with only one SDS phenotype. Samples that keyed to *R. flavipes* were represented by three SDS phenotypes, similar to ones previously reported by Clément et al. (1985) and Bagnères et al. (1990). Quintana et al. (2003) described species-

[1] There are several incorrect references in these papers. Szalanski et al. (2006) acknowledged the "identification of new species from northern California ...reported by Haverty and Nelson (1997), Haverty et al. (1999) [our Haverty et al. 1999b] applying CHCs, and further investigated with ethological data (Getty et al. 2000a, b)." However, there was no new information on species of *Reticulitermes* from northern California reported in the cited Haverty et al. (1999) reference. This paper (Haverty et al. 1999b) reported a number of new CHC phenotypes from Georgia, New Mexico, Arizona, and Nevada. Furthermore, the ethological information that supports species designations is not found in the Getty et al. (2000a, b) papers, but in Haverty et al. (1999a) and Delphia et al. (2003) in which we examined agonistic behavior; and in Haverty et al. (2003) we studied flight phenology as a reproductive isolating mechanism. This same referencing error was repeated by Tripodi et al. (2006).

specific SDS phenotypes for seven European *Reticulitermes* species.

Our findings for *Reticulitermes* from southern California show that the two primary CHC phenotypes (SC-A and SC-B) have qualitatively similar SDS mixtures, although they are quantitatively dissimilar (Table 3). Possible explanations for this lack of correspondence include (a) different rates of evolution among the character sets and/or (b) this particular SDS phenotype is adaptive to the conditions of southern California. However, the samples collected at Lake Arrowhead, which is the type locality for *R. hesperus*, had the same SDS profile as CA-A/A′ from northern California.

Taken together, CHC and SDS profiles appear to be complementary character sets that have similar, although not always identical, sensitivity in discriminating putative *Reticulitermes* taxa.

Genetics of Reticulitermes Within the past decade, numerous genetic analyses have been published that indicate additional species or taxa, potential synonymies, and the degree of genetic variation within a species. Copren et al. (2005) used the COII gene in a phylogenetic analysis that indicated at least six clades or species of *Reticulitermes* in California, including the two CHC phenotypes presented here from southern California. Szalanski et al. (2006) and Tripodi et al. (2006) provided genetic evidence of an undescribed species in western North America and a phylogenetic analysis of *Reticulitermes* by applying the 16S mtDNA gene, and validated the prior genetic analysis by Copren et al. (2005). Furthermore, Tripodi et al. (2006) and Su et al. (2006) identified an apparent exotic species, *R. flavipes*, in California via genetic analyses. McKern et al. (2006, 2007) identified *R. flavipes* and *R. hageni* in Oregon by using the 16S mtDNA, which is the first report of these species in that state.

Recent genetic evaluations of North American, European, and Chilean *Reticulitermes* have resulted in intriguing conclusions: (1) North American *R. flavipes* and *R. arenincola*, and European *R. santonensis* are likely conspecific (Ye et al. 2004), and (2) the invasive *Reticulitermes* in Chile and Uruguay is *R. flavipes* (Austin et al. 2005b; Su et al. 2006). One Indiana population of *R. arenincola* shares identical DNA sequences with one *R. santonensis* population from France and one *R. flavipes* population from Indiana (Ye et al. 2004). Even though *R. flavipes* = *R. arenincola* by DNA sequence, there appeared to be morphological differences between the respective soldier castes, and the authors decided that the synonymy of these two species would be premature (Ye et al. 2004). Su et al. (2006) demonstrated that all 13 samples of Chilean *Reticulitermes* termites collected from four cities were *R. flavipes* and had identical gene sequences for all loci examined, thus suggesting a single geographic introduction. A similar conclusion was reached by Austin et al. (2005b).

As an adjunct to population studies of *Reticulitermes* in northern California, Copren (2007) found that three microsatellite loci, specifically developed for *R. hesperus*, did not amplify with the three other species of *Reticulitermes* from northern California. Finding fixed genetic differences among cryptic species that result in reciprocal monophyly, particularly among sympatric species, provides information useful for taxonomic studies (Copren 2007).

We think that GenBank is an important tool for phylogenetic analyses, but its use is not without problems. The use of GenBank data submitted by others requires one to accept the submitter's species designations as accurate or authoritative. With refractory genera, such as *Reticulitermes* or *Heterotermes*, GenBank sequences should be used with caution.

Use of Multiple Characters in Reticulitermes *Taxonomy* In a difficult genus like *Reticulitermes*, taxonomic conclusions are greatly strengthened when two or more features, CHC mixtures, SDS mixtures, genetic markers, morphology, behavioral characteristics (such as agonism or flight times), etc., correlate. The more differences that exist among groups of organisms, the greater the likelihood of their being separate species (Futuyma 1998). Jenkins et al. (2000) and Copren et al. (2005) demonstrated the association of CHC phenotypes and mtDNA haplotypes, illustrating that CHCs are useful in separating known species, determining new species, and understanding termite evolution, and adding robustness to the phylogenetic analyses (Jenkins et al. 2000; Page et al. 2002). Additional examples of multiple, independent data sets for determining species of *Reticulitermes* include the CHC and morphological work of Takematsu and Yamaoka (1999), the CHC and SDS study of Nelson et al. (2001), and the use of microsatellites to assess sympatric populations of *R. hesperus* and cryptic species of *Reticulitermes* in northern California (Copren 2007).

In contrast to this approach, Ye et al. (2004) relied heavily on morphological information and did not address contradictory genetic information. Genetic data gathered from three mitochondrial DNA genes (COII, 16S, and 12S) indicated that *R. flavipes*, *R. santonensis*, and *R. arenincola* are conspecific. Others reached the same conclusion about *R. flavipes* and *R. santonensis* (Austin et al. 2005b; Su et al. 2006). However, Ye et al. (2004) felt it was premature to synonymize *R. arenincola* and *R. flavipes* due to the morphological separation of *R. arenincola* and *R. flavipes* by soldier characteristics.

Reticulitermes *in Southern California: the Current Study* In early studies of Californian termites, *Reticulitermes* was

determined by various authors as consisting of just two species, *R. hesperus* and *R. tibialis* (Light and Pickens 1934; Pickens 1934a, b; Snyder 1949, 1954). There is a persistent assumption that these are the only *Reticulitermes* species in California and that they have distributions that are allopatric over most of their ranges (Weesner 1970).

We were among the first to question the number of species of *Reticulitermes* in California, and we reported five CHC phenotypes of *Reticulitermes* (CA-A, CA-A′, CA-B, CA-C, and CA-D) in northern California (Haverty and Nelson 1997). In the current study, we report a wider distribution of previously described phenotypes SC-A and SC-B and a variant of one of them (SC-B′) that occurs in Riverside County. Correlation of CHC phenotypes with mtDNA (COII) genotypes supported elevating phenotypes CA-B, CA-C, CA-D, and SC-B to the status of new species, but suggested that CA-A, CA-A′, and SC-A represent one species, which we postulated to be *R. hesperus*, with the CA and SC phenotypes comprising geographic subspecies (Copren et al. 2005).

Banks and Snyder (1920) originally described *R. hesperus* based on samples from California, Nevada, Oregon, and Washington and designated the type locality as Little Bear Lake, San Bernardino Mountains, California (Snyder 1949). Many of these sites are distant from the Pacific Coast and east of the Cascade Range and Sierra Nevada. In a case like this, with an old species designation based on morphology of a limited number of specimens (Banks and Snyder 1920), it is difficult to associate chemical or genetic determinations of taxa with the species in question. We collected at the type locality of *R. hesperus* so that we could associate one of our CHC phenotypes with a species name. The type specimen of *R. hesperus* (winged, Cat. No. 21864, U.S.N.M.) was reported from Little Bear Lake, CA, now known as Lake Arrowhead (Robinson 1989), in the San Bernardino Mountains (see Banks and Snyder 1920; Snyder 1949). Haverty et al. (2003) and Copren et al. (2005) reported that topotype samples from Lake Arrowhead all had one CHC phenotype, CA-A′. Copren et al. (2005) used the geographic nomenclature SC-A to describe this phenotype, but the lack of 9,11-dimethylalkanes, the presence of C25:3, and γ-cadinene in the SDSs puts it in the same group as CA-A′ from northern California. Thus, this phenotype was postulated to represent *R. hesperus*.

Studies by Szalanski et al. (2006) and Tripodi et al. (2006) that used 16S mtDNA, have identified a genetically distinct species in the western United States, which they have named *R.* n. sp. "*R. okanaganensis*." We contend that is incorrect, and the collections designated as *R.* n. sp. "*R. okanaganensis*" are actually the common, and widely distributed, *R. hesperus*. Austin et al. (2002) were likely correct, and the revision by Szalanski et al. (2006) and Tripodi et al. (2006) simply confused the status of *Reticulitermes* in California. Szalanski et al. (2006) and Tripodi et al. (2006) apparently disregarded the value of collections at the type locality. Instead, they designated a sample from Lake Arrowhead as their putative new species, *R. okanaganensis*, but did not find what they now consider to be *R. hesperus* at the type locality. Actually, the distribution presented for *R. okanaganensis* (Szalanski et al. 2006) closely resembles the original distribution map of *R. hesperus* reported by Banks and Snyder (1920) and reinforced by Weesner (1970) and Nutting (1990).

One particular mitochondrial DNA sequence is key to our study, especially as it relates to Tripodi et al. (2006): a fragment of the mtDNA cytochrome oxidase (COII) from *R. hesperus* [GenBank AF525329] (Austin et al. 2002, 2004a; Copren et al. 2005). The sequence for *R. hesperus* from Los Angeles, California [GenBank AF525329] was useful for the demonstration of the correlation of species determinations based on CHCs and COII sequences, as this sequence was similar to that of the collections from the type locality for *R. hesperus* (Copren et al. 2005). Now, based on the mtDNA 16S sequence, Tripodi et al. (2006) state that the sample identified by Austin et al. (2002) as *R. hesperus* [GenBank AF525329] was a mistake, and it truly represents a new species, "*R. okanaganensis*." We think that Tripodi et al. (2006) are wrong and that Austin et al. (2002) attached the correct species name to sequence AF525329, possibly relying on location to assign the species name since the references they cited did not have keys or had keys that were inappropriate for specimens from California. The confusion would be compounded if, based on Tripodi et al. (2006), someone "corrected" the species name associated with AF525329.

If our analysis is correct, *R. hesperus* is the most common subterranean termite throughout California. It is found sympatrically in northern California with three unnamed species: *R.* sp. CA-B, *R.* sp. CA-C, and *R.* sp. CA-D. *R.* sp. CA-B and *R.* sp. CA-C are rare compared to *R. hesperus* and *R.* sp. CA-D (Copren 2007). In southern California, *R. hesperus* (SC-A) occurs sympatrically with *R.* sp. SC-B over most of their distributions. Furthermore, in Arizona and Nevada, we collected samples (phenotype AZ-D) that resemble specimens (designated CA-A′) from the type locality of *R. hesperus*. Therefore, it is likely that *R. hesperus* also occurs in northwestern Arizona and mountainous areas of southern Nevada, but is somewhat rare (Haverty and Nelson 2007). The CA-A/A′, SC-A, and AZ-D phenotypes may each represent a geographic subspecies of *R. hesperus*, as the CHCs are similar, but the SDSs are dissimilar, with AZ-D having SDSs consisting of mostly geranyl linalool (Haverty and Nelson 2007).

Today, the distribution, or even presence, of *R. tibialis* in California is not as clear as it apparently was to early researchers (Banks and Snyder 1920; Light and Pickens 1934; Pickens 1934b; Snyder 1954). Tripodi et al. (2006) only reported samples of *R. tibialis* in the southern portion of California in areas that border the Mojave Desert. Likewise, Copren et al. (2005) found specimens in Inyo County, California, on the xeric east side of the Sierra Nevada in the northern portion of the Mohave Desert that closely resembled (by COII mtDNA) the sample of "*R. tibialis*" from Cochise County, Arizona, USA, (GenBank AF525355) reported by Austin et al. (2002).

The type specimen for *R. tibialis* (winged, Cat. No. 21861 U.S.N.M.) was collected in Beeville, Texas (Banks and Snyder 1920; Snyder 1949). This species is purported to be the most widely distributed species, covering diverse habitats in North America (Banks and Snyder 1920; Snyder 1934; Weesner 1970). However, Weesner (1970) recognized that many of the alate specimens collected from the range of the species (Colorado, Illinois, Kansas, Missouri, Oklahoma, and Texas) were "typical" for *R. tibialis*, yet additional collections from the same area, particularly Texas, were extremely variable and could not be designated as *R. tibialis*. This suggests that there is more than one species in this distribution.

One of the GenBank COII sequences assigned to *R. tibialis* [AF525355] has been used in many studies (Austin et al. 2002, 2004a; Szalanski et al. 2003; Copren et al. 2005) and was collected from Cochise County, Arizona. This specimen was collected from an area that is likely to be the same as the species designated as our CHC phenotype AZ-B because only this phenotype has been found at the lower elevations in this region of Arizona. AZ-B is the most common CHC phenotype/species found in Arizona from Fairbank in Cochise County (≈1,200 m) to the North Rim of the Grand Canyon in Coconino County (≈2,500 m; Haverty and Nelson 2007). Using this sequence, *R. tibialis* [AF525355] is similar to, yet significantly different from, other *Reticulitermes* taxa in California (Copren et al. 2005).

Other COII sequences assigned to *R. tibialis* [GenBank AY168206 & AY168207, see Ye et al. 2004; GenBank AY808094, see Su et al. 2006] have not been used together with AF525355 in a comparative study and have likely been assigned to *R. tibialis* based on the location of the collection, as neither present a source for identification of any species (Ye et al. 2004) or the *R. tibialis* samples (Su et al. 2006). Furthermore, none of the samples identified as *R. tibialis* have been collected within 1500 km of the type locality of this species or in habitats resembling the type locality. As we see it, the actual genotype(s) or CHC phenotype of *R. tibialis* remains unknown.

Other Reticulitermes *Taxa in California* Su et al. (2006) and Tripodi et al. (2006) reported finding *R. flavipes* in California on the basis of mtDNA. We, too, have found *R. flavipes* in California based on CHC and SDS composition (Haverty and Nelson, unpublished), as have Ripa et al. (2002). Tripodi et al. (2006) were not able to confirm this based on morphology, whereas Su et al. (2006) identified their California population from Sacramento as *R. flavipes* by using the key in Snyder (1954). One unexpected finding was that the *R. flavipes* sample collected from Sacramento, California, closely resembled the introduced Chilean *R. flavipes* in combined mitochondrial DNA (COII, 16S, and 12S) sequences (Su et al. 2006). Su et al. (2006) hypothesized that the Chilean *R. flavipes* may have been introduced from California, or vice versa, or that both Chilean and Californian *R. flavipes* populations may have the same origin in North America. The well-established *R. flavipes* infestation in Sacramento suggests the ability of this east coast species to survive in a Mediterranean climate and that it could be more widespread in California than is known (Su et al. 2006). Tripodi et al. (2006) opined that the establishment of *R. flavipes* in Sacramento and El Cajon probably represents either extreme western distributions of the species or accidental introductions from anthropogenic sources. We believe the latter explanation is more likely.

Additional exotic or undescribed species of *Reticulitermes* are likely present in southern California given the numerous coastal, montane, and desert habitats. Based on mtDNA sequences, Su et al. (2006) found two populations of *Reticulitermes* from California (US27 and US42) that were similar to a sample labeled *R. tibialis* (US5), but one (US27) differed from it by 61 bp, the other (US42) by 52 bp, and they differed from each other by 34 bp. These samples may thus represent two undescribed species. Furthermore, Tripodi et al. (2006) also reported two undescribed species from southern California; one from Arrowbear, San Bernardino County, and another from Mission Gorge, San Diego County.

To summarize, the total count of probable species of *Reticulitermes* in California is at least seven: (1) *R. hesperus* (our hydrocarbon phenotypes CA-A, CA-A′, and SC-A); (2) *R.* sp. CA-B; (3) *R.* sp. CA-C; (4) *R.* sp. CA-D; (5) *R.* sp. SC-B [and SC-B′]; (6) "*R. tibialis*" = *R.* unknown sp. (Copren et al. 2005); (7) *R. flavipes* (Su et al. 2006, Tripodi et al. 2006); as well as *R.* unknown I (Su et al. 2006) and *R.* unknown II (Su et al. 2006). It is possible that the two unknown species identified by Tripodi et al. (2006) are the same as those found by Su et al. (2006). Genetic comparison of these samples will resolve this question. It is also possible that one of the unknown species of Su et al. (2006) and Tripodi et al. (2006) equates with our SC-B/B′.

Reproductive isolation is the most convincing if populations are sympatric (Freeman and Herron 1998). Exam-

ples of valid species of *Reticulitermes* with sympatric distributions have been reported numerous times for *R. flavipes*, *R. virginicus*, and *R. hageni* (Weesner 1970 and others), as well as *R. banyulensis* and *R. grassei* (Uva et al. 2004). Haverty et al. (2003) provided evidence of reproductive isolation in sympatric populations of different CHC phenotypes of *Reticulitermes* in northern California. Alates of phenotype CA-A/A′ (identified here as *R. hesperus*) take flight in the spring, whereas alates of phenotype CA-D fly only in the fall. These disparate flight times make reproductive isolation, and thus species status, complete. In our sampling of *Reticulitermes* in southern California, alates with the hydrocarbon phenotype SC-A were collected in March 2001 and 2002, while SC-B alates were collected in September and November 2001. This sampling is not extensive and warrants further corroboration, but the trend matches earlier reports for *Reticulitermes* species in northern California.

We (MIH and LJN) and our colleagues have been cautious, perhaps too cautious, in delineating and naming new termite species. We have taken this approach in order to avoid further confusing the taxonomy of *Reticulitermes* in North America before appropriate suites of taxonomic characters are agreed upon, and the status of previously published species is resolved. We do not propose naming species based on CHC phenotypes alone, but once taxa are sorted by this method, we can corroborate or refute the groupings with other evidence, such as morphological, chemical, behavioral, and genetic data. When there is agreement of two or more character sets, we can be more confident assigning species status to the phenotypes. The evidence for two predominant species, *R. hesperus* and *R.* sp. SC-B, in southern California is strong, and we believe our inference regarding *R. hesperus* and its type locality is correct. Finally, we urge researchers in this field to be cautious in applying species names based on inappropriate, unspecified, or incorrectly cited reference sources, and to take care in accurately describing both their own methodology and research performed by others.

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References

AUSTIN, J. W., SZALANSKI, A. L., UVA, P., BAGNÈRES, A. -G., and KENCE, A. 2002. A comparative genetic analysis of the subterranean termite genus *Reticulitermes* (Isoptera: Rhinotermitidae). *Ann. Entomol. Soc. Am.* 95:753–760.

AUSTIN, J. W., SZALANSKI, A. L., and CABRERA, B. J. 2004a. Phylogenetic analysis of the subterranean termite family Rhinotermitidae (Isoptera) by using the mitochondrial cytochrome oxidase II gene. *Ann. Entomol. Soc. Am.* 97:548–555.

AUSTIN, J. W., SZALANSKI, A. L., GOLD, R. E., and FOSTER, B. T. 2004b. Genetic variation and geographic distribution of the subterranean termite genus *Reticulitermes* in Texas. *Southwest. Entomol.* 29:1–11.

AUSTIN, J. W., SZALANSKI, A. L., and KARD, B. M. 2004c. Distribution and genetic variation of *Reticulitermes* (Isoptera: Rhinotermitidae) in Oklahoma. *Southwest. Entomol.* 29:1–11.

AUSTIN, J. W., SZALANSKI, A. L., SCHREFFAHN, R. H., and MESSENGER, M. T. 2005a. Genetic variation of *Reticulitermes flavipes* (Isoptera: Rhinotermitidae) in North America applying the mitochondrial rRNA 16S gene. *Ann. Entomol. Soc. Am.* 98:980–988.

AUSTIN, J. W., SZALANSKI, A. L., SCHREFFAHN, R. H., MESSENGER, M. T., DRONNET, S., and BAGNÈRES, A.-G. 2005b. Genetic evidence for the synonymy of two *Reticulitermes* species: *Reticulitermes flavipes* and *Reticulitermes santonensis*. *Ann. Entomol. Soc. Am.* 98:395–401.

AUSTIN, J. W., BAGNÈRES, A. -G., SZALANSKI, A. L., SCHEFFRAHN, R. H., HEINTSCHEL, B. P., MESSENGER, M. T., CLÉMENT, J. -L., and GOLD, R. E. 2007. *Reticulitermes malletei* (Isoptera: Rhinotermitidae): a valid Nearctic subterranean termite from Eastern North America. *Zootaxa* 1554:1–26.

BAGNÈRES, A. -G., LANGE, C., CLÉMENT, J. -L., and JOULIE, C. 1988. Les hydrocarbures cuticulaires: variation spècifiques et colonials. *Actes Colloq. Insect. Soc.* 4:34–42.

BAGNÈRES, A. -G., CLÉMENT, J. -L., BLUM, M. S., SEVERSON, R. F., JOULIE, C., and LANGE, C. 1990. Cuticular hydrocarbons and defensive compounds of *Reticulitermes flavipes* (Kollar) and *R. santonenesis* (Feytaud): polymorphism and chemotaxonomy. *J. Chem. Ecol.* 16:3213–3244.

BAGNÈRES, A. -G., KILLIAN, A., CLÉMENT, J. -L., and LANGE, C. 1991. Interspecific recognition among termites of the genus *Reticulitermes*: evidence for a role for the cuticular hydrocarbons. *J. Chem. Ecol.* 17:2397–2420.

BAKER, P. B., and HAVERTY, M. I. 2007. Foraging populations and distances of the desert subterranean termite, *Heterotermes aureus* (Isoptera: Rhinotermitidae), associated with structures in southern Arizona. *J. Econ. Entomol.* 100:1381–1390.

BAKER, R., PARTON, A. H., and HOWSE, P. E. 1982. Identification of an acyclic diterpene alcohol in the defense secretion of soldiers of *Reticulitermes lucifugus*. *Experientia* 38:297–298.

BANKS, F. A. 1946. Species Distinction in *Reticulitermes* (Isoptera: Rhinotermitidae). University of Chicago, Chicago, IL.

BANKS, N., and SNYDER, T. E. 1920. A revision of the Nearctic termites. *Bull. US Natl. Mus.* 108:1–228.

BLOMQUIST, G. J., and DILLWITH, J. W. 1985. Cuticular lipids, pp. 117–154, in G. A. Kerkut, and L. I. Gilbert (eds.). Comprehensive Insect Physiology, Biochemistry and Pharmacology, vol. 3. Integument, Respiration and Circulation. Pergamon, Oxford, United Kingdom.

BLOMQUIST, G. J., NELSON, D. R., and DE RENOBALES, M. 1987. Chemistry, biochemistry, and physiology of insect cuticular waxes. *Arch. Insect Biochem. Physiol.* 6:227–265.

CLÉMENT, J. -L., and BAGNÈRES, A. -G. 1998. Nestmate recognition in termites, pp. 126–155, in R. K. Vander Meer, M. D. Breed, M. L. Winston, and K. E. Espelie (eds.). Pheromone Communication in Social Insects, Ants, Wasps, Bees and Termites. Westview Press, Colorado.

CLÉMENT, J. -L., LANGE, C., BLUM, M. S., and LLOYD, H. 1985. Chimiosystematique du genre *Reticulitermes* (Isoptères) aux U.S.A. et en Europe. *Actes Coll. Ins. Soc.* 2:123–131.

CLÉMENT, J. -L., HOWARD, R. W., BLUM, M. S., and LLOYD, H. 1986. L'isolement spécifique des termites du genre *Reticulitermes* (Isoptera) du sudest des Etats-Unis. Mise en èvidence grâce à la chimie et au comportement d'une espèce jumelle de *R. virginicus* = *R. malletei* sp. nov. et d'une semi-species de *R. flavipes*. *C. R. Acad. Sci. Paris* 302:67–70.

CLÉMENT, J. -L., LEMAIRE, M., NAGNAN, P., ESCOUBAS, P., BAGNÈRES, A. -G., and JOULIE, C. 1988. Chemical ecology of European termites of the genus *Reticulitermes*: Allomones, Pheromones and Kairomones. *Sociobiology* 14:165–174.

CLÉMENT, J. -L., BAGNÈRES, A. -G., UVA, P., WILFERT, L., QUINTANA, A., REINHARD, J., and DRONNET, S. 2001. Biosystematics of *Reticulitermes* termites in Europe: Morphological, chemical and molecular data. *Ins. Soc.* 48:202–215.

COPREN, K. A. 2004. The genetic and social structure of the western subterranean termite, *Reticulitermes hesperus* (Isoptera: Rhinotermitidae). PhD dissertation. University of California, Davis.

COPREN, K. A. 2007. Characterization of microsatellite loci in the western subterranean termite, *Reticulitermes hesperus*, and cross-amplification in closely related cryptic species. *J. Insect Sci.* 7:17 5pp. [http://insectscience.org/7.17].

COPREN, K. A., NELSON, L. J., VARGO, E. L., and HAVERTY, M. I. 2005. Phylogenetic analyses of mtDNA sequences corroborate taxonomic designations based on cuticular hydrocarbons in subterranean termites. *Mol. Phylogenet. Evol.* 35:689–700.

DELPHIA, C. M., COPREN, K. A., and HAVERTY, M. I. 2003. Agonistic behavior between individual worker termites from three cuticular hydrocarbon phenotypes of *Reticulitermes* (Isoptera: Rhinotermitidae) from northern California. *Ann. Entomol. Soc. Am.* 96:585–593.

DONOVAN, S. E., JONES, D. T., SANDS, W. A., and EGGLETON, P. 2000. Morphological phylogenetics of termites (Isoptera). *Biol. J. Linnean Soc.* 70:467–513.

FLORANE, C. B., BLAND, J. M., HUSSENEDER, C., and RAINA, A. K. 2004. Diet-mediated inter-colonial aggression in the Formosan subterranean termite *Coptotermes formosanus*. *J. Chem. Ecol.* 30:2559–2574.

FOSTER, B. T., COGNATO, A. I., and GOLD, R. E. 2004. DNA-based identification of the eastern subterranean termite, *Reticulitermes flavipes* (Isoptera: Rhinotermitidae). *J. Econ. Entomol.* 97:95–101.

FREEMAN, S., and HERRON, J. C. 1998. Evolutionary Analysis, 2nd edn. Prentice Hall, Upper Saddle River, New Jersey.

FUTUYMA, D. J. 1998. Evolutionary Biology. Sinauer Associates, Sunderland, Massachusetts.

GETTY, G. M., HAVERTY, M. I., COPREN, K. A., and LEWIS, V. R. 1999a. Seasonal foraging behavior of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in northern California, USA, pp. 347–352, in W. H. Robinson, F. Rettich, and G. W. Rambo (eds.). Proceedings of the 3rd International Conference on Urban Pests (ICUP), Czech University of Agriculture, Prague, Czech Republic, 19–22 July 1999.

GETTY, G. M., HAVERTY, M. I., LEWIS, V. R., and SBRAGIA, R. J. 1999b. Interfacing basic biology of *Reticulitermes* spp. and the Sentricon Termite Colony Elimination System in northern California, USA, pp. 601–604, in W. H. Robinson, F. Rettich, and G. W. Rambo (eds.). Proceedings of the 3rd International Conference on Urban Pests (ICUP), Czech University of Agriculture, Prague, Czech Republic, 19–22 July 1999.

GETTY, G. M., HAVERTY, M. I., and LEWIS, V. R. 2000a. Agonistic behavior between recently collected and laboratory cultured *Reticulitermes* spp. (Isoptera: Rhinotermitidae) from northern California. *Pan-Pacific Entomol.* 76:243–250.

GETTY, G. M., HAVERTY, M. I., COPREN, K. A., and LEWIS, V. R. 2000b. Response of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in northern California to baiting with hexaflumuron with Sentricon Termite Colony Elimination System. *J. Econ. Entomol.* 93:1498–1507.

GOELLNER, E. J. 1931. A new species of termite, *Reticulitermes arenincola*, from the sand dunes of Indiana and Michigan, along the shores of Lake Michigan. *Proc. Entomol. Soc. Wash.* 33:227–234.

HAVERTY, M. I., and NELSON, L. J. 1997. Cuticular hydrocarbons of *Reticulitermes* (Isoptera: Rhinotermitidae) from northern California indicate undescribed species. *Comp. Biochem. Physiol.* 118B:869–880.

HAVERTY, M. I., and NELSON, L. J. 2007. *Reticulitermes* (Isoptera: Rhinotermitidae) in Arizona: Multiple cuticular hydrocarbon phenotypes indicate additional taxa. *Ann. Entomol. Soc. Am.* 100:206–221.

HAVERTY, M. I., NELSON, L. J., and PAGE, M. 1991. Preliminary investigations of the cuticular hydrocarbons from North American *Reticulitermes* and tropical and subtropical *Coptotermes* (Isoptera: Rhinotermitidae) for chemotaxonomic studies. *Sociobiology* 19:51–76.

HAVERTY, M. I., FORSCHLER, B. T., and NELSON, L. J. 1996a. An assessment of the taxonomy of *Reticulitermes* (Isoptera: Rhinotermitidae) from the southeastern United States based on cuticular hydrocarbons. *Sociobiology* 28:287–318.

HAVERTY, M. I., GRACE, J. K., NELSON, L. J., and YAMAMOTO, R. T. 1996b. Intercaste, intercolony, and temporal variation in cuticular hydrocarbons of *Coptotermes formosanus* Shiraki (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 22:1813–1834.

HAVERTY, M. I., COPREN, K. A., GETTY, G. M., and LEWIS, V. R. 1999a. Agonistic behavior and cuticular hydrocarbon phenotypes of colonies of *Reticulitermes* (Isoptera: Rhinotermitidae) from northern California. *Ann. Entomol. Soc. Am.* 92:269–277.

HAVERTY, M. I., NELSON, L. J., and FORSCHLER, B. T. 1999b. New cuticular hydrocarbon phenotypes of *Reticulitermes* (Isoptera: Rhinotermitidae) from the United States. *Sociobiology* 34:1–21.

HAVERTY, M. I., GETTY, G. M., COPREN, K. A., and LEWIS, V. R. 1999c. Seasonal foraging and feeding behavior of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in a wildland and a residential location in northern California. *Environ. Entomol.* 28:1077–1084.

HAVERTY, M. I., GETTY, G. M., COPREN, K. A., and LEWIS, V. R. 2000. Size and dispersion of colonies of *Reticulitermes* spp. (Isoptera: Rhinotermitidae) in a wildland and a residential location in northern California. *Environ. Entomol.* 29:241–249.

HAVERTY, M. I., GETTY, G. M., NELSON, L. J., and LEWIS, V. R. 2003. Flight phenology of sympatric populations of *Reticulitermes* (Isoptera: Rhinotermitidae) in northern California: disparate flight intervals indicate reproductive isolation among cuticular hydrocarbon phenotypes. *Ann. Entomol. Soc. Am.* 96:828–833.

HAVERTY, M. I., WOODROW, R. J., NELSON, L. J., and GRACE, J. K. 2005. Identification of termite species by the hydrocarbons in their feces. *J. Chem. Ecol.* 31:2119–2151.

HOSTETTLER, N. C., HALL, D. W., and SCHEFFRAHN, R. H. 1995. Intracolony morphometric variation and labral shape in Florida *Reticulitermes* (Isoptera: Rhinotermitidae) soldiers: significance for identification. *Fla. Entomol.* 78:119–129.

HOWARD, R. W. 1993. Cuticular hydrocarbons and chemical communication, pp. 177–226, in D. W. Stanley-Samuelson, and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

HOWARD, R. W., and BLOMQUIST, G. J. 1982. Chemical ecology and biochemistry of insect hydrocarbons. *Annu. Rev. Entomol.* 27:149–172.

HOWARD, R. W., MCDANIEL, C. A., and BLOMQUIST, G. J. 1978. Cuticular hydrocarbons of the eastern subterranean termite, *Reticulitermes flavipes* (Kollar) (Isoptera: Rhinotermitidae). *J. Chem. Ecol.* 4:233–245.

HOWARD, R. W., MCDANIEL, C. A., NELSON, D. R., BLOMQUIST, G. J., GELBAUM, L. T., and ZALKOW, L. H. 1982. Cuticular hydrocarbons of *Reticulitermes virginicus* (Banks) and their role as potential species and caste recognition cues. *J. Chem. Ecol.* 8:1227–1239.

JENKINS, T. M., HAVERTY, M. I., BASTEN, C. J., NELSON, L. J., and PAGE, M. 2000. Correlation of mitochondrial haplotypes with cuticular hydrocarbon phenotypes of sympatric *Reticulitermes* species from the southeastern United States. *J. Chem. Ecol.* 26:1525–1542.

JENKINS, T. M., DEAN, R. E., VERKERK, R., and FORSCHLER, B. T. 2001. Phylogenetic analyses of two mitochondrial genes and one nuclear intron region illuminate European subterranean termite (Isoptera: Rhinotermitidae) gene flow, taxonomy, and introduction dynamics. *Mol. Phylogenet. Evol.* 20:286–293.

KAIB, M., BRANDL, R., and BAGINE, R. K. N. 1991. Cuticular hydrocarbon profiles: a valuable tool in termite taxonomy. *Naturwissenschaften* 78:176–179.

KIM, Y. -K., COOL, L. G., and ZAVARIN, E. 1994. *cis*-Calamenene-related sesquiterpenoids from *Cupressus bakeri* foliage. *Phytochemistry* 36:961–965.

KING, S. W., AUSTIN, J. W., and SZALANSKI, A. L. 2007. Use of soldier pronotal width and mitochondrial DNA sequencing to distinguish the subterranean termites, *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) (Isoptera: Rhinotermitidae), on the Delmarva Peninsula: Delaware, Maryland, and Virginia, U.S.A. *Entomol. News* 118:41–48.

KRISHNA, K., and WEESNER, F. M. 1969. Biology of Termites, vol. I. Academic Press, New York.

LEMAIRE, M., NAGNAN, P., CLÉMENT, J. -L., LANGE, C., PERU, L., and BASSELIER, J. -J. 1990. Geranyllinalool (diterpene alcohol). An insecticidal component of pine wood and termites (Isoptera: Rhinotermitidae) in four European ecosystems. *J. Chem. Ecol.* 16:2067–2079.

LIANG, D., and SILVERMAN, J. 2000. "You are what you eat": diet modifies cuticular hydrocarbons and nestmate recognition in the Argentine ant, *Linepithema humile*. *Naturwissenschaften* 87:412–416.

LIGHT, S. F. 1934. The termite fauna of North America with special reference to the United States, pp. 127–135, in C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe (eds.). Termites and Termite Control, 2nd edn. University of California Press, Berkeley, California.

LIGHT, S. F., and PICKENS, A. L. 1934. American subterranean termites and their distribution, pp. 150–156, in C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe (eds.). Termites and Termite Control, 2nd edn. University of California Press, Berkeley, California.

MCKERN, J. A., SZALANSKI, A. L., and AUSTIN, J. W. 2006. First record of *Reticulitermes flavipes* and *Reticulitermes hageni* in Oregon (Isoptera: Rhinotermitidae). *Fla. Entomol.* 89:541–542.

MCKERN, J. A., SZALANSKI, A. L., AUSTIN, J. W., MESSENGER, M. T., MAHN, J., and GOLD, R. E. 2007. Phylogeography of termites (Isoptera) from Oregon and Washington. *Sociobiology* 50:607–622.

MILLER, E. M. 1949. A Handbook of Florida Termites. University of Miami Press, Coral Gables, Florida.

NELSON, D. R. 1993. Methyl-branched lipids in insects, pp. 271–315, in D. W. Stanley-Samuelson, and D. R. Nelson (eds.). Insect Lipids: Chemistry, Biochemistry and Biology. University of Nebraska Press, Lincoln.

NELSON, L. J., COOL, L. G., FORSCHLER, B. T., and HAVERTY, M. I. 2001. Correspondence of soldier defense secretion mixtures with cuticular hydrocarbon phenotypes for chemotaxonomy of the termite genus *Reticulitermes* in North America. *J. Chem. Ecol.* 27:1449–1479.

NUTTING, W. L. 1990. Insecta: Isoptera, pp. 997–1032, in D. L. Dindal (ed.). Soil Biology Guide. John Wiley and Sons Inc., New York, New York.

PAGE, M., NELSON, L. J., BLOMQUIST, G. J., and SEYBOLD, S. J. 1997. Cuticular hydrocarbons as chemotaxonomic characters of pine engraver beetles (*Ips* spp.) in the *grandicollis* subgeneric group. *J. Chem. Ecol.* 23:1053–1099.

PAGE, M., NELSON, L. J., FORSCHLER, B. T., and HAVERTY, M. I. 2002. Cuticular hydrocarbons suggest three lineages in *Reticulitermes* (Isoptera: Rhinotermitidae) from North America. *Comp. Biochem. Physiol.* 131B:305–324.

PARTON, A. H., HOWSE, P. E., BAKER, R., and CLÉMENT, J. -L. 1981. Variation in the chemistry of the frontal gland secretion of European *Reticulitermes* species, pp. 193–209, in P. E. Howse, and J. L. Clément (eds.). Biosystematics of Social Insects. Academic Press, London.

PICKENS, A. L. 1934a. The biology and economic significance of the western subterranean termite, *Reticulitermes hesperus*, pp. 157–183, in C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe (eds.). Termites and Termite Control, 2nd edn. University of California Press, Berkeley, CA.

PICKENS, A. L. 1934b. The barren-lands subterranean termite, *Reticulitermes tibialis*, pp. 184–186, in C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe (eds.). Termites and Termite Control, 2nd edn. University of California Press, Berkeley, CA.

PRESTWICH, G. D. 1979. Chemical defense by termite soldiers. *J. Chem. Ecol.* 5:459–480.

PRESTWICH, G. D. 1983. Chemical systematics of termite exocrine secretions. *Annu. Rev. Ecol. Syst.* 14:287–311.

QUINTANA, A., REINHARD, J., FAURE, R., UVA, P., BAGNÈRES, A. -G., MASSIOT, G., and CLÉMENT, J. -L. 2003. Interspecific variation in terpenoid composition of defensive secretions of European *Reticulitermes* termites. *J. Chem Ecol.* 29:639–652.

R DEVELOPMENT CORE TEAM. 2004. A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. [http:/www.R-project.org].

REINHARD, J., QUINTANA, A., SRENG, L., and CLÉMENT, J.-L. 2003. Chemical signals inducing attraction and alarm in European *Reticulitermes* termites (Isoptera, Rhinotermitidae). *Sociobiology* 42:675–691.

RIPA, R., CASTRO, L., SU, N.-Y., and PALMA, P. 2002. Laboratory estimate of wood consumption rates by *Reticulitermes* sp. (Isoptera: Rhinotermitidae) in Chile. *Sociobiology* 39:285–290.

ROBINSON, J. W. 1989. The San Bernardinos. The mountain country from Cajon Pass to Oak Glen. Two centuries of changing use. Big Santa Anita Historical Society, Arcadia, CA.

SCHEFFRAHN, R. H., and SU, N. -Y. 1994. Keys to soldier and winged adult termites (Isoptera) of Florida. *Fla. Entomol.* 77:460–474.

SNYDER, T. E. 1934. American subterranean termites other than those of the Pacific Coast, pp. 187–195, in C. A. Kofoid, S. F. Light, A. C. Horner, M. Randall, W. B. Herms, and E. E. Bowe (eds.).

Termites and Termite Control, 2nd edn. University of California Press, Berkeley, California.

SNYDER, T. E. 1949. Catalog of the termites (Isoptera) of the world. *Smithsonian Misc. Coll.* 112:1–490.

SNYDER, T. E. 1954. Order Isoptera. The Termites of the United States and Canada. National Pest Control Association, New York.

SU, N.-Y., YE, W., RIPA, R., SCHEFFRAHN, R. H., and GIBLIN-DAVIS, R. M. 2006. Identification of Chilean *Reticulitermes* (Isoptera: Rhinotermitidae) inferred from three mitochondrial gene DNA sequences and soldier morphology. *Ann. Entomol. Soc. Am.* 99: 352–363.

SZALANSKI, A. L., AUSTIN, J. W., and OWENS, C. B. 2003. Identification of *Reticulitermes* spp. (Isoptera: Reticulitermatidae [sic]) from South Central United States by PCR-RFLP. *J. Econ. Entomol.* 95:1514–1519.

SZALANSKI, A. L., AUSTIN, J. W., MCKERN, J., and MESSENGER, M. T. 2006. Genetic evidence of a new subterranean termite species (Isoptera: Rhinotermitidae) from western United States and Canada. *Fla. Entomol.* 89:299–304.

TAKEMATSU, Y. 1999. The genus *Reticulitermes* (Isoptera:Rhinotermitidae) in Japan, with description of a new species. *Entomol. Sci.* 2:231–243.

TAKEMATSU, Y., and YAMAOKA, R. 1999. Cuticular hydrocarbons of *Reticulitermes* (Isoptera:Rhinotermitidae) in Japan and neighboring countries as chemotaxonomic characters. *Appl. Entomol. Zool.* 34:179–188.

TRIPODI, A. D., AUSTIN, J. W., SZALANSKI, A. L., MCKERN, J., CARROLL, M. K., SARAN, R. K., and MESSENGER, M. T. 2006. Phylogeography of *Reticulitermes* termites (Isoptera:Rhinotermitidae) in California inferred from mitochondrial DNA sequences. *Ann. Entomol. Soc. Am.* 99:697–706.

UVA, P., CLÉMENT, J. -L., AUSTIN, J. W., AUBERT, J., ZAFAGNINI, V., QUINTANA, A., and BAGNÈRES, A. -G. 2004. Origin of a new *Reticulitermes* termite (Isoptera: Rhinotermitidae) inferred from mitochondrial and nuclear DNA data. *Mol. Phylogenet. Evol.* 30:344–353.

WEESNER, F. M. 1965. The Termites of the United States. A Handbook. The National Pest Control Association, Elizabeth, New Jersey.

WEESNER, F. M. 1970. Termites of the Nearctic region, pp. 477–525, in K. Krishna, and F. M. Weesner (eds.). Biology of Termites, Vol. II. Academic Press, New York.

YE, W., LEE, C. -Y., SCHEFFRAHN, R. H., ALEONG, J. M., SU, N. -Y., BENNETT, G. W., and SCHARF, M. E. 2004. Phylogenetic relationships of nearctic *Reticulitermes* species (Isoptera:Rhinotermitidae) with particular reference to *Reticulitermes arenincola* Goellner. *Mol. Phyl. Evol.* 30:815–822.

ZALKOW, L. H., HOWARD, R. W., GELBAUM, L. T., GORDON, M. M., DEUTSCH, H. M., and BLUM, M. S. 1981. Chemical ecology of *Reticulitermes flavipes* (Kollar) and *R. virginicus* (Banks) (Rhinotermitidae): Chemistry of the soldier cephalic secretions. *J. Chem. Ecol.* 7:717–731.

Sympatric species with similar pheromone blends and conspecific male preference functions are hypothesized to be the major evolutionary forces acting on female signal traits (i.e., the amount and ratio of components emitted; Cardé and Haynes 2004). The former could impose directional selection on the pheromone signal and result in either reproductive character displacement via communication interference or reinforcement if hybridization is possible, but at a fitness cost (sensu Butlin 1987; Cardé and Haynes 2004). The evolutionary forces associated with male preference functions depend on preference function shape, which can be open ended, flat, or unimodal (Bentsen et al. 2006). Open-ended and flat preference functions result in directional and no selection, respectively. The type of selection that results from a unimodal preference function depends on the relationship between the preference function maximum and mean signal value (Bentsen et al. 2006). If the maximum and mean are coincident, selection will be stabilizing. If there is an asymmetry, selection will be directional or directional and stabilizing depending on whether or not the maximum occurs within the range of signal phenotypes produced. Open-ended, flat, and unimodal male preference functions for pheromone signal traits all have been documented (Allison and Cardé 2008).

In the short term, the potential for a trait to respond to selection will be determined by the amount of heritable variation. Empirical studies of the genetics of moth pheromones suggest the existence of both polygenic (Collins and Cardé 1985; Hunt et al. 1990; Gemeno et al. 2001; Svensson et al. 2002; Allison and Cardé 2006; Schek et al. 2006) and major gene effects (Klun and Maini 1979; Roelofs et al. 1987; Haynes and Hunt 1990) on female pheromone signaling traits in several species. Selection experiments have observed predictable changes in both the relative and absolute amounts of components (Sreng et al. 1989; Collins and Cardé 1989; Collins et al. 1990; Allison and Cardé 2007). A genetic basis for male response traits has been demonstrated in several lepidopteran species, including the European corn borer, *Ostrinia nubilalis* (Hübner) (Hansson et al. 1987; Roelofs et al. 1987); the brownheaded leafroller, *Ctenopseustis obliquana* (Walker) (Hansson et al. 1989); the pink bollworm, *Pectinophora gossypiella* (Saunders) (Collins and Cardé 1990); and the cabbage looper, *Trichoplusia ni* (Hübner) (Liu and Haynes 1994; Evenden et al. 2002). These studies suggest that both female signaling and male response traits have the potential to respond to selection.

Traits are often associated with each other and as a consequence, are seldom inherited independently (Lande 1979; Lande and Arnold 1983). Positive and negative genetic correlations can result in unexpected phenotypic changes. Negative correlations indicate trade-offs among traits and are a fundamental assumption underlying some evolutionary theory [e.g., life history theory (Roff 1992; Stearns 1992; Reznick et al. 2000)]. Few studies have examined the genetic architecture of moth pheromone signals. Significant genetic correlations were documented among the pheromone components of the cabbage looper, *T. ni* (both positive and negative, Gemeno et al. 2001) and the almond moth, *Cadra cautella* (Walker) (positive, Allison and Cardé 2006). These correlations suggest that the potential evolutionary trajectories of the female pheromone blend are genetically constrained. Variation among males in breadth of response and sensitivity to female pheromone blends has been hypothesized to be one potential source of variation in male mating success (Cardé and Charlton 1984). Negative correlations between the male response traits sensitivity and breadth of response have been the subject of speculation for over 20 years (see Cardé 1986). For example, it has been suggested that a negative correlation between sensitivity and the breadth of response profile may be a common feature of the insect peripheral nervous system and may explain the rarity of generalist natural enemies able to use moth sex pheromones as foraging cues (Cardé and Haynes 2004). This hypothesis is an extension of the extraordinary sensitivity and high specificity observed for moth pheromone signals.

The objective of this study was to examine empirically the relationship between two male pheromone response traits: sensitivity and breadth of response. The cabbage looper, *T. ni* is a model system for examination of this relationship because: (1) studies of variation in the female pheromone blend led to the discovery of a single gene mutation with major effects on the female pheromone blend but no effects on male preference (Haynes and Hunt 1990); and (2) after 49 generations of rearing pure mutant cultures, mutant male response levels to the mutant and wild-type pheromone blends were equivalent (Liu and Haynes 1994). The existence of mutant males with an increased breadth of response provides an opportunity to test for a trade-off between sensitivity and breadth of response.

Methods and Materials

Insect Rearing The wild-type colony of *T. ni* was initiated with insects obtained from a colony maintained at the USDA-ARS Insect Attractants, Behavior, and Basic Biology Research Laboratory in Gainesville, FL, USA and were maintained without further introductions for about 3 years until the time of these experiments. The mutant colony of *T. ni* was established from a rare pheromone phenotype discovered in a laboratory colony that originated from a field population in Riverside, CA, USA (Haynes and Hunt 1990). The pure mutant colony had been maintained in the laboratory for approximately 10 years at the time of

experimentation. Both colonies were reared on a pinto bean-based diet (Shorey and Hale 1965) at ambient room temperature, humidity, and light conditions. Pupae were sexed and males were transferred to 3.8 l paper cartons. The cartons were placed in an environmental chamber with a photoperiod of L/D 14:10 h and temperature regime of 27:25°C (photophase/scotophase). Eclosed males were collected daily and maintained on a 10% sugar solution until they were bioassayed.

Wind Tunnel Bioassays The wind tunnel used for male flight bioassays was designed based on the wind tunnel described by Miller and Roelofs (1978). Air was pushed through the tunnel with a variable speed fan controlled with a rheostat to 50 cm/s for all experiments. The pheromone plume was exhausted out of the assay room by a second fan at the downwind end of the wind tunnel. The flight section of the wind tunnel was approximately 1.5 m long by 1 m high at its center and was illuminated by overhead red and white lights at an intensity of 0.4 lx (measured 20 cm above the wind tunnel floor at its longitudinal center). A pheromone-loaded septum was suspended from a hook attached to the front of a 15×15 cm metal plate on top of a 20-cm tall metal platform located 50 cm from the upwind end of the tunnel. At the beginning of the fourth hour of the scotophase, males 3–4 days old were placed individually in 4 cm diam×8 cm high hardware cloth cages with aluminum foil lids and allowed to acclimate to the conditions of the wind tunnel room for 1 h. During the fifth to eighth hour of scotophase, caged males were placed on another 20 cm tall metal platform 150 cm downwind from the pheromone source. The lid was removed and the male was given 1 min to respond to the pheromone and scored (+ or −) for the following behaviors: wing fanning, taking flight (leaving the wire cage), locking on (zigzag flight in the pheromone plume), upwind flight, flying within 10 cm of the source, and source contact. Males were discarded after being assayed. If any males failed to respond after 1 min, they were tested to make sure they were capable of flight. Males incapable of flight were removed from the analysis.

Dose–Response Experiment Stock solutions of wild-type and mutant pheromone blends were formulated in HPLC grade hexane with synthetic pheromone components. These solutions consisted of a mixture of 12:Ac, *Z*5-12:Ac, *Z*7-12:Ac, 11-12:Ac, *Z*7-14:Ac, and *Z*9-14:Ac (see Table 1 for emission ratios). Blends were checked for accuracy by using a Hewlett Packard Series II Plus Gas Chromatograph connected to a Hewlett Packard 5972 Series Mass Selective Detector (Palo Alto, CA, USA) with a 30-m DB-Wax capillary column (0.25 mm ID, J&W Scientific, Folsom, CA, USA). Serial dilutions of both blends were prepared from this stock solution. Fifty microliters of each blend was loaded onto a hexane-extracted rubber septa (Thomas Scientific, Swedesboro, NJ, USA; 5×9 mm, red). Blends differed in the amount of the major pheromone component *Z*7-12:Ac with either 100 μg, 33 μg, 10 μg, 1 μg, or 100 ng of *Z*7-12:Ac per septum. After loading, septa were left in a fume hood overnight to allow solvent evaporation and then stored at −10°C until use. Loaded septa were treated in the same way in all subsequent experiments. Volatiles emitted from the highest dose septa of both blends were determined following the procedure of Haynes and Hunt (1990). These emission rates given in Table 1 are similar to that of wild-type and mutant females (Haynes and Hunt 1990).

Each day that moths were bioassayed, one or two males of each type (mutant or wild type) were flown individually to each blend/concentration combination, resulting in 20–40 males flown each day. A total of 67 mutant and 71 wild-type males were tested to each blend–concentration combination, with the exception that 65 mutant and 69 wild-type males were flown to the mutant blend at the 0.1-μg dose because of equipment malfunction. Results were analyzed with logistic regression (SAS Institute 1996) with moth–blend combination, concentration, and the interaction as model variables. This linear model allowed

Table 1 Targeted (and measured) emitted blend ratios of *Trichoplusia ni* sex pheromone components from septa

	Wild-type blend (measured)	Intermediate blends			Mutant blend (measured)
	W	WWM	WM	WMM	M
12:Ac	15 (12.4)	15	15	15	15 (18.6)
*Z*5-12:Ac	12 (14.5)	5.7	2.7	1.3	0.6 (0.7)
*Z*7-12:Ac	100 (100)	100	100	100	100 (100)
11-12:Ac	6 (8.9)	6	6	6	6 (8.2)
*Z*7-14:Ac	1.4 (2.1)	1.4	1.4	1.4	1.4 (1.5)
*Z*9-14:Ac	0.6 (0.6)	2.1	7.6	27	100 (101.9)

*Z*7-12:Ac is set at 100 for each blend. In order to achieve the desired emitted blend ratio 7-fold excess, *Z*7-14:Ac and *Z*9-14:Ac were loaded onto the septa. Measurements were made once (i.e., no replication) on the aged septa

treatments to be compared in terms of their overall level of response (tests for similar intercepts) and rates of response increase (tests for similar slope).

Intermediate Blend Response Experiment Five artificial blends were formulated: a wild-type, a mutant, and three intermediate blends (ratios of compounds are given in Table 1). For all five blends, the amounts of 12:Ac, 11-12:Ac, and *Z*7-14:Ac relative to *Z*7-12:Ac was 15:6:1.4:100. The amount of *Z*5-12:Ac gradually decreased from an optimum relative amount of 12 to 100 parts *Z*7-12:Ac in the wild-type blend through three intermediate blends to a minimum of 0.6 to 100 parts *Z*7-12:Ac in the mutant blend. Conversely, the amount of *Z*9-14:Ac gradually increased from a minimum of 0.6 to 100 parts *Z*7-12:Ac in the wild-type blend through three intermediate blends to a maximum of 100 to 100 parts *Z*7-12:Ac in the mutant blend. Fifty microliters of a blend was loaded onto a hexane-extracted rubber septum (Thomas Scientific, 5×9 mm, red) resulting in 33 μg of *Z*7-12:Ac.

During each day bioassays were run, three or four males of each type (mutant or wild type) were flown to each blend, resulting in 30 or 40 males flown each day. Sixty-six wild-type and mutant moths were flown to each blend. Results for mutant and wild-type males were analyzed separately by using logistic regression (SAS Institute 1996) looking for effects of blend. After these analyses, mutant and wild-type male responses were compared. This was done by combining data sets and adding moth-type and moth-type × blend interaction terms into the model.

Hybrid Response Experiment Male and female moths from the pure wild-type and mutant colonies were used to generate the F1 hybrid experimental moths. The rearing protocol was the same as outlined above. The following crosses were made (male × female): a pure wild-type line, W × W; a pure mutant line, M × M; and two hybrid lines, W × M, and M × W. Crosses were created by placing one male and female (each 2–5 days old) in 0.47 l cartons lined with blotter paper. Eggs were collected 3, 5, and 7 days after pairing. Male pupae were separated from females and kept in styrofoam containers. Upon emergence, males were transferred to 0.47 l cartons with access to a 10% sucrose solution until testing. A total of 23 W × W families were tested, 24 M × M families, 26 W × M families, and 30 M × W families.

Four of the blends from the intermediate blend response experiment above were used: the wild-type blend, the mutant blend, the WM blend, and the WMM blend (see Table 1 for emission ratios). Thirty-three micrograms of *Z*7-12:Ac (with other compounds in the corresponding amounts) was loaded onto a hexane-extracted rubber septum (Thomas Scientific, 5×9 mm, red). During each day of testing, one to three males of each cross were flown to each blend. Fifty W × W, 51 M × M, 55 W × M, and 55 M × W moths were flown to each blend. Results for each cross were compared by using logistic regression (SAS Institute 1996) comparing treatments in terms of their overall level of response (tests of intercepts) and blend preference (tests for slope). Linear model variables included the amount of *Z*9-14:Ac (to test for blend effect), the effect of cross type, and *Z*9-14:Ac by cross-type interactions.

Results

Dose–Response Experiment: Mutant Males Overall, mutant males did not respond differently to the wild-type or mutant pheromone blend at any behavior category (Fig. 1). For the behaviors wing fanning and taking flight, there were no significant concentration effects ($P=0.092$ and $P=0.056$, respectively; data not shown). For all other behaviors, strong concentration effects were observed ($P<0.001$; Fig. 1). For mutant males, there were no significant differences among blend types in terms of level of response (i.e., intercept), or rate of response increase with concentration (i.e., slope) for any of the behaviors measured: wing fanning ($P=0.643$ for a test of similar intercept and $P=0.165$ for a test of similar slope), taking flight ($P=0.815$ for a test of similar intercept and $P=0.323$ for a test of similar slope), locking on ($P=0.056$ for a test of similar intercept and $P=0.355$ for a test of similar slope), flying upwind ($P=0.067$ for a test of similar intercept and $P=0.150$ for a test of similar slope), coming within 10 cm of the source ($P=0.311$ for a test of similar intercept and $P=0.699$ for a test of similar slope), and source contact ($P=0.330$ for a test of similar intercept and $P=0.475$ for a test of similar slope; Fig. 1). These results indicate that there is no discrimination by the mutant males between the wild-type and mutant pheromone blends and that the effects of concentration on response level were parallel. Because the behavioral responses of the mutant males to the mutant and wild-type blends did not differ, their responses to these two treatments were combined for comparison to the wild-type male responses to the wild-type and mutant pheromone blends.

Wild-Type Males The response level of wild-type males to the mutant pheromone blend was lower compared to the level of response of wild-type males to the wild-type pheromone blend, or when compared to the mutant response to either blend across all behaviors ($P<0.001$ for all tests of similar intercept; Fig. 1). The rate of response increase of wild-type males to the mutant pheromone blend was greater than the combined mutant male responses for the behaviors of flying upwind and coming within 10 cm of the pheromone source ($P=0.031$ and $P=0.038$, respectively,

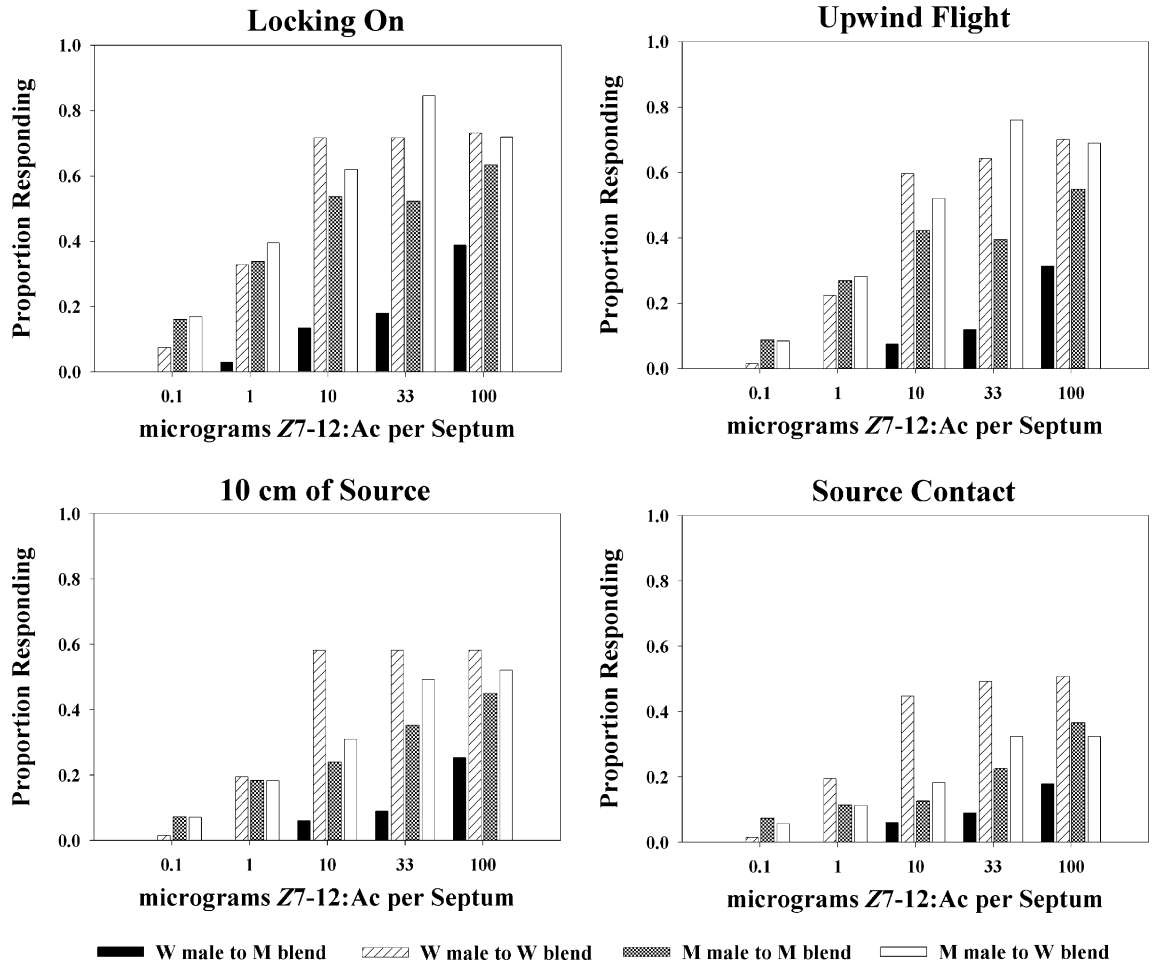

Fig. 1 The effect of pheromone dose on the proportion of male *Trichoplusia ni* engaging in the behaviors of "locking on", "upwind flight", "coming within 10 cm of the source", and "source contact" in response to synthetic pheromone sources. Wild-type male responses to the mutant (*solid black bars*) and wild-type (*hatched bars*) pheromone blends and mutant male responses to the mutant (*stippled bars*) and wild-type (*open bars*) pheromone blends are shown

for tests of similar slope; Fig. 1). For all other behaviors, the slopes were not significantly different ($P>0.05$ for tests of similar slope; Fig. 1). When the wild-type male response to the wild-type blend was compared to the mutant male responses to both blends, there were no significant differences in the rate of increase of response [tests for similar slope: $P=0.090$ for wing fanning; $P=0.212$ for taking flight; $P=0.306$ for locking on; $P=0.188$ for upwind flight; $P=0.853$ for coming within 10 cm of the source; and $P=0.940$ for source contact (Fig. 1)]. When the overall level of response was examined, there were differences between mutant male and wild-type male responses to the wild-type pheromone blend. Wild-type males had lower levels of response than mutant males for the behaviors wing fanning and taking flight ($P<0.001$ and $P=0.001$, respectively, for tests of similar intercept). There were no significant differences between wild-type and mutant male responses for the behaviors locking on and upwind flight ($P=0.909$ and $P=0.948$, respectively, tests of similar intercept; Fig. 1). Wild-type males were more likely to fly within 10 cm and contact the wild-type pheromone source than mutant males ($P=0.011$ and $P<0.001$, respectively, test of similar intercept; Fig. 1).

Intermediate Blend Response Experiment: Mutant Males Mutant male moths did not discriminate among blends. For none of the behaviors observed did blend variation explain the variation seen [wing fanning ($P=0.624$), taking flight ($P=0.382$), locking on ($P=0.115$), upwind flight ($P=0.238$), coming within 10 cm of the source ($P=0.116$), and source contact ($P=0.775$)] (Fig. 2; data for the behaviors wing fanning and taking flight not shown). These results indicate that mutant males do not discriminate among the mutant, wild-type, or intermediate pheromone blends at any behavioral category.

Wild-Type Males Wild-type males showed the highest response to the wild-type blend. Their responses decreased

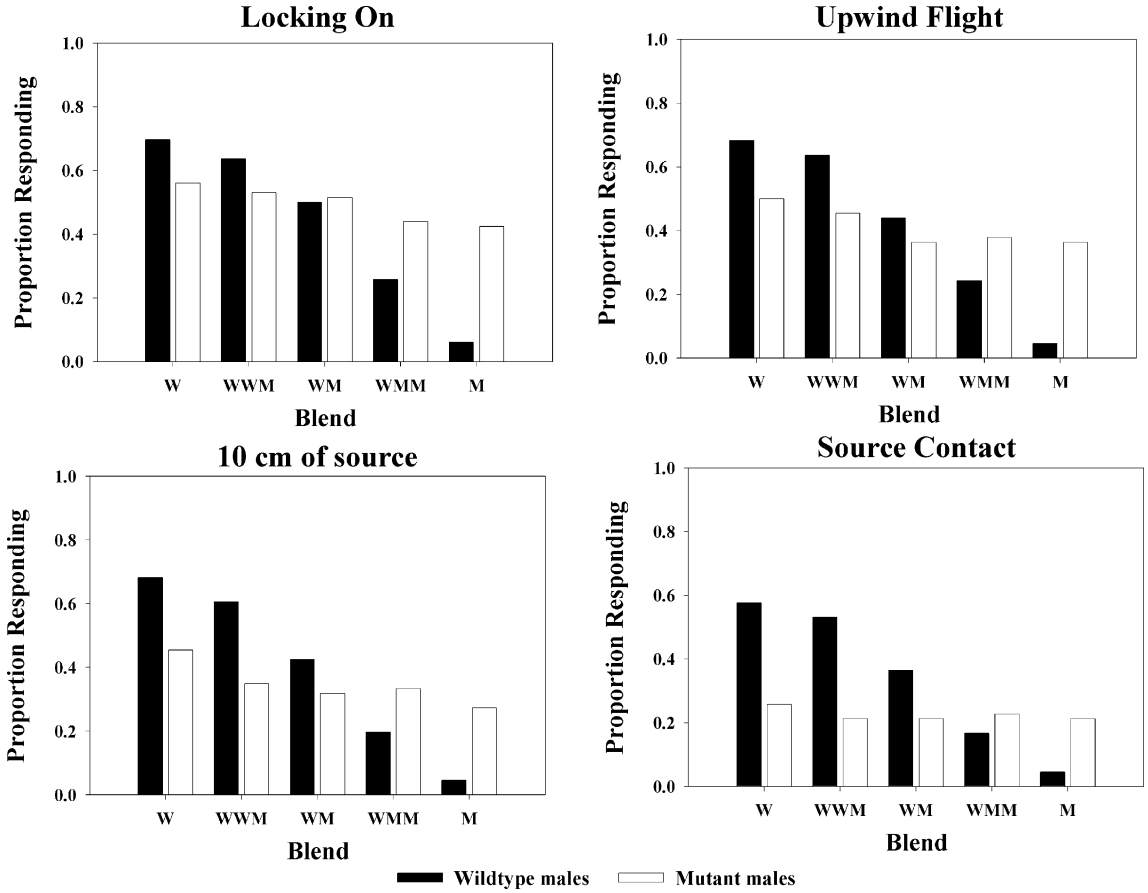

Fig. 2 The effect of pheromone blend on the proportion of male *Trichoplusia ni* engaging in the behaviors of "locking on", "upwind flight", "coming within 10 cm of the source", and "source contact" in response to synthetic pheromone sources. Wild-type and mutant male responses are shown by the *solid black* and *open bars*, respectively. For all five blends (see Table 1), the amounts of 12:Ac, 11-12:Ac, and *Z*7-14:Ac relative to *Z*7-12:Ac were 15:6:1.4:100. The amount of *Z*5-12:Ac gradually decreased from an optimum in the wild-type blend through three intermediate blends to a minimum in the mutant blend. Conversely, the amount of *Z*9-14:Ac gradually increased from a minimum in the wild-type blend through three intermediate blends to a maximum in the mutant blend

as the blend gradually changed to that of mutant females. For the behaviors wing fanning (P=0.002), taking flight (P=0.005), locking on (P<0.001), upwind flight (P< 0.001), coming within 10 cm of source (P<0.001), and source contact (P<0.001), blend had a significant effect (Fig. 2). For all behaviors, wild-type males were most responsive to the wild-type blend, and response decreased progressively through the WWM, WM, and WMM and reached a minimum for the mutant blend. These results illustrate the discrimination of wild-type males for the wild-type blend over the mutant blend, and that this discrimination is graded becoming more evident as the blend diverges from the wild type.

The responses of the wild-type and mutant males to the five pheromone blends were also compared directly. No differences in the responses of the wild-type and mutant males were observed for the behavioral categories wing fanning (P=0.503 for a test of similar intercept and P= 0.242 for a test of similar slope) or taking flight (P=0.302 for a test of similar intercept and P=0.220 for a test of similar slope). There was no difference among wild-type and mutant males in the level of response (P=0.056 for a test of similar intercept) for the behavior locking on, but there was a significant difference in blend preference (P< 0.001 for a test of similar slope; Fig. 2). For the behaviors upwind flight, coming within 10 cm, and source contact, there was a difference in blend preference (P<0.001 for all three behaviors for a test of similar slope) with wild-type males showing a preference for the wild-type blend. Similarly, wild-type males had higher levels of response to the wild-type blend for the behaviors upwind flight (P< 0.001 for a test of similar intercept), coming within 10 cm (P<0.001 for a test of similar slope), and source contact (P<0.001 for a test of similar slope; Fig. 2).

Hybrid Response Experiment: Hybrid Males Offspring of both types of hybrid crosses, W × M and M × W (male × female), behaved similarly across behavioral categories

(Fig. 3). For the behaviors wing fanning and taking flight, there was no blend effect (P=0.864 and P=0.300). For the behavior wing fanning, there were no significance differences between crosses (P=0.790 for a test of similar intercept and P=0.212 for a test of similar slope). At the next behavioral category, taking flight, there were no significant differences in the level of response (P=0.090 for a test of similar intercept), but there was a difference in how the crosses reacted to the different blends (P=0.024 for a test of similar slopes). For the behavior locking on, there was a strong and significant blend effect (P<0.001) with hybrids showing a preference for the wild-type blend (Fig. 3). There was no significant hybrid-type (maternal wild-type or mutant crosses) effect (P=0.334 for a test of similar intercept and P=0.602 for a test of similar slope). For the remaining behaviors, significant preferences for the wild-type blend and no hybrid effects were observed [upwind flight (P<0.001 for a blend effect, P=0.322 for a test of similar intercept, and P=0.858 for a test of similar slope); approach within 10 cm (P<0.001 for a blend effect, P=0.876 for a test of similar intercept, and P=0.285 for a test of similar slope); and source contact (P=0.022 for a blend effect, P=0.676 for a test of similar intercept, and P=0.656 for a test of similar slope)].

Hybrid vs. Parental Males For those behaviors where there were no significant differences between the responses of the two hybrid strains, the two strains were combined and compared to the pure wild-type and mutant lines. For the behavior taking flight, the W × M and M × W (male × female) hybrid offspring were tested separately. No significant differences between the combined hybrid treatment and either of the pure wild-type or mutant crosses (hybrids vs. pure wild-type males, P=0.066 for a test of similar

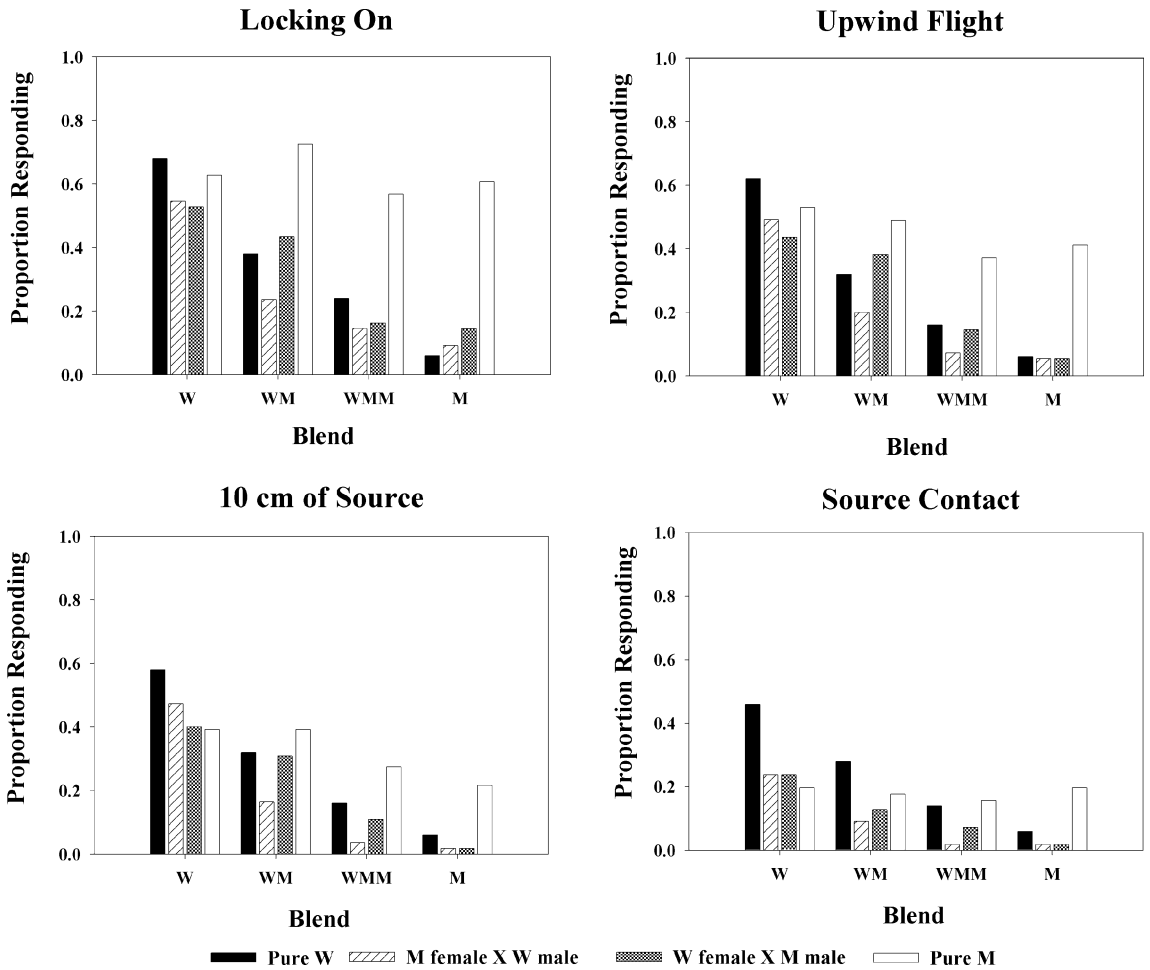

Fig. 3 The effect of pheromone blend on the proportion of male *Trichoplusia ni* engaging in the behaviors of "locking on", "upwind flight", "coming within 10 cm of the source", and "source contact" in response to synthetic pheromone sources. Pure wild-type (*solid black bars*), mutant female × wild-type male hybrid (*hatched bars*), wild-type female × mutant male (*stippled bars*), and pure mutant (*open bars*) male responses to the differing pheromone blends are shown. For all four blends, see Table 1

intercept and $P=0.190$ for a test of similar slope; hybrids vs. pure mutant males, $P=0.946$ for a test of similar intercept and $P=0.950$ for a test of similar slope) were observed for the behavior wing fanning. Similarly, for the behavior taking flight, no differences were observed between the M × W hybrid males and the pure wild-type ($P=0.558$ for a test of similar intercept and $P=0.198$ for a test of similar slope) and mutant ($P=0.600$ for a test of similar intercept and $P=0.519$ for a test of similar slope) males. There were no differences between the W × M hybrid males and the pure mutant males ($P=0.208$ for a test of similar intercept and $P=0.060$ for a test of similar slope). The W × M males were less sensitive than wild-type males ($P=0.031$ for a test of similar intercept), but there was no significant difference in blend preference ($P=0.087$ for a test of similar slope). For all subsequent behavioral categories, both hybrid strains were combined for statistical analysis as in the wing fanning behavioral category. Hybrid males did not differ from the pure wild-type moths in blend discrimination ($P=0.092$ for a test of similar slope), both hybrids and pure wild-type males preferred the wild-type blend over the mutant blend. The hybrids did show a decreased level of response compared to wild-type moths ($P=0.027$ for a test of similar intercept). Hybrid and pure mutant moths differed in both blend discrimination and level of response ($P<0.001$ for a test of similar intercept and $P<0.001$ for a test of similar slope). For the behavior upwind flight, the hybrid male response did not differ from the pure wild-type male response ($P=0.099$ for a test of similar intercept and $P=0.697$ for a test of similar slope) indicating similar levels of response and blend discrimination. The hybrid males did differ from the pure mutant males in blend discrimination ($P<0.001$ for a test of similar slope), and there was a borderline non-significant difference in the level of response ($P=0.052$ for a test of similar intercept). For the behavior coming within 10 cm of the source, there was no difference in the blend preference or level of response of the hybrid and pure wild-type males ($P=0.194$ for a test of similar intercept and $P=0.124$ for a test of similar slope). Although there was no difference in the level of response of hybrid and pure mutant males ($P=0.846$ for a test of similar intercept), there was a difference in blend discrimination ($P<0.001$ for a test of similar slope). Hybrid males exhibited a preference for the normal blend, while pure mutant males did not. For the final behavior, source contact, there was no difference in the blend discrimination of hybrid and pure wild-type males ($P=0.550$ for a test of similar intercept); both had a preference for the wild-type over the mutant blend. Hybrids did have a reduced level of response compared to wild-type males ($P<0.001$ for a test of similar intercept). The level of hybrid male response was more similar to the level of response of pure mutant males ($P=0.930$ for a test of similar intercept). Hybrid males did show greater blend discrimination than pure mutant males ($P=0.001$ for a test of similar slope). These results indicate that hybrid males have wild-type male preferences for the wild-type over the mutant pheromone blend, but like mutant males have reduced pheromone sensitivity.

Discussion

Because the adaptive value of pheromone communication is mate location, selection should favor male preferences for (Butlin and Ritchie 1989; Groot et al. 2006; Allison and Cardé 2008) and maximum male sensitivity to (Roelofs 1978; Linn et al. 1987) the most common blend emitted by females. As a result, variation in the presence and relative amounts of components in the pheromone blend may contribute to variation in female mating success. The resultant variation in mating success is hypothesized to impose stabilizing selection on the pheromone signal and ultimately constrain signal evolution (Butlin and Ritchie 1989). This paradigm is the basis for a model of moth sex pheromone evolution that has been referred to as the "stasis" model (Allison and Cardé 2008). An alternate model, asymmetric tracking, emphasizes an expected asymmetry in the strength of sexual selection that acts on female signal and male response traits due to differential investment in the zygote (Phelan 1997). Accordingly, males are predicted to optimize individual fitness by rapidly locating females and mating multiply, whereas females are not expected to be limited in mating opportunities. As a consequence, this model predicts that the female pheromone blend is free to change via non-adaptive evolutionary processes (i.e., drift) and that male response traits (e.g., breadth of response and sensitivity) will be selected simultaneously to track changes in the female pheromone blend.

The primary difference between the two models involves the predicted shape of the male preference function. The stasis model emphasizes unimodal male preference functions that constrain signal evolution, whereas the asymmetric tracking hypothesis does not (see Butlin and Ritchie 1989; Phelan 1997). The stasis model predicts a male response phenotype with maximal sensitivity to the most common blend and a narrow preference function with an optimum coincident with the most common pheromone blend. Similarly, the asymmetric tracking hypothesis predicts maximal sensitivity to, and an optimum coincident with, the most common blend. It differs from the stasis model in that it predicts a broad male preference function. Implicit to the prediction of maximal sensitivity to the most common pheromone blend is a trade-off in sensitivity among blends

(i.e., high sensitivity to one signal comes at the expense of reduced sensitivity to other signals). In the absence of this trade-off, the optimal male phenotype would appear to be high sensitivity to the complete range of signals defined by the male preference function.

Little empirical data on the relationship between the traits breadth of response and sensitivity in male moths to pheromone blends exist. The two traits are hypothesized to be negatively correlated, and the optimization of male response phenotypes limited (i.e., increases in sensitivity to one or more blends results in reduced sensitivity to other blends; Cardé 1986; Cardé and Haynes 2004). Independent studies have documented that mutant males have a broader response profile (Liu and Haynes 1994) and are less sensitive at the peripheral level (Baker and Domingue, pers. comm.) than wild-type males. Simulations of a population genetics model predict that if changes in sensitivity are not associated with trade-offs in breadth of male response, that selection will favor males with broad response phenotypes (Butlin and Trickett 1997). When a negative correlation between breadth of response and sensitivity was included in the model, the simulations predicted that selection would favor males with narrow response phenotypes and high sensitivity.

In this study, mutant male sensitivity and rate of response increase to increasing pheromone concentrations were independent of the pheromone blend. In contrast, wild-type males were more responsive to the wild-type than the mutant pheromone blend (Figs. 1 and 2). When the responses of wild-type males to the wild-type pheromone blend were compared to the combined mutant male responses, the level of response sensitivity differed among strains (Fig. 1). These results are consistent with the results of earlier wind tunnel and field trapping studies (Liu and Haynes 1994; Zhu et al. 1997) and suggest a trade-off between sensitivity and breadth of response.

The terminal behaviors in the mate location sequence (coming within 10 cm and source contact) are likely the best indicator of the ability of an individual male to locate a mate. For both of the behaviors, coming within 10 cm and source contact, wild-type males were more likely to complete the behaviors to the wild-type blend than mutant males to either blend (Fig. 1). Similar results have been reported for *Grapholita molesta* (Baker et al. 1981) and *P. gossypiella* (Linn and Roelofs 1985) in which males were most sensitive to the female produced pheromone blend. Because wild-type males are more sensitive to the wild-type pheromone, mutant males may be at a competitive disadvantage when competing with wild-type males for mating opportunities with wild-type females [an argument supported empirically by Evenden and Haynes (2001)]. Conversely, wild-type males do not respond well to mutant pheromone and may be at a disadvantage when competing with mutant males for mutant females. Interestingly, although wild-type and mutant hybrid males and pure wild-type males have similar blend preferences at the level of source contact, wild-type males are more sensitive than hybrid males (Fig. 3). These data suggest that assortative mating could occur in mixed populations (Zhu et al. 1997) and provide a mechanism for reinforcement and diversification of pheromone signals that could ultimately result in speciation.

The mutant males did not show a preference for any blend at any behavioral category. They responded equally to the normal, intermediate, and mutant blends. This lends support to the hypothesis that the mutant males underwent a general broadening of response as they became equally responsive to the normal and mutant pheromone blends.

Overall, it appears that at the same time that mutant males experienced a broadening of response, they simultaneously experienced a reduction in sensitivity. This documents a trade-off between sensitivity and breadth of response in *T. ni*. Population genetic simulations run by Butlin and Trickett (1997) suggest that when a trade-off between sensitivity and breadth of response exists, selection should favor a narrower, more sensitive response profile. It remains to be seen if this trade-off between sensitivity and breadth of response is present in other moth pheromone systems. The trade-off in sensitivity was observed with lures releasing up to 2 ng/min of *Z*7-12:Ac; the mean amount of *Z*7-12:Ac emitted by calling females has been estimated to range from 2 to 11 ng/min of *Z*7-12:Ac (see Hunt and Haynes 1990; Liu and Haynes 1994). If trade-offs are a general phenomenon, it could mean that the evolution of novel pheromone signals could involve an intermediate step during which some individuals have a broad response, but lower sensitivity. Presumably, selection could then act on the male response phenotype, narrowing the response peak and increasing sensitivity to the new pheromone blend. For now, our ability to make such generalizations is limited by a lack of data for other species.

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References

ALLISON, J. D., and CARDÉ, R. T. 2006. Heritable variation in the sex pheromone of the almond moth, *Cadra cautella*. *J. Chem. Ecol.* 32:621–641.

ALLISON, J. D., and CARDÉ, R. T. 2007. Bidirectional selection for novel pheromone blend ratios in the almond moth, *Cadra cautella*. *J. Chem. Ecol.* 33:2293–2307.

ALLISON, J. D., and CARDÉ, R. T. 2008. Male pheromone blend preference function measured in choice and no-choice wind-tunnel trials with *Cadra cautella*. *Anim. Behav.* 75:259–266.

BAKER, T. C., MEYER, W., and ROELOFS, W. L. 1981. Sex-pheromone dosage and blend specificity of response by oriental fruit moth males. *Entomol. Exp. Appl.* 30:269–279.

BENTSEN, C. L., HUNT, J., JENNIONS, M. D., and BROOKS, R. 2006. Complex multivariate sexual selection on male acoustic signaling in a wild population of *Teleogryllus commodus*. *Am. Nat.* 167: E102–E116.

BUTLIN, R. K. 1987. Speciation by reinforcement. *Trends Ecol. Evol.* 2:8–13.

BUTLIN, R. K., and RITCHIE, M. G. 1989. Genetic coupling in mate recognition systems: what is the evidence? *Biol. J. Linn. Soc.* 37:237–246.

BUTLIN, R. K., and TRICKETT, A. J. 1997. Can population genetic simulations help to interpret pheromone evolution?, pp. 548–562, in R. T., Cardé, A. K., and Minks (eds.). Insect Pheromone Research: New Directions Chapman and Hall, New York.

CARDÉ, R. T. 1986. The role of pheromones in reproductive isolation and speciation of insects, pp. 303–317, in M. D., and Huettel (eds.). Genetics of Invertebrate Behavior: Progress and Prospects-Plenum, New York.

CARDÉ, R. T., and CHARLTON, R. E. 1984. Olfactory sexual communication in Lepidoptera: strategy, sensitivity and selectivity, pp. 241–265, in T. Lewis (ed.). Insect Communication Academic, London.

CARDÉ, R. T., and HAYNES, K. F. 2004. Structure of the pheromone communication channel in moths, pp. 283–332, in R. T., Cardé, K. F., and Haynes (eds.). Advances in Insect Chemical Ecology. Cambridge University Press, Cambridge.

COLLINS, R. D., and CARDÉ, R. T. 1985. Variation in and heritability of aspects of pheromone production in the pink bollworm moth, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Ann. Entomol. Soc. Am.* 78:229–234.

COLLINS, R. D., and CARDÉ, R. T. 1989. Selection for altered pheromone-component ratios in the pink bollworm moth, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *J. Insect Behav.* 2:609–621.

COLLINS, R. D., and CARDÉ, R. T. 1990. Selection for increased pheromone response in the male pink-bollworm, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Behav. Genet.* 20:325–331.

COLLINS, R. D., ROSENBLUM, S. L., and CARDÉ, R. T. 1990. Selection for increased pheromone titre in the pink bollworm moth, *Pectinophora gossypiella* (Lepidoptera: Gelechiidae). *Physiol. Entomol.* 15:141–147.

EVENDEN, M. L., and HAYNES, K. F. 2001. Potential for the evolution of resistance to pheromone-based mating disruption tested using two pheromone strains of the cabbage looper, *Trichoplusia ni*. *Entomol. Exp. Appl.* 100:131–134.

EVENDEN, M. L., SPOHN, B. G., MOORE, A. J., PREZIOSI, R. F., and HAYNES, K. F. 2002. Inheritance and evolution of male response to sex pheromone in *Trichoplusia ni* (Lepidoptera: Noctuidae). *Chemoecology* 12:53–59.

GEMENO, C., MOORE, A. J., PREZIOSI, R. F., and HAYNES, K. F. 2001. Quantitative genetics of signal evolution: a comparison of the pheromonal signal of two populations of the cabbage looper, *Trichoplusia ni*. *Behav. Genet.* 31:157–165.

GROOT, A. T., HOROVITZ, J. L., HAMILTON, J., SANTANGELO, R. G., SCHAL, C., and GOULD, F. 2006. Experimental evidence for interspecific directional selection on moth pheromone communication. *Proc. Natl. Acad. Sci. U. S. A.* 103:5858–5863.

HANSSON, B. S., LÖFSTEDT, C., and ROELOFS, W. L. 1987. Inheritance of olfactory response to sex pheromone components in *Ostrinia nubilalis*. *Naturwissenchaften* 74:497–499.

HANSSON, B. S., LÖFSTEDT, C., and FOSTER, S. P. 1989. Z-linked inheritance of male olfactory response to sex pheromone components in two species of tortricid moths, *Ctenopseustis obliquana* and *Ctenopseustis* sp. *Entomol. Exp. Appl.* 53:137–145.

HAYNES, K. F., and HUNT, R. E. 1990. A mutation in pheromonal communication system of cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:1249–1257.

HUNT, R. E., and HAYNES, K. F. 1990. Periodicity in the quantity and blend ratios of pheromone components in glands and volatile emissions of mutant and normal cabbage looper moths, *Trichoplusia ni*. *J. Insect Physiol.* 36:769–774.

HUNT, R. E., ZHAO, B., and HAYNES, K. F. 1990. Genetic aspects of interpopulational differences in the pheromone blend of the cabbage looper moth, *Trichoplusia ni*. *J. Chem. Ecol.* 16:2935–2946.

KLUN, J. A., and MAINI, S. 1979. Genetic basis of an insect chemical communication system: the European corn borer. *Environ. Entomol.* 8:423–426.

LANDE, R. 1979. Quantitative genetic analysis of multivariate evolution applied to brain: body area allometry. *Evolution* 33:402–416.

LANDE, R., and ARNOLD, S. J. 1983. The measurement of selection on correlated characters. *Evolution* 37:1210–1226.

LINN, C. E., and ROELOFS, W. L. 1985. Response specificity of male pink bollworm moths to different blends and dosages of sex pheromone. *J. Chem. Ecol.* 11:1583–1590.

LINN, C. E. Jr., CAMPBELL, M. G., and ROELOFS, W. L. 1987. Pheromone components and active spaces: what do moths smell and where do they smell it? *Science* 237:650–652.

LIU, Y. -B., and HAYNES, K. F. 1994. Evolution of behavioral responses to sex pheromone in mutant laboratory colonies of *Trichoplusia ni*. *J. Chem. Ecol.* 20:231–237.

LÖFSTEDT, C., and KOZLOV, M. 1997. A phylogenetic analysis of pheromone communication in primitive moths, pp. 473–489, in R. T., Cardé, A. K., and Minks (eds.). Insect Pheromone Research: New Directions. Cambridge University Press, Cambridge.

MCELFRESH, J. S., and MILLAR, J. G. 2001. Geographic variation in the pheromone system of the saturniid moth *Hemileuca eglanterina*. *Ecology* 82:3505–3518.

MILLER, J. R., and ROELOFS, W. L. 1978. Sustained-flight tunnel for measuring insect responses to wind-borne sex pheromones. *J. Chem. Ecol.* 4:187–198.

PHELAN, P. L. 1997. Evolution of mate-signaling in moths: phylogenetic considerations and predictions from the asymmetric tracking hypothesis, pp. 240–256, in J. C., Choe, B. J., and Crespi (eds.). The Evolution of Mating Systems in Insects and Arachnids. Cambridge University Press, New York.

REZNICK, D., NUNNEY, L., and TESSIER, A. 2000. Big houses, big cars, superfleas and the cost of reproduction. *Trends Ecol. Evol.* 15:421–425.

ROELOFS, W. L. 1978. Threshold hypothesis for pheromone perception. *J. Chem. Ecol.* 4:685–699.

ROELOFS, W. L., and COMEAU, A. 1969. Sex pheromone specificity: taxonomic and evolutionary aspects in Lepidoptera. *Science* 165:398–399.

ROELOFS, W. L., GLOVER, T., TANG, X., SRENG, I., ROBBINS, P., ECKENRODE, C., LÖFSTEDT, C., HANSSON, B. S., and BENGTSSON, B. O. 1987. Sex pheromone production and perception in European corn borer moths is determined by both autosomal and sex-linked genes. *Proc. Natl. Acad. Sci. U. S. A.* 84:7585–7589.

ROFF, D. A. 1992. The Evolution of Life Histories. Chapman and Hall, London.

SAS 1996. SAS User's Guide: Basics. 6.03th edn.SAS Institute Inc, Cary, NC.

SCHEK, A. L., GROOT, A. T., WARD, C. M., GEMENO, C., WANG, J., BROWNIE, C., SCHAL, C., and GOULD, F. 2006. Genetics of sex

pheromone blend differences between *Heliothis viriscens* and *Heliothis subflexa*: a chromosome mapping approach. *J. Evol. Biol.* 19:600–617.

SHOREY, H. H., and HALE, R. L. 1965. Mass-rearing of the larvae of nine noctuid species on a simple artificial medium. *J. Econ. Entomol.* 58:522–524.

SRENG, I., GLOVER, T., and ROELOFS, W. 1989. Canalization of the redbanded leafroller moth sex pheromone blend. *Arch. Insect Biochem. Physiol.* 10:73–82.

STEARNS, S. C. 1992. The Evolution of Life Histories. Oxford University Press, Oxford.

SVENSSON, G. P., RYNE, C., and LÖFSTEDT, C. 2002. Heritable variation of sex pheromone composition and the potential for evolution of resistance to pheromone-based control of the Indian meal moth, *Plodia interpunctella*. *J. Chem. Ecol.* 28:1447–1461.

TUMLINSON, J. H., YONCE, C. E., DOOLITTLE, R. E., HEATH, R. R., GENTRY, C. R., and MITCHELL, E. R. 1974. Sex pheromones and reproductive isolation of the lesser peachtree borer and the peachtree borer. *Science* 185:614–616.

ZHU, J., CHASTAIN, B. C., SPOHN, B. G., and HAYNES, K. F. 1997. Assortative mating in two pheromone strains of the cabbage looper moth, *Trichoplusia ni*. *J. Insect Behav.* 10:805–808.

Aquatic animals often use chemical alarm cues to signal the risks associated with predation (Chivers and Smith 1998; Wisenden 2000). In many teleost fishes, olfactory cues are released following mechanical damage to the skin, which in turn stimulates a stereotypic avoidance behavior; the fright reaction (von Frisch 1938; Schutz 1956; Mathis and Smith 1993; Brown and Smith 1997). Specialized epidermal cells, club cells, are believed to contain the alarm substances responsible for eliciting this behavior (Pfeiffer 1963). In several species, the number of club cells decreases in sexually mature or testosterone-treated male fish (Smith 1973, 1976). This was suggested to be an adaptive mechanism to prevent the release of alarm substances during abrasive male–male interactions that commonly occur during the breeding season. In this way, the display of aggressive spawning behaviors such as abrasive contact and fighting would not result in the release of false alarm signals. Although less pronounced than in males, females may also experience physical abrasion during the breeding season as a result of active courtship behavior and nest-building. Taken together, this indicates that the potential to display chemical alarm cues is suppressed during the breeding season in fishes. However, to our knowledge the effect of sexual maturation on the response to olfactory cues from damaged conspecifics has not been reported previously.

The present study reports the occurrence of a suppressed olfactory induced fright reaction in late stages of sexual maturation in crucian carp, *Carassius carassius* (L.) (Cypriniformes: Cyprinidae). Maturation of gonads and plasma levels of gonadal steroids suggest that this suppression occurs around the time of ovulation in females.

Methods and Materials

Experimental Animals In May 2005, crucian carp were caught in a fishing pot with corn and bread as bait in a small lake on the outskirts of the city of Oslo, Norway, and transported to the aquaria facilities at the Department of Molecular Biosciences, University of Oslo. The fish were kept in 1,000 l aquaria supplied with through-flowing freshwater, and maintained under a photoperiod of light–dark (L–D) 12:12 h and a temperature of 10°C. The fish were fed three times a week with commercial pelleted feed (Modulfôr, Ewos, Norway). All experimental procedures were made in accordance with national legislation and institutional guidelines at the University of Oslo.

Skin Extract Three specimens of crucian carp were killed by decapitation and flayed. Skin samples were immediately homogenized manually in cold distilled water (50 g/l). The homogenate was centrifuged, and the supernatant frozen immediately at –20°C in 1 ml aliquots.

Experimental Procedure In June 2006, 24 female and 18 male specimens (body weight, 6–12 g) were transferred from the holding tank to a 25 l observation aquarium at approximately 4 PM, where they were kept singly. The photoperiod was set to L–D 13:11 h and the temperature was changed gradually to room temperature (approximately 18°C), in order to induce the onset of ovulation and increase milt volume (Dulka et al. 1987). Between 9:30 AM and 12:00 PM on the following day, the fish were exposed to 0.2 ml of skin extract, injected into the water through a polyethylene tube. The subsequent reaction to the skin extract was categorized as either a fright reaction or no fright reaction, by using behavioral characteristics previously reported for this species (Hamdani et al. 2000; Höglund et al. 2005). In short, the fright response in crucian carp is characterized by fish displaying rapid tail movements and swimming to the bottom floor of the aquaria. After 12 min of skin extract exposure, the fish were netted and blood samples taken for the measurement of plasma levels of sex steroid hormones. Gonadal maturation of these fish was characterized as either strippable or non-strippable, depending on whether milt/eggs could be readily stripped following gentle abdominal pressure. This showed that 18 of 24 females had strippable eggs (ovulation) and 12 of 18 males had strippable milt.

Measurement of Sex Steroid Hormones The major sex steroid hormones in mature male and female teleost fish are 11-ketotestosterone (11-KT) and testosterone (T), and T and 17β-estradiol (E_2), respectively (Borg 1994). The plasma levels of 11-KT, T, and E_2 were measured by specific radioimmunoassay (RIA) according to Pall et al. (2002), a method validated for the use of small volumes of plasma. In brief, individual plasma samples (15–50 µl) were diluted to 300 µl with RIA buffer and heated at 80°C for 60 min. The samples were centrifuged at 3,400×*g* (Hettich bench centrifuge, Model Rotanta 46 R, Hettich Instruments, Tuttlingen, Germany) for 15 min, after which the supernatant was removed and stored at 4°C until assay. Fifty µl aliquots of the sample, followed by 50 µl of RIA buffer were added first to incubation tubes. Fifty µl of the radio-labelled steroid were added to each tube (^{3}H-T and ^{3}H-E_2 was purchased from Amersham International and ^{3}H 11-KT was a gift from Dr. A.P. Scott, CEFAS, UK) (30,000–35,000 dpm/50 µl). Two hundred µl of steroid antiserum were also added to the tube (a gift from Dr. Helge Tveiten, University of Tromsø). All samples were run in duplicate and a mean plasma level of steroid was calculated for each fish and used in further statistical analyses (see below). The vials were vortexed and incubated overnight at 4°C. Free, unbound steroid was separated from bound steroid with dextran charcoal suspension (activated charcoal, acid-washed, 100–400 mesh (Product No. C.5385) and Dextran T-70

(average molecular weight 64,000–76,000; Product No. D.4751) both purchased from Sigma-Aldrich Sweden AB, Stockholm, Sweden). Following a 5-min centrifugation, the supernatant was poured into scintillation vials that contained 4 ml scintillation fluid (OptiPhase Hi Safe II, LKB Wallac) and run for 5 min in the counter (1214 Rackbeta liquid scintillation counter, LKB Wallac). The detection limit for the assay was approximately 2 ng/ml and the intra- and interassay coefficients of variance were 5.4 and 7.0%, respectively.

Statistical Analyses Fisher's exact probability test (two-tailed) was applied (http://statpages.org/ctab2x2.html) to analyze for significant differences in the number of males or females with and without strippable gonads performing or not performing a fright reaction. An unpaired Student's *t* test (two-tailed) was applied to analyze the differences in the mean levels of plasma sex hormones (Excel, Microsoft Office 2003). Individuals responding with a fright reaction to skin extract were compared to those not responding for each gender separately. A critical value of $\alpha=0.05$ was used for all comparisons.

Results

Maturation of Gonads, Females Following exposure to skin extract, female crucian carp could be divided into two groups, those performing (10) and those not performing (14) a fright reaction. As judged by the presence or absence of strippable eggs, maturation status was more advanced in females not performing a fright reaction (Fig. 1). In this group, 13 out of 14 females had strippable eggs. In the group performing a fright reaction, this ratio was five out of 10. This difference in relative frequency of fully mature individuals between the two groups is significant ($P=0.05$).

Maturation of Gonads, Males Following exposure to skin extract, only six out of 18 males displayed the fright reaction. Among males not performing a fright reaction, nine of 12 had running milt. In the group performing a fright reaction, three of six had running milt (Fig. 1). Hence, in contrast to the situation in females, among males the occurrence of mature individuals was not significantly different between the responding and not responding groups ($P=0.34$).

Steroid Hormones, Females The mean plasma levels of E_2 (Fig. 2) were significantly higher in those females performing a fright reaction (54.3±22.5 ng/ml) compared to those that did not (34.3±14.8 ng/ml; $P=0.03$). The same was observed with plasma T levels, where means were significantly higher in the individuals performing a fright reaction (7.1±2.2 ng/ml) compared to those that did not (4.8±1.3 ng/ml; $P=0.01$).

Steroid Hormones, Males In contrast to the females, there were no significant differences in steroid hormone levels between those males that performed a fright reaction and those that did not perform a fright reaction. Plasma T levels were not significantly different between responders (7.2±3.3 ng/ml) compared to non-responders (6.4±1.7 ng/ml; $P=0.32$). Similarly, plasma 11-KT levels (Fig. 2) were not significantly different in responders (5.0±4.0 ng/ml), compared to non-responders (7.0±2.3 ng/ml; $P=0.38$).

Discussion

We report a suppression of the fright reaction in female crucian carp during the final stages of sexual maturation. That is, among the females failing to perform a fright reaction, a large majority had ovulated, as indicated by the presence of strippable eggs. In contrast, in mature male fish, the failure to respond to alarm substances was not associated with the presence of strippable milt.

Steroid measurements showed that the group of females displaying a fright reaction had significantly higher mean plasma levels of both E_2 and T compared to the females without fright reaction. The relatively low levels of E_2 and T observed in females not performing a fright reaction are in accordance with the endocrine changes that occur just

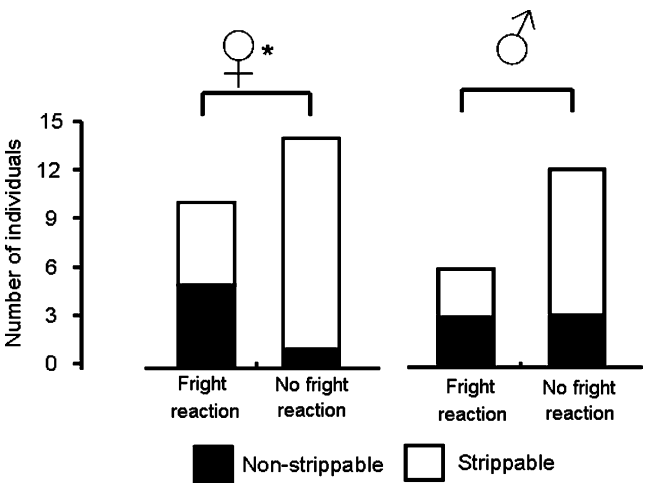

Fig. 1 Number of individuals grouped by behavioral response to skin extract as either a fright reaction or no fright reaction. Maturation of gonads was characterized as either strippable or non-strippable, depending on whether milt/eggs could be stripped following gentle abdominal pressure. The *asterisk* indicates a significant difference in the status of gonad maturation ($P=0.05$) between the two behavioral response groups of females (Fisher's exact probability test)

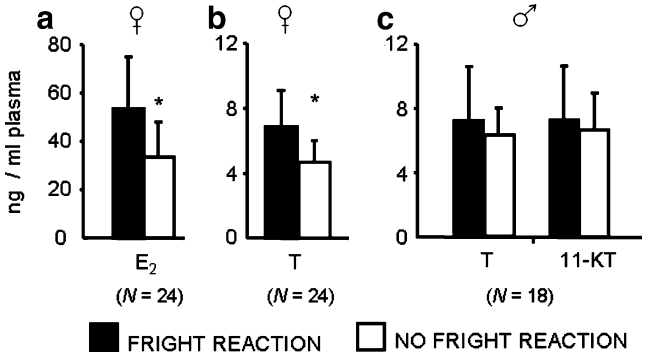

Fig. 2 Mean plasma levels+SD of gonadal steroids in individuals performing and individuals not performing a fright reaction upon skin extract exposure. **a** *E_2* 17*β*-estradiol, **b** *T* testosterone in females (*N*=24) and **c** *T* testosterone and *11-KT* 11-ketotestosterone in males (*N*=18). Note that the scale for E_2 values differs from that of the androgens (T and 11-KT). The *asterisks* indicate significant differences in E_2 values (*P*=0.03) and T values (*P*=0.01) between the two behavioral response groups of females (Student's *t* test)

before ovulation in the goldfish, *Carassius auratus*, a close relative of the crucian carp (Kobayashi et al. 2002). These results are in accordance with a suppression of the alarm response during final oocyte maturation and ovulation, a time when plasma levels of the sex steroid hormones E_2 and T are declining.

The majority of all males, 12 of 18, did not show an alarm response. Still, there were no significant differences among males responding or not responding with a fright reaction to skin extract, both with respect to strippable milt, and mean plasma levels of those androgens (T and 11-KT) that influence both male sexual behavior and spermatogenesis (Borg 1994). Unfortunately, the low sample-size could potentially make the comparisons between responding and non-responding individuals problematic. There may be a suppressed alarm response induced by factors not taken into account in this study. In male fish, spermatogenesis as well as courtship behavior are dependent on socio-sexual cues from conspecifics (Poling et al. 2001; Stacey et al. 2001; Kobayashi et al. 2002), and it is possible that a suppression of a fright reaction is affected by the same socio-sexual cues, which were not taken into account in the present study. Female preovulatory steroids, such as 17α, 20β-dihydroxypregnenone, and postovulatory pheromones such as prostaglandins are possible candidates.

By presenting the steroid levels as we have, we attempted to highlight the reproductive state of the fish, rather than to show the role of these hormones in the suppression of the behavioral response. However, we feel that our data are robust enough to suggest the possible involvement of gonadal hormones in the modulation of the fright reaction in female crucian carp. This assumption is strengthened when we compare responding and non-responding females in the ovulated group; the mean T level in the group with fright reaction is higher when compared to the group without the reaction. There is also a strong trend for higher mean E_2 level in the group with fright reaction when compared to the group without the reaction (data not shown). Thus, we hypothesize that endocrine variation may account for the loss of predator avoidance.

Previous studies have demonstrated the absence or reduction of alarm substance cells in the skin of sexually mature fish, where it was suggested that this could prevent the release of false alarm signals that result from fighting and abrasive contact (courtship, nest building) that commonly occurs during spawning behavior (Smith 1976). Our results demonstrate that female crucian carp lose the behavioral response to these predation cues during the final stages of sexual maturation, which may have the same ultimate function, i.e., to prevent interruption of spawning. To reduce predator avoidance in favor of reproduction may be necessary, since the fright reaction includes hiding and freezing (Hamdani et al. 2000; Höglund et al. 2005); behaviors that are incompatible with typical spawning behavior (Stacey and Liley 1974; Bjerselius et al. 1995; Weltzien et al. 2003). The suppression of either sending or responding to information about potential predators may seem risky from an evolutionary point of view. Predation eliminates all future reproductive opportunities, and animals have been suggested to maximize lifetime and reproductive success by attaining an optimal balance between reproduction and predator avoidance (Lima and Dill 1990; Magnhagen 1991). In the present study, the loss of the fright reaction seems to occur during a relatively short time window in relation to the release of gametes, thus indicating that the increased risk-taking is minimized.

Mechanisms involved in suppression of a behavioral response may be related to modification of the olfactory organ and/or the central mechanisms involved in processing received information. Interestingly, there is plasticity in the olfactory organ in crucian carp, showing seasonal variation in the number of sensory cells that are believed to be involved in the detection of sex pheromones (Hamdani and Døving 2006; Lastein et al. 2006; Hamdani et al. 2008). However, such a mechanism might be too slow to account for the suppression of the fright reaction. Central modifications induced by endocrine alterations are more likely to be involved.

In summary, we have demonstrated that the fright reaction is suppressed during the final stages of sexual maturation in female crucian carp. This seems to occur during a relatively short time window corresponding to ovulation and the release of gametes. Having only a short window during which the fright reaction is suppressed would minimize the overall risk of predation. Since the fright reaction in crucian carp consists of behavior incompatible with spawning behavior, we hypothesize that

this short-term suppression has evolved to enable spawning to occur uninterrupted.

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References

BJERSELIUS, R., OLSEN, K. H., and ZHENG, W. H. 1995. Endocrine, gonadal and behavioral-responses of male crucian carp to the hormonal pheromone 17α,20β-dihydroxy-4-pregnen-3-one. *Chem. Senses* 20:221–230.

BORG, B. 1994. Androgens in teleost fishes. *Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol.* 109:219–245.

BROWN, G. E., and SMITH, R. J. F. 1997. Conspecific skin extracts elicit antipredator responses in juvenile rainbow trout (*Oncorhynchus mykiss*). *Can. J. Zool.* 75:1916–1922.

BROWN, G. E., RIVE, A. C., FERRARI, M. C. O., and CHIVERS, D. P. 2006. The dynamic nature of antipredator behavior: prey fish integrate threat-sensitive antipredator responses within background levels of predation risk. *Behav. Ecol. Sociobiol.* 61:9–16.

CANDOLIN, U. 1997. Predation risk affects courtship and attractiveness of competing threespine stickleback males. *Behav. Ecol. Sociobiol.* 41:81–87.

CHIVERS, D. P., and SMITH, R. J. F. 1998. Chemical alarm signalling in aquatic predator-prey systems: a review and prospectus. *Ecoscience* 5:338–352.

DILL, L. M., HEDRICK, A. V., and FRASER, A. 1999. Male mating strategies under predation risk: do females call the shots? *Behav. Ecol.* 10:452–461.

DULKA, J. G., STACEY, N. E., SORENSEN, P. W., and VANDERKRAAK, G. J. 1987. A steroid sex-peromone synchronizes male-female spawning readiness in goldfish. *Nature* 325:251–253.

ENDLER, J. A. 1987. Predation, light intensity and courtship behavior in *Poecilia reticulata* (*Pisces- Poeciliidae*). *Anim. Behav.* 35:1376–1385.

FARR, J. A. 1975. Role of predation in evolution of social-behavior of natural-populations of guppy, *Poecilia reticulata* (*Pisces-Poeciliidae*). *Evolution* 29:151–158.

GODIN, J. G. J., and DUGATKIN, L. A. 1996. Female mating preference for bold males in the guppy, *Poecilia reticulata*. *Proc. Natl. Acad. Sci. U S A* 93:10262–10267.

HAMDANI, E. H., and DØVING, K. B. 2006. Specific projection of the sensory crypt cells in the olfactory system in crucian carp, *Carassius carassius*. *Chem. Senses* 31:63–67.

HAMDANI, E. H., STABELL, O. B., ALEXANDER, G., and DØVING, K. B. 2000. Alarm reaction in the crucian carp is mediated by the medial bundle of the medial olfactory tract. *Chem. Senses* 25:103–109.

HAMDANI, E. H., LASTEIN, S., GREGERSEN, F., and DØVING, K. B. 2008. Seasonal variations in olfactory sensory neurons—fish sensitvity to sex pheromones explained? *Chem. Senses* 33:119–123.

HÖGLUND, E., WELTZIEN, F. A., SCHJOLDEN, J., WINBERG, S., URSIN, H., and DØVING, K. B. 2005. Avoidance behavior and brain monoamines in fish. *Brain Res.* 1032:104–110.

KAVALIERS, M., and CHOLERIS, E. 2001. Antipredator responses and defensive behavior: ecological and ethological approaches for the neurosciences. *Neurosc. Biobehav. Rev.* 25:577–586.

KAVALIERS, M., CHOLERIS, E., and COLWELL, D. D. 2001. Brief exposure to female odors "emboldens" male mice by reducing predator-induced behavioral and hormonal responses. *Horm. Behav.* 40:497–509.

KOBAYASHI, M., SORENSEN, P. W., and STACEY, N. E. 2002. Hormonal and pheromonal control of spawning behavior in the goldfish. *Fish Physiol. Biochem.* 26:71–84.

KOSS, W. A., GEHLERT, D. R., and SHEKHAR, A. 2004. Different effects of subchronic doses of 17-beta estradiol in two ethologically based models of anxiety utilizing female rats. *Horm. Behav.* 46:158–164.

LASTEIN, S., HAMDANI, E. H., and DØVING, K. B. 2006. Gender distinction in neural discrimination of sex pheromones in the olfactory bulb of crucian carp, *Carassius carassius*. *Chem. Senses* 31:69–77.

LIMA, S. L., and DILL, L. M. 1990. Behavioral decisions made under the risk of predation—a review and prospectus. *Can. J. Zool.* 68:619–640.

LUNGA, P., and HERBERT, J. 2004. 17 beta-oestradiol modulates glucocorticoid, neural and behavioural adaptations to repeated restraint stress in female rats. *J. Neuroendocrinol.* 16:776–785.

MAGNHAGEN, C. 1991. Predation risk as a cost of reproduction. *Trends Ecol. Evolution* 6:183–185.

MATHIS, A., and SMITH, R. J. F. 1993. Intraspecific and cross-superorder responses to chemical alarm signals by brook stickleback. *Ecology* 74:2395–2404.

PALL, M. K., MAYER, I., and BORG, B. 2002. Androgen and behavior in the male three-spined stickleback, *Gasterosteus aculeatus* I.—Changes in 11-ketotestosterone levels during the nesting cycle. *Horm. Behav.* 41:377–383.

PFEIFFER, W. 1963. Alarm substances. *Experientia* 19:1–11.

POLING, K. R., FRASER, E. J., and SORENSEN, P. W. 2001. The three steroidal components of the goldfish preovulatory pheromone signal evoke different behaviors in males. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 129:645–651.

SCHUTZ, F. 1956. Vergleichende Untersuchungen über die Schreckreaktion bei Fischen und deren Verbreitung. *Z. vegleich. Physiol.* 38:84–135.

SMITH, R. J. F. 1973. Testosterone eliminates alarm substance in male fathead minnows. *Can. J. Zool.* 51:875–876.

SMITH, R. J. F. 1976. Seasonal loss of alarm substance cells in North-American cyprinoid fishes and its relation to abrasive spawning behavior. *Can. J. Zool.* 54:1172–1182.

STACEY, N. E., and LILEY, N. R. 1974. Regulation of spawning behavior in female goldfish. *Nature* 247:71–72.

STACEY, N., FRASER, E. J., SORENSEN, P., and VAN DER KRAAK, G. 2001. Milt production in goldfish: regulation by multiple social stimuli. *Comp. Biochem. Physiol. C Toxicol. Pharmacol.* 130:467–476.

STRONG, D. R. 1973. Amphipod-amplexus, significance of ecotypic variation. *Ecology* 54:1383–1388.

VON FRISCH, K. 1938. Zur Psychologie des Fisch-schwarmes. *Naturwissenschaften* 26:601–606.

WELTZIEN, F. A., HÖGLUND, E., HAMDANI, E. H., and DØVING, K. B. 2003. Does the lateral bundle of the medial olfactory tract mediate reproductive behavior in male crucian carp? *Chem. Senses* 28:293–300.

WISENDEN, B. D. 2000. Olfactory assessment of predation risk in the aquatic environment. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 355:1205–1208.

diterpenoids (approximately 400) are the main natural products in this genus and are especially abundant in fresh branches and leaves (Sun et al. 2001). Some of these compounds possess diverse bioactivities that include among other properties: antitumor activity (Tengchaisri et al. 1998), induction of apoptosis (Suzuki et al. 2004), and cytotoxicity (Jiang et al. 1999; Han et al. 2004). Being perennial deciduous herbs, shrubs, or sub-shrubs, the aerial parts of *Isodon* plants wilt, fall to the ground, and are incorporated into the rhizosphere via degradative processes on an annual basis. However, whether these chemical components mediate interactions in natural surroundings is still unknown.

Leukamenin E (Fig. 1) was first isolated from the leaves of *Isodon umbrosa* var. *leucantha* f. *Kameba* (Takeda et al. 1981); however, little information is available concerning its bioactivity. Our investigation of *Isodon racemosa* (Hemsl) Hara obtained from Gansu, China, led to the isolation of leukamenin E from branches and leaves (Ding et al. 2006). Leukamenin E is abundant, and yields can reach as high as 0.13% DW. Preliminary screening of several *ent*-kaurene diterpenoids (e.g., leukamenin E, weisinensin B, rabdosin B, and epinodosin; data not shown) confirmed that leukamenin E is most phytotoxic toward the growth of lettuce, radish, ryegrass, and wheat seedlings. Consequently, it was selected to probe for possible modes of action.

Plant root systems are most likely to be affected by certain allelochemicals because they have direct contact with soil and can absorb many compounds. Mitotic activity can be used to evaluate root growth resulting from cell division of meristematic cells and cell expansion in the elongation zone of roots. Root hairs, projections from root epidermal cells that increase the effective surface area available for nutrient and water uptake, can be used to evaluate the influence of compounds on root development.

The purpose of our experiments was: (1) to determine the phytotoxic effects of leukamenin E on root growth and root hair development in lettuce seedlings and (2) to probe possible modes of action. Lettuce has been used extensively as a test organism because of its fast germination and high sensitivity (Rasmussen and Einhellig 1979) and, thus, was selected as the target plant species in our study.

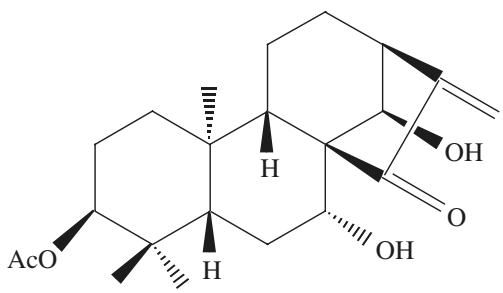

Fig. 1 Chemical structure of leukamenin E, an *ent*-kaurene diterpenoid from species of *Isodon*

Methods and Materials

Plant Materials and Chemicals Lettuce (*Lactuca sativa* L.) seeds were obtained from Dadi Seed Service (Lanzhou, China). Leukamenin E was isolated previously in our laboratory from *I. racemosa* (Hemsl) Hara (Ding et al. 2006). Ethephon was purchased from BBI, USA. Silver thiosulfate solutions were prepared by mixing $AgNO_3$ with $Na_2S_2O_3$ in a 1:4 molar ratio (Kamaluddin and Zwiazek 2002). All other chemicals were from Sango Biological Engineering Technology and Service Co., Ltd. (Shanghai, China).

Culture Conditions and Treatments Lettuce seeds were rinsed in tap water (×3), surface-sterilized with 0.1% mercuric chloride (8 min), followed by rinsing in distilled water. Seeds were placed into Petri dishes that contained two sheets of filter paper soaked with distilled water and allowed to germinate for 48 h at 22±2°C and a photoperiod of 16:8 h L:D. Uniform, 2-day-old seedlings (ten per treatment) were transferred into Petri dishes containing different concentrations of treatment solutions and incubated for 48 h under the same conditions.

Leukamenin E was dissolved in DMSO and diluted to the desired concentrations with distilled water. The amount of DMSO varied for each treatment but never exceeded 0.1%—i.e., below a level where root growth and root hair developments were affected (data not shown). Net growth rates (NGR) were measured to evaluate the influence of leukamenin E and Ag^+ on root growth according to the method of Pan et al. (2001). The seminal root lengths of the seedlings in each treatment were measured before and after treatment. NGR was calculated as follows: (final length-initial length)/initial length.

Mitotic Activity and Aberrations Squash techniques were modified from Akinboro and Bakare (2007). Primary roots of lettuce seedlings after leukamenin E treatments were fixed in freshly prepared 3:1 (*v*/*v*) ethanol–acetic acid for 24 h, transferred into 70% EtOH, and stored at 4°C until use. Roots were hydrolyzed in 1 M HCl for 8–10 min at 60°C followed by rinsing in distilled water (3–4 rinses). Root tips (approximately 1–2 mm) were excised, stained with carbol fuchsin (10 min), and squashed onto slides. Five slides were prepared for each treatment, and at least 1,000 cells were randomly counted per slide. The mitotic index was calculated as the number of dividing cells per 1,000 observed cells. All slides were examined for aberrant

mitosis with a Nikon Eclipse E400 optical microscope. Digital images were captured with a Canon A460 digital camera.

Measurements of Root Hairs Root hairs were evaluated with slight modifications according to the method of Yang et al. (2004). Seedlings (ten) were randomly selected from each treatment. Root segments (1 cm) were excised from each seedling at 0.5 cm behind the root tip, fixed in 70% EtOH, and placed onto microscope slides for microscopic observation. The number and length of root hairs present in certain regions (424 μm root segment) were measured (×3) from the ends and the middle of root segments with an ocular micrometer. Measurements for each treatment were determined from the ten root segments, and mean values were calculated.

Statistical Analysis All data are presented as means ± SD. Data were subjected to analysis of variance (ANOVA) with significant differences among means identified by LSD multiple range tests using SPSS 11.0. Differences were considered significant at $P \leq 0.05$. To verify the relationship between pairs of data (root NGR/mitotic index; mitotic index/prophase index) at the same leukamenin E concentrations, linear regression analysis was calculated, and the coefficient of regression (r) and its level of significance was determined.

Results

Leukamenin E Effects on Root Growth The inhibitory effect of increasing concentrations of leukamenin E on lettuce seedling roots is evident in Fig. 2A. NGR over 48 h were calculated to quantify the effects of leukamenin E on root growth. The NGR resulting from treatment with a low concentration of leukamenin E (10 μM) was no different from control seedlings exposed to DMSO. However, the inhibitory effects of higher concentrations of leukamenin E (50–200 μM) were significantly different. At all concentrations above 50 μM, the NGR of lettuce seedlings decreased, but the inhibitory effects at 100 and 200 μM leukamenin E resulted in similar levels of inhibition. When seedlings were treated with the highest concentration of leukamenin E, the NGR remained at about 37% of the control (Fig. 2B).

Effects of Leukamenin E on Mitotic Activity Leukamenin E affected the mitotic index in a pattern that was similar to the NGR (Fig. 3A). The mitotic index for root tip cells treated with 10 μM leukamenin E did not differ from controls. At higher concentrations (i.e., 50–200 μM), the mitotic index

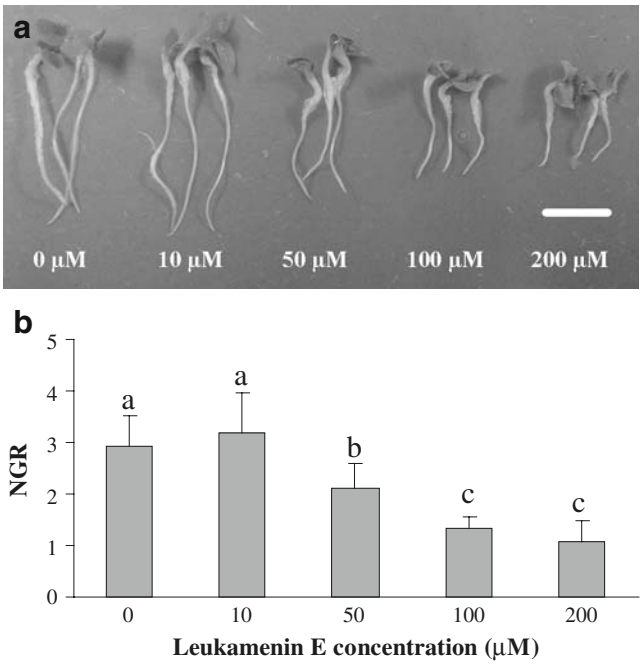

Fig. 2 **A** Effect of increasing concentrations of leukamenin E on the root growth of lettuce seedlings 48 h after treatment. **B** Root NGR in lettuce seedlings calculated after 48 h of treatment. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD. *Bar*= 1 cm

decreased, and the inhibition was positively correlated with leukamenin E concentration. The mitotic index of root cells treated with 200 μM was approximately 62% of the control value. Regression analysis revealed a positive correlation between NGR and mitotic index (r=0.97, P=0.006). In addition, leukamenin E affected the relative proportion of the various stages of mitosis in meristematic cells with different stages showing different sensitivity to leukamenin E (Fig. 3B). The decrease in the number of meristematic cells in prophase was analogous to the mitotic index (r= 0.98, P=0.004), while the percentages of other phases were less affected.

The distribution of mitotic phases relative to mitotic cells was also influenced (Fig. 3C). After treatment with higher concentrations of leukamenin E (100 and 200 μM), the percentage of cells in metaphase increased compared to cells in other mitotic phases while the number of cells in prophase decreased. Finally, a low incidence of aberrant mitosis, (e.g., chromosome stickiness, C-colchicine metaphase, chromosome bridges, multipolar division, lagging chromosome, etc.) was observed in plants treated with 100 and 200 μM leukamenin E (Fig. 4). These phenomena were not observed in control seedling cells or cells treated with lower concentrations of leukamenin E.

Inhibition of Root Hair Development by Leukamenin E Root hair development in lettuce seedlings (i.e., length

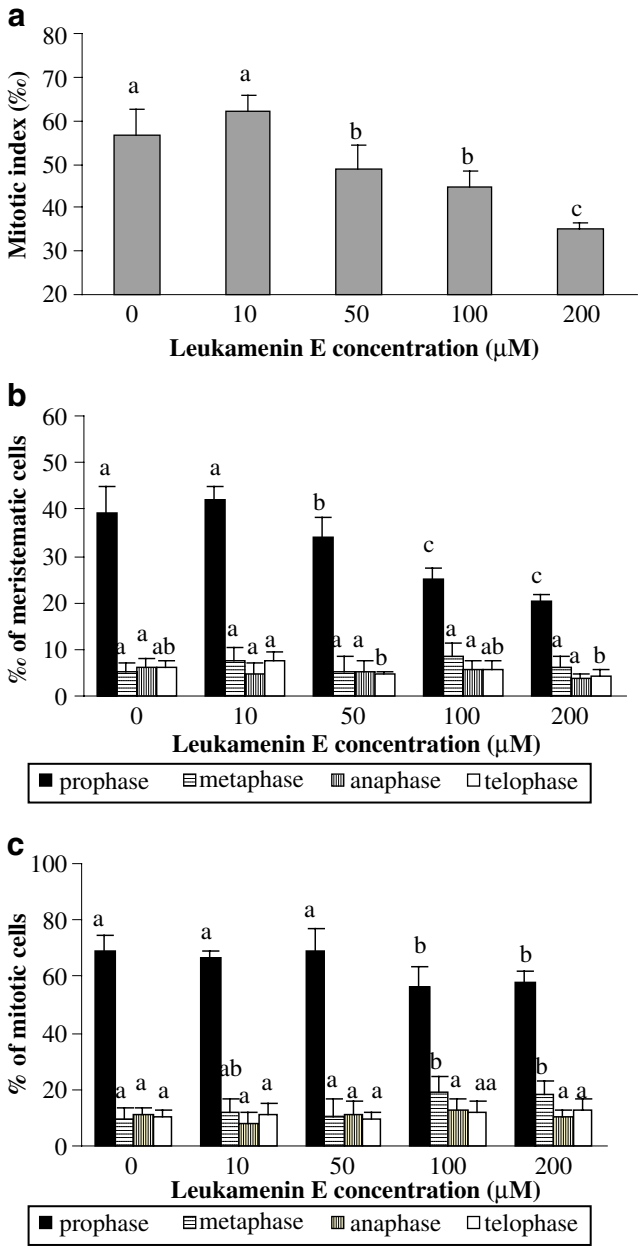

Fig. 3 Effect of leukamenin E on the mitotic activity of lettuce seedling root apical meristem cells after treatment (48 h). **A** The mitotic index of root apical cells; **B** percentage of mitotic phases calculated relative to the total number of meristematic cells; and **C** the distribution of mitotic phases calculated relative to mitotic cells. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD

and density) was inhibited by leukamenin E (Fig. 5). Concentrations between 10–80 μM curbed root hair elongation and decreased the average root hair length in a dose-dependent manner (Fig. 5A). In contrast, root hair density remained stable up to 20 μM and only declined at concentrations above 40 μM (Fig. 5B). A few bulges were observed on root surfaces when leukamenin E concentrations were raised to 40 μM; at 80 μM, the roots were rendered smooth and hairless. The effect of leukamenin E on root hair length in lettuce seedlings compared to control root hairs is shown in (Fig. 6).

Effects of Ag^+ on Lettuce Seedling Roots The effects of Ag^+ on root growth and root hair development are shown in Fig. 7. Lower levels of Ag^+ (0.5–2 mM) promoted root growth, and the root NGR reached a maximum at 1 mM (Fig. 7A). Inhibitory effects were not observed at the Ag^+ concentrations tested. Significant inhibitory effects on root hair length were found at all Ag^+ concentrations at or above 0.5 mM, but the inhibition did not increase linearly at the higher concentrations (Fig. 7B). The density of root hairs remained constant at all concentration tested (Fig. 7C).

Reversion of Ethephon Enhanced Root Hairs by Leukamenin E Lettuce seedlings were treated simultaneously with ethephon (40 μM), an ethylene-releasing agent, and different concentrations of either leukamenin E or Ag^+ to determine whether leukamenin E might act as an ethylene antagonist similar to Ag^+. Treatment of lettuce seedlings with ethephon alone stimulated root hair growth (Fig. 8). Simultaneous treatment of seedlings with ethephon and Ag^+ (0.5 and 4 mM) inhibited the stimulatory effect of ethephon on root hair growth. Increasing concentrations of leukamenin E and simultaneous treatment with ethephon also inhibited the stimulatory effect of ethephon on root hair growth.

Treatment of lettuce seedlings with ethephon resulted in a significant reduction in the NGR of control seedlings. Increasing concentrations of Ag^+ were able to reverse this effect. The NGRs of lettuce seedlings simultaneously treated with ethephon and leukamenin E were significantly lower than seedlings treated with Ag^+—i.e., leukamenin E was not able to reverse the inhibitory effect of ethephon (Fig. 9).

Discusssion

For the first time, we have demonstrated that leukamenin E is phytotoxic toward root growth and root hair development in lettuce seedlings. Parker et al. (2000) reported that root hairs contribute as much as 77% to the total root surface area in cultivated crops, being the major point of contact between plants and the rhizosphere. Among other physiological functions, plant roots are important in water and nutrient uptake, growth regulation, and anchoring in soil (Ryan et al. 2001; Bibikova and Gilroy 2003). Root length is important in the exploitation of soil resources (Aerts et al. 1991). Short, hairless roots that result from exposure to leukamenin E would undoubtedly hamper a plant's ability

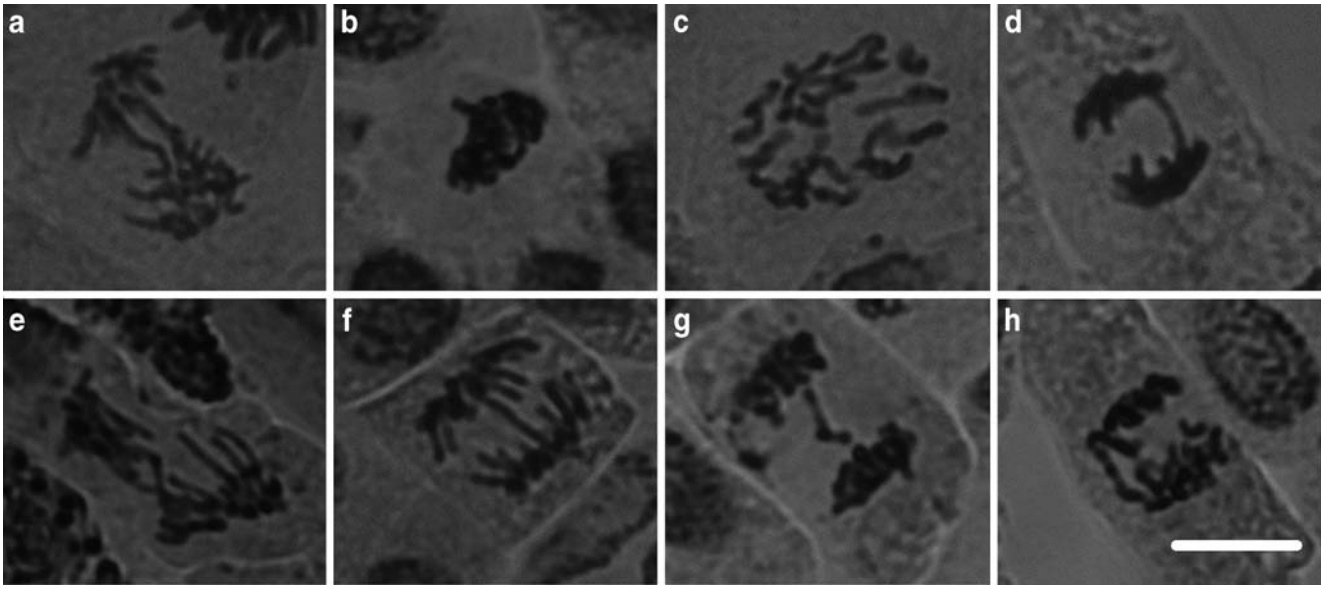

Fig. 4 Representative images showing aberrant mitosis in root apical meristem cells from lettuce seedlings 48 h after treatment with leukamenin E. **A** Anaphase with double bridges; **B** chromosome stickiness; **C** C-colchicine metaphase; **D** telophase with a bridge; **E** multipolar division; **F** anaphase with a bridge; **G** lagging chromosome; **H** chromosome bridge and fragment. **A**–**C** Treatments with 100 μM leukamenin E; **D**–**H** treatments with 200 μM leukamenin E. *Bar*=10 μm

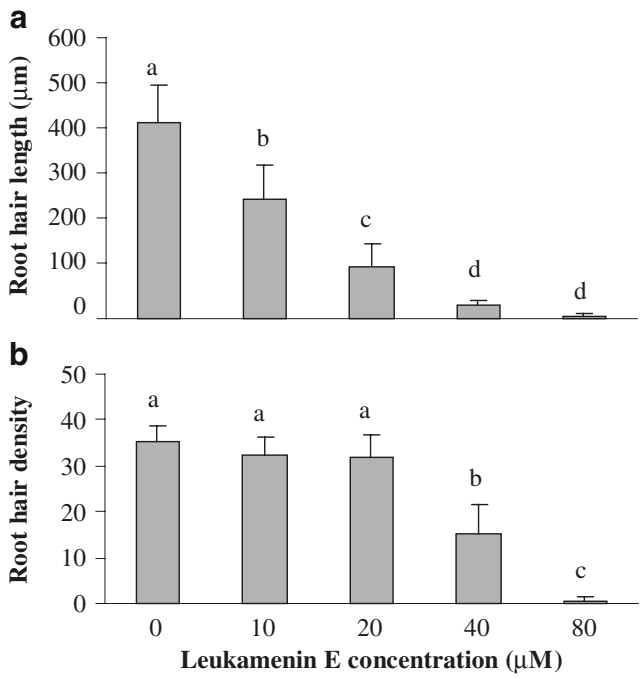

Fig. 5 Effect of leukamenin E on root hair development in lettuce seedlings 48 h after treatment. Root hair development was evaluated by average root hair length (**A**) and root hair density (**B**). Root hair density was measured as the number of root hairs per 424 μm root segment. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD

to compete for resources and, as a result, could lead to decreased shoot growth. This suggests that this abundant *ent*-kaurene diterpenoid may play a role in mediating ecological interactions between *Isodon* and other species in nature.

Root growth arises from the proliferation of meristematic cells followed by cell expansion that results in root elongation. Our results demonstrate that lettuce seedlings treated with leukamenin E experience a reduction in root NGR and that this is correlated with the mitotic index of root meristematic cells. This suggests that inhibition of root growth may result from suppressed mitotic activity. Along with a reduction in the number of mitotic cells, leukamenin E alters the percentage of cells in the various phases of mitosis calculated relative to meristematic cells. A reduction in the number of cells in prophase (Fig. 3B) follows a pattern that is similar to the mitotic index (Fig. 3A); however, the percentage of cells in other mitotic phases did not change. Romagni et al. (2000) reported that treatment with a monoterpene—1,4-cineole—caused similar decreases in the number of cells in prophase. It is hypothesized that such a phenomenon depends on the general mechanism responsible for arresting the mitotic cycle in cells (Fusconi et al. 2007). Upon induction of DNA damage or after alteration of the intracellular redox homeostasis, the cell cycle slows down at the G_1/S and G_2/M transition points (Den Boer and Murray 2000). This may reduce the percentage of prophases and eventually the

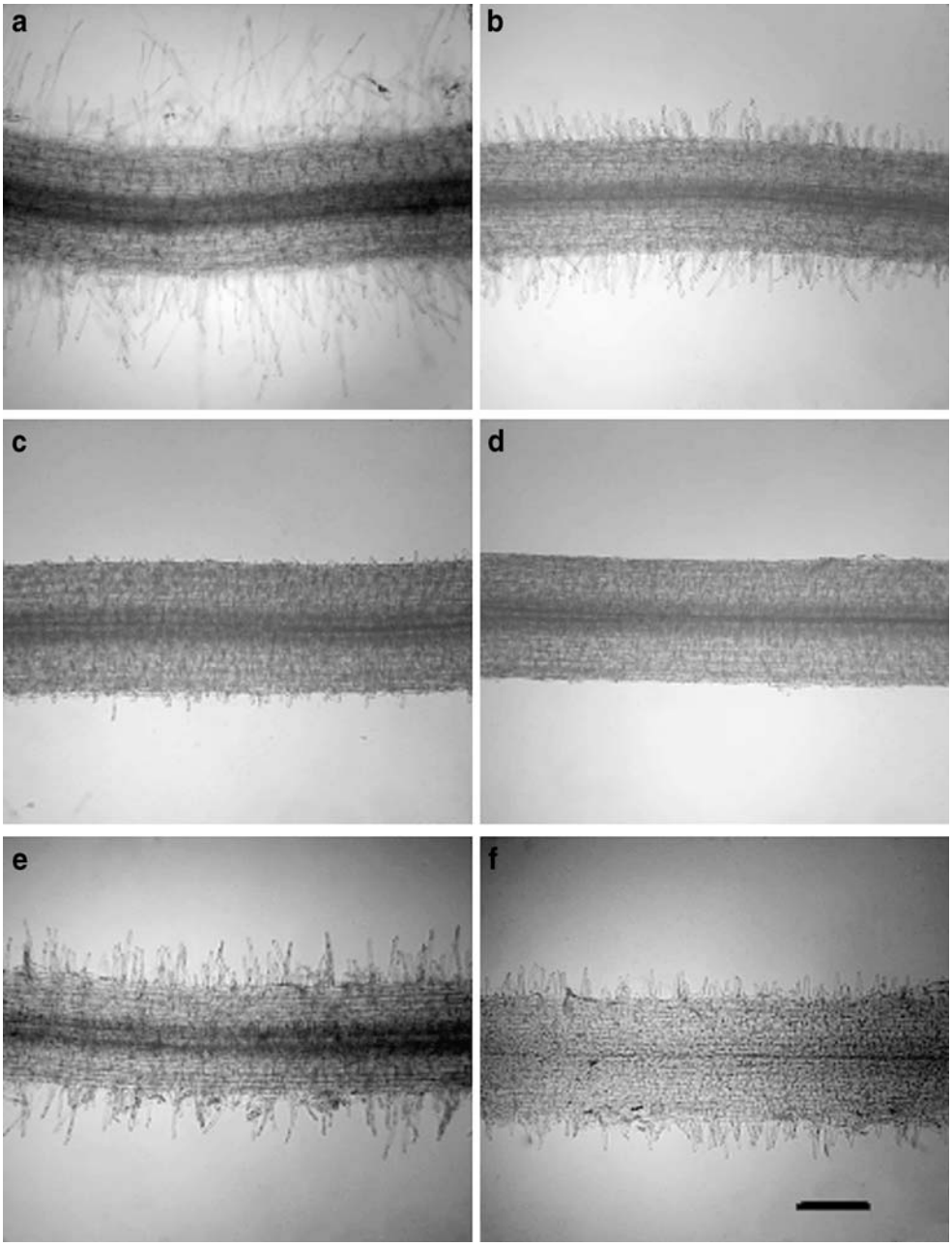

Fig. 6 Representative images showing the inhibitory effects of leukamenin E and Ag^{+} on root hair development in lettuce seedlings 48 h after treatment. **A** Control; **B**–**D** treatments with 20, 40, and 80 μM leukamenin E, respectively; **E** and **F** treatments with 0.5 and 4 mM Ag^{+}, respectively. *Bar*=200 μm

mitotic index. However, according to Borboa and De La Torre (2000), plant cells with unrepaired chromosomal damage can overcome the G_2/M-checkpoint pathway and enter mitosis, giving rise to chromosomal aberrations (e.g., chromosome bridges in anaphase). Our results support this since a low incidence of aberrant mitosis was observed following leukamenin E treatments (at 100 and 200 μM), some of which may be related to DNA damage. In plants treated with the two highest concentrations of leukamenin E (100 and 200 μM), the percentage of cells in metaphase relative to the number of mitotic cells increased, while the percentage of cells in prophase decreased compared with controls. Such a phenomenon might be accounted for by alterations in mitotic spindle microtubules, which could delay chromosome segregation and increase the percentage of metaphases (Fusconi et al. 2007). However, interference with tubulin or microtubules in general often causes a large increase in metaphase that was not observed. This may be explained by speculation that higher concentrations of leukamenin E (100 and 200 μM) are insufficient to induce large increases in metaphase, since aberrant mitosis that may arise from abnormal microtubule pattern did not occur in higher percentage at these two concentrations. In short, root growth inhibition caused by leukamenin E was mainly

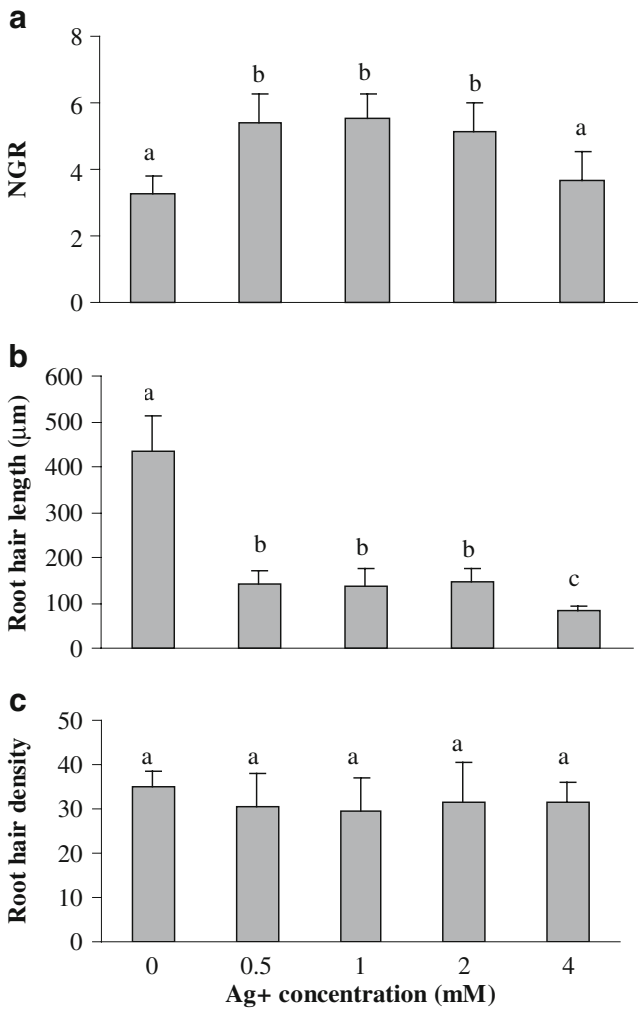

Fig. 7 Effect of Ag^+ on root growth and root hair development in lettuce seedlings 48 h after treatment. NGR were calculated to evaluate the effects on root growth (**A**); root hair development was evaluated in terms of root hair length (**B**) and root hair density (**C**). Root hair density was measured as the number of root hairs per 424 μm of root segment. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD

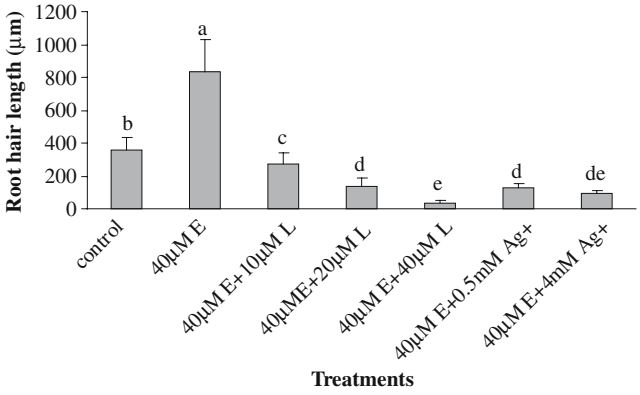

Fig. 8 Enhanced root hair length caused by 40 μM ethephon could be counteracted by addition of both leukamenin E and Ag^+. Ethephon (40 μM) and different concentrations of leukamenin E or Ag^+ were applied simultaneously to lettuce seedlings; the average root hair length was evaluated after 48 h. *E* represents ethephon and L leukamenin E. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD

the result of a decreased mitotic index, possibly by arresting the cell cycle.

Many terpenes are antagonistic to ethylene action (Sisler et al. 2006). Consequently, we investigated whether leukamenin E could act as an ethylene antagonist and inhibit root hair development. Our results suggest that the diterpene may be an ethylene antagonist. Experiments that involve the simultaneous addition of ethephon and leukamenin E or ethephon and Ag^+ confirmed that enhanced root hair length induced by ethephon could be reversed by addition of either Ag^+ or leukamenin E. Seedlings with suppressed root hairs that were treated with leukamenin E (40 or 80 μM) could not be reversed by exogenous application of ethephon (10–80 μM; data not shown), suggesting that leukamenin E may interfere with the ethylene response pathway instead of ethylene biosynthesis as with Ag^+. Leukamenin E inhibited root hair elongation in a dose-dependent manner and completely blocked root hair initiation at 80 μM. In contrast, the inhibitory effect of Ag^+ on root hairs length was not dose-dependent. Leukamenin E (above 40 μM) decreased root hair density while remaining stable with increasing concentrations of Ag^+. Differences between the interfering mechanism of Ag^+ and leukamenin E are apparent even though both inhibit root hair length by interfering with ethylene action.

Inhibition of root growth is a characteristic ethylene response. A simple explanation for ethylene-triggered root inhibition is that ethylene induces *WEI2/7* expression

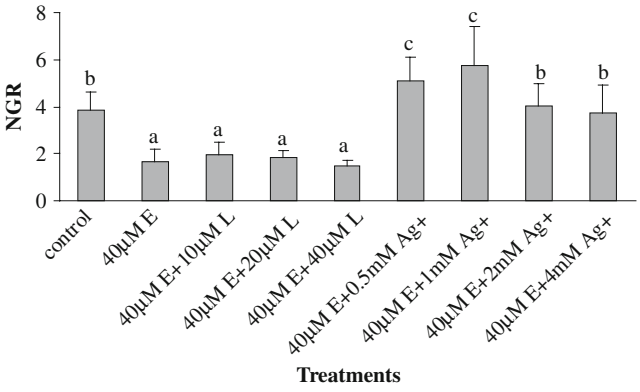

Fig. 9 Effects of leukamenin E and Ag^+ on root growth caused by ethephon. Ethephon (40 μM) and different concentrations of leukamenin E or Ag^+ were applied simultaneously to lettuce seedlings. Root NGR were calculated 48 h after treatment; *E* represents ethephon and L leukamenin E. *Different letters* indicate significant differences at $P \leq 0.05$. *Bars* indicate SD

specifically in root tips, which in turn accelerates auxin biosynthesis and consequently inhibits root elongation (Li and Guo 2007). Investigations with Ag^+, a specific ethylene action inhibitor, showed that lower concentrations of Ag^+ (0.5–2 mM) promoted root growth. Since ethylene generally inhibits root growth, it is reasonable to suspect that interference of ethylene action by Ag^+ could result in root elongation, which is supported by our finding that ethephon-shortened roots were reversed by simultaneous application of Ag^+. In contrast, root growth in lettuce seedlings was significantly reduced when treated with leukamenin E through the suppression of mitotic activity. Stimulatory effects on root growth were not detected at lower concentrations, indicating that the diterpenoid may affect root growth by a mechanism different from Ag^+. Moreover, addition of leukamenin E did not overcome the inhibitory effect on root growth caused by ethephon as Ag^+ did. It is possible that leukamenin E interferes with the downstream portion of the ethylene pathway, thus, specifically controlling root hair development instead of affecting root growth. Its inhibitory effects on root growth are largely attributed to the decreased mitotic index, while Ag^+ may interact with an ethylene receptor (Rodríguez et al. 1999) at a point upstream in the ethylene pathway, thus, both affecting root hair development and root growth.

In summary, our research demonstrates the potential phytotoxic effects of leukamenin E on root growth and root hair development in lettuce seedlings and offers a plausible mechanism for this action. Studies are needed to confirm the role of leukamenin E in mediating ecological interactions. In addition, further investigation is necessary to determine whether leukamenin E possesses activity that is antagonistic to other ethylene responses. If so, it is possible that such research may have commercial relevance, such as prevention of fruit ripening and flower senescence.

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References

ABRAHIM, D., BRAGUINI, W. L., KELMER-BRACHT, A. M., and ISHII-IWAMOTO, E. L. 2000. Effects of four monoterpenes on germination, primary root growth, and mitochondrial respiration of maize. *J. Chem. Ecol.* 26:611–624.

ABRAHIM, D., TAKAHASHI, L., KELMER-BRACHT, A. M., and ISHII-IWAMOTO, E. L. 2003. Effects of phenolic acids and monoterpenes on the mitochondrial respiration of soybean hypocotyls axes. *Allelopathy J.* 11:21–30.

AERTS, R., BOOT, R. G. A., and VAN DER AART, P. J. M. 1991. The relation between above- and belowground biomass allocation patterns and competitive ability. *Oecologia* 87:4551–559.

AKINBORO, A., and BAKARE, A. A. 2007. Cytotoxic and genotoxic effects of aqueous extracts of five medicinal plants on *Allium cepa* Linn. *J. Ethnopharmacol.* 112:470–475.

BIBIKOVA, T., and GILROY, S. 2003. Root hair development. *J. Plant Growth Regul.* 21:383–415.

BORBOA, L., and DE LA TORRE, C. 2000. Adaptation to Cd (II) and Zn (II), and the caffeine-potentiated override of the G_2 block induced by the checkpoint activated by DNA damage. *Plant Biosyst.* 134:3–9.

CANGIANO, T., DELLAGRECA, M., FIORENTINO, A., ISIDORI, M., MONACO, P., and ZARRELLI, A. 2002. Effect of ent-labdane diterpenes from Potamogetonaceae on *Selenastrum capricornutum* and other aquatic organisms. *J. Chem. Ecol.* 28:1091–1102.

DEN BOER, B. G. W., and MURRAY, J. A. H. 2000. Triggering the cell cycle in plants. *Trends Cell Biol.* 10:245–250.

DING, L., LIU, G. A., WANG, L., SUN, K., and WANG, H. 2006. Cytotoxic *ent*-kaurane diterpenoids from *Isodon racemosa* (Hemsl) Hara. *Indian J. Chem.* 45:548–551.

FUSCONI, A., GALLO, C., and CAMUSSO, W. 2007. Effects of cadmium on root apical meristems of *Pisum sativum* L.: cell viability, cell proliferation and microtubule pattern as suitable markers for assessment of stress pollution. *Mutat. Res.* 632:9–19.

HAN, Q. B., ZHANG, J. X., LU, Y., WU, Y. S., ZHENG, Q. T., and SUN, H. D. 2004. A novel cytotoxic oxetane *ent*-kauranoid from *Isodon japonicus*. *Planta Med.* 70:581–584.

JIANG, B., LU, Z. Q., HOU, A. J., ZHAO, Q. S., and SUN, H. D. 1999. *ent*-Kaurane diterpenoids from *Isodon lungshengensis*. *J. Nat. Prod.* 62:941–945.

KAMALUDDIN, M., and ZWIAZEK, J. J. 2002. Ethylene enhances water transport in hypoxic aspen. *Plant Physiol.* 128:962–969.

KOITABASHI, R., SUZUKI, T., KAWAZU, T., SAKAI, A., KUROIWA, H., and KUROIWA, T. 1997. 1,8-cineole inhibits root growth and DNA synthesis in the root apical meristem of *Brassica campestris* L. *J. Plant Res.* 110:1–6.

KÖNIG, G. M., WRIGHT, A. D., AUST, H.-J., DRAEGER, S., and SCHULZ, B. 1999. Geniculol, a new biologically active diterpene from the endophytic fungus *Geniculosporium* sp. *J. Nat. Prod.* 62:155–157.

LI, H. J., and GUO, H. W. 2007. Molecular basis of the ethylene signaling and response pathway in *Arabidopsis*. *J. Plant Growth Regul.* 26:106–117.

MACÍAS, F. A., MOLINILLO, J. M. G., GALINDO, J. C. G., VARELA, R. M., TORRES, A., and SIMONET, A. M. 1999. Terpenoids with potential use as natural herbicide templates, pp. 15–31, in H. G. Cutler, and S. J. Cutler (eds.). Biologically Active Natural Products: AgrochemicalsCRC, Boca Raton.

MACÍAS, F. A., VARELA, R. M., SIMONET, A. M., CUTLER, H. G., CUTLER, S. J., ROSS, S. A., DUNBAR, D. C., DUGAN, F. M., and HILL, R. A. 2000. (+)-Brevione A. The first member of a novel family of bioactive spiroditerpenoids isolated from *Penicillium brevicompactum* Dierckx. *Tetrahedron Lett.* 41:2683–2686.

MORALES-FLORES, F., AGUILAR, M. I., KING-DÍAZ, B., DE SANTIAGO-GÓMEZ, J.-R., and LOTINA-HENNSEN, B. 2007. Natural diterpenes from *Croton ciliatoglanduliferus* as photosystem II and photosystem I inhibitors in spinach chloroplasts. *Photosynth. Res.* 91:71–80.

NISHIDA, N., TAMOTSU, S., NAGATA, N., SAITO, C., and SAKAI, A. 2005. Allelopathic effects of volatile monoterpenoids produced by *Salvia leucophylla*: inhibition of cell proliferation and DNA synthesis in the root apical meristem of *Brassica campestris* seedlings. *J. Chem. Ecol.* 31:1187–1203.

PAN, J. W., ZHU, M. Y., and CHEN, H. 2001. Aluminum-induced cell death in root-tip cells of barley. *Envir. Exp. Bot.* 46:71–79.

PARKER, J. S., CAVELL, A. C., DOLAN, L., ROBERTS, K., and GRIERSON, C. S. 2000. Genetic interactions during root hair morphogenesis in *Arabidopsis*. *Plant Cell* 12:1961–1974.

RASMUSSEN, J. A., and EINHELLIG, F. A. 1979. Inhibitory effects of combinations of three phenolic acids on grain sorghum germination. *Plant Sci. Lett.* 14:69–74.

RODRÍGUEZ, F. I., ESCH, J. J., HALL, A. E., BINDER, B. M., SCHALLER, G. E., and BLEECKER, A. B. 1999. A copper cofactor for the ethylene receptor ETR1 from *Arabidopsis*. *Science* 283:996–998.

ROMAGNI, J. G., ALLEN, S. N., and DAYAN, F. E. 2000. Allelopathic effects of volatile cineoles on two weedy plant species. *J. Chem. Ecol.* 26:303–313.

RYAN, E., STEER, M., and DOLAN, L. 2001. Cell biology and genetics of root hair formation in *Arabidopsis thaliana*. *Protoplasma* 215:140–149.

SISLER, E. C., GRICHKO, V. P., and SEREK, M. 2006. Interaction of ethylene and other compounds with the ethylene receptor: Agonists and antagonists, in N. A. Khan (ed.). Ethylene Action in PlantsSpringer, Heidelberg.

SUN, H. D., XU, Y. L., and JIANG, B. 2001. Diterpenoids from Isodon Species. Science, Beijing.

SUZUKI, I., KONDOH, M., NAGASHIMA, F., FUJII, M., ASAKAWA, Y., and WATANABE, Y. 2004. A comparison of apoptosis and necrosis induced by *ent*-kaurene type diterpenoids in HL-60 cells. *Planta Med.* 70:401–406.

TAKEDA, Y., FUJITA, T., and UENO, A. 1981. Structure of Leukamenins. *Chem. Lett.* 10:1229–1232.

TENGCHAISRI, T., CHAWENGKIRTTIKUL, R., RACHAPHAEW, N., REUTRAKUL, V., SANGSUWAN, R., and SIRISINHA, S. 1998. Antitumor activity of triptolide against cholangiocarcinoma growth *in vitro* and in hamsters. *Cancer Lett.* 133:169–175.

YANG, X. H., OWENS, T. G., SCHEFFLER, B. E., and WESTON, L. A. 2004. Manipulation of root hair development and sorgoleone production in sorghum seedlings. *J. Chem. Ecol.* 30:199–213.

ZUNINO, M. P., and ZYGADLO, J. A. 2004. Effect of monoterpenes on lipid oxidation in maize. *Planta* 219:303–309.

serious impacts on plant communities, but the extent of the effect is largely unknown (Ro et al. 2001; Pagel Brown et al. 2007).

Acid rain, as a result of atmospheric pollution, can have detrimental effects on terrestrial and aquatic ecosystems (US-NAPAP 1991). For example, in forest ecosystems, soil acidification is thought to be responsible for a decline in plant health (Aber et al. 1982; Xin et al. 2007). Acidification of soil causes damage to the fine roots of plants that results in leakage of nutrients and directly impacts the soil microbial communities required for plant health and maintenance (Malinowski et al. 1998; Illmer et al. 2003). For example, studies have shown that mycorrhizal associations present in healthy forest trees are poorly developed or absent in unhealthy trees exposed to acid rain (Dighton and Skeffington 1987).

Industrial pollution is pervasive and may comprise organic and inorganic chemicals that may be toxic to plants and their supporting microbes (Kasurinen et al. 2007). Acidification of soils, a byproduct of industrial pollution, increases metal bioavailability (Bergholm et al. 2003; Wang et al. 2006) and exacerbates the detrimental effects of metal pollutants on plants and microbes. All plants have the ability to perceive stresses such as high CO_2, soil acidification, and heavy metal exposure and can initiate complex physiological responses that mitigate impacts of these stresses (Bohnert et al. 1995; Kasurinen et al. 2007). However, few plants are capable of thriving in high stress habitats. The adaptation to stressful habitats is considered to involve changes in the genome (Robe and Griffiths 2000; Schurr et al. 2006) as well as associations with various microbes that are important for health and maintenance of plant systems (Petrini 1996; Rodriguez et al. 2008). Over the last half century, researchers have become concerned about hyperaccumulation of heavy metals in plants due to the alarming increase in industry induced heavy metal contamination and soil acidification (Wang et al. 2005). These metals are easily taken up by roots and translocated to different plant organs (Baker et al. 1994), and high accumulation generally causes growth inhibition and even plant death (Khan and Khan 1983).

The aim of this study was to compare the stress tolerance ability of one plant species growing in industrially contaminated and noncontaminated soils. In South Korea, the invasive weed *Phytolacca americana* has become established in both contaminated and noncontaminated areas (Park et al. 1999, Kim et al. 2005a, b). Here, we present the first chemical studies of *P. americana* plants from two different locales: industrially contaminated soils of Ulsan (acidified soil, metals, and heavy metals) and noncontaminated soils from Suwon, South Korea. Collectively, field and laboratory-generated plants were analyzed and compared for chemical composition and plant response to abiotic stresses associated with global warming (elevated temperature and CO_2) and acid rain (soil pH).

Methods and Materials

Plant and Soil Collection P. americana plants and seeds were collected from contaminated Ulsan (Kyunnam Province) and noncontaminated Suwon (Kyunggi Province) soils, South Korea. The organic plant debris layer surrounding the plants was removed and discarded, and the upper 5 cm of soil (approximately 200 g) was collected in plastic baggies and maintained at 4°C until processed.

Endophyte Profile Analysis of Plants and Soil Greenhouse studies were conducted by propagation of PaU and PaS plants generated from seeds collected from Ulsan and Suwon sites, respectively. For endophyte profile analysis, seeds with their seed coats and plant tissues were surface sterilized in 2% sodium hypochlorite for 10–30 min and rinsed in ten volumes of sterile distilled water. Seeds (N=100) and plant tissues (N=6; cut into roots, stem, and leaf sections) were placed on 0.1× potato dextrose agar (PDA) medium supplemented with antibiotics (100 ug/ml ampicillin and streptomycin, 50 μg/ml tetracycline; Redman et al. 2002a). Soils and plant tissues were processed for endophyte colony forming units (CFU) and percentage colonization of plant tissues, respectively. Soil was homogenized in plastic baggies, passed through a 2-mm soil sieve, 3 g resuspended in 30 ml of sterile water, and 100 μg/ml plated onto 0.1× PDA plates. One gram of surface sterilized plant tissues (Redman et al. 2002a) was homogenized, resuspended in 3 ml of STC (1.2 M Sorbitol, 10 mM TRIS pH 8.0, 10 mM $CaCl_2$) and 500 μg/ml were plated onto 0.1× PDA medium. All plates were maintained at 28°C with a 12-h light regime for 10 days and assessed for fungal colonization. Fungi were identified by using standard taxonomic, microscopic, and molecular techniques (Barnett and Hunter 1998; Redman et al. 2002a). CFU analysis was repeated a minimum of three times.

Soil and Plant Chemical Analysis Soil from Ulsan and Suwon were air-dried and passed through a 2-mm soil sieve. Root and leaf samples of PaU and PaS field plants were oven-dried at 60°C for 48 h and then ground and passed through a 2-mm sieve. Soil and plant tissues were analyzed by using standard protocols for pH, total nitrogen, Cl (Piper 1966), various compounds and metals (B, Al, Co, Cd, Cu, Fe, Mn, Ni, Pb, Zn, As; Agricultural Improving Institute 1988), available phosphate, (Agricultural Improving Institute 1988), suggested exchangeable ions ($(Na^+$, K^+, Mg^{++}, $NO3^-$) Page et al. 1982), and total phenolic compounds (Swain and Hillis 1959) see below.

Total Phenolic Compounds Two hundred grams of fresh PaU and PaS leaf and root tissues were extracted in 1 L of distilled water at room temperature for 48 h and centrifuged at 15,000 rpm for 30 min (Centrikon T-1045, Kontron Co). For soil sampling, the upper 5 cm of soil was collected under a patch of *P. americana* after removing the organic layer. Ten g of PaU and PaS soils were resuspended in 50 ml of sterile water. The supernatant was collected and stored at 4°C (Inderjit 1996). Total phenolic compounds in plant materials (leaves and roots) and soils were analyzed by the Folin–Denis reagent method (Swain and Hillis 1959). All assays were repeated a minimum of three times.

Chlorophyll Content The total chlorophyll content (chlorophyll a and b) of leaves was determined by using standard protocols (MacKinney 1941). Four mature leaves that were 15 cm above the pot soil base for each treatment were measured for chlorophyll content. A minimum of ten measurements per leaf was taken and recorded.

Simulated Acid Rain Stress Uniformly sized PaU and PaS seeds were sterilized for 3 min in 5% sodium hypochlorite solution, and then rinsed with 7–10-fold volume of distilled water. Using a modified bioassay of Lodhi (1976), 30 seeds were sown in a petri dish (diameter of 90 mm) and treated with simulated acid rain (pH 2 to 8) solutions (Park et al. 1999). Seeds were placed in an incubator at 28°C under 400 μmol/m^2/s fluorescent lighting with 16/8 h L/D period for 6 days. Seed germination, growth, and biomass were assessed daily. All assays were replicated a minimum of three times.

Statistical Analysis Data were normally distributed, and significant differences between treatments and controls were calculated with Duncan's mean separation test for the measured parameters (SAS INSTITUTE 2000). The data given in tables and figures are the mean ± SE.

Results

Chemical analysis of the soils and plants (roots and leaves) from contaminated Ulsan and noncontaminated Suwon sites was performed to measure various elements and metals, pH, and total phenolics (Table 1). Statistical analysis revealed that all of the elements analyzed and total phenolics were significantly higher in Ulsan soils. In addition, Ulsan soil pH (3.84) was significantly more acidic than Suwon soil pH (5.82). Of the metals analyzed, Al, Fe, and Mn were in highest abundance (approximately 3, 129, and 14 times, respectively) in Ulsan soils compared to Suwon soils. A similar pattern was observed in the plant leaf tissues with elevated levels of Al, Fe, and Mn at 50.41, 18.30, and 69.45 mg/l in Ulsan plants (PaU) compared to 1.49, 2.80, and 3.11 mg/l in Suwon plants (PaS). Overall levels of these compounds in roots was lower than in leaf tissues; however, differences between PaU and PaS root

Table 1 Chemical characteristics and analysis of soil and plant tissues from Ulsan and Suwon field sites

	Soil		Plant leaf		Plant root	
	Ulsan	Suwon	PaU	PaS	PaU	PaS
Heavy metals (ppm)						
As	**3.62^a±0.40**	0.21^b±0.17	**0.20^a±0.063**	0.04^b±0.01	0.01^c±0.001	0.02^c±0.005
Cd	**0.82^a±0.40**	ND	**0.01^a±0.003**	ND	ND	ND
Co	**0.68^a±0.05**	ND	**0.12^a±0.01**	ND	ND	ND
Cu	**2.13^a±0.24**	0.06^b±0.09	**0.12^a±0.034**	0.04^b±0.008	0.03^b±0.005	0.02^b±0.006
Mn	**93.98^a±0.67**	6.52^b±0.92	**69.45^a±0.45**	3.11^b±0.24	1.10^c±0.065	0.88^c±0.037
Ni	**0.41^a±0.02**	ND	**0.13^a±0.026**	ND	ND	ND
Pb	**3.82^a±0.37**	1.24^b±0.06	**0.14^a±0.018**	ND	ND	ND
An	**5.60^a±0.03**	ND	**3.95^a±0.077**	0.72^b±0.024	ND	0.10^b±0.013
Metals (ppm)						
Al	**1116.23^a±20.67**	432.09^b±15.23	**50.41^a±0.346**	1.49^b±0.089	0.98^b±0.147	0.88^b±0.113
B	**0.24^a±0.07**	ND	**0.61^a±0.059**	0.39^b±0.05	0.08^c±0.004	0.06^c±0.006
Fe	**32.21^a±4.06**	0.25^b±1.10	**18.30^a±0.46**	2.80^b±0.166	0.14^c±0.026	0.39^c±0.052
pH	3.84^b±0.74	**5.82^a±0.69**	**5.42^b±0.236**	5.92^b±0.136	**7.47^a±0.15**	**7.55^a±0.017**
TP	**0.22^a±0.08**	0.07^b±0.02	**1.24^a±0.135**	**0.69^b±0.017**	0.15^c±0.023	0.08^c±0.004

Values represent mean ± SE (N=4) and mean values with the same letter are not significantly different (Duncan's multiple range test, P<0.001). Values in bold indicate significant differences between Ulsan and Suwon soils or *P. americana* plants (roots and leaves) harvested from Ulsan (PaU) and Suwon (PaS) field sites

TP total phenolic compound levels, *ND* not detected

tissues were not significant (Table 1). The pH of leaves and plant tissues were similar in both PaS and PaU plants with the pH ranging from 5.92–5.42 and 7.55–7.47 in the leaf and root tissues, respectively (Table 1). Analysis of total phenolic compunds indicated that they were more than three times higher in Ulsan soils compared to Suwon soils. Similarly, total phenolic compounds were approximately twice as high in both the plant leaf and root tissues of PaU plants compared to PaS plants (Table 1).

Nutrient composition of PaU and PaS plant tissues were also analyzed (Table 2). PaU leaf tissues had elevated levels of Cl, NH_4, N, P, and S compared to PaS plants with N, Cl and S being 846, 11.8, and 1.98 times higher in PaU leaves, respectively. In contrast, PaS plant leaves had elevated levels of K, Na, Ca, and Mg compared to PaU leaf tissues. With the exception of Ca, Na, Mg, and K, all elements tested were higher in PaU roots compared to PaS root tissues. Of these elements, Cl, N, P, and S were notably higher in PaU roots compared to PaS roots (Table 2).

The effects of simulated acid rain (pH 2–6.4) on PaU and PaS plants was measured by determining the percentage seed germination, total phenolic compounds, and chlorophyll levels in plants, as well as plant growth and biomass (Fig. 1). With the exception of chlorophyll content, the overall pattern in each of these assays was similar: PaU plants had higher seed germination ($P<0.001$), plant growth, and biomass compared to PaS plants for all simulated acid rain treatments ($P<0.001$). The highest seed germination of 100% and 78% and chlorophyll content of 37 and 22 mg/m^3 occurred at simulated acid rain pH 2.0 for both PaU and PaS plants, respectively (Fig. 1A and B). The same pattern was observed with plant growth and biomass of shoot at simulated acid rain pH 2.0, with the highest plant growth of 45 and 38 cm, and plant biomass of 5.4 and 4.2 mg occurred in PaU and PaS plants, respectively (Fig. 1C and D). Assessment of plant seed germination, chlorophyll content, plant growth and biomass over a simulated acid rain pH range (2.0–6.4) showed a similar pattern in that the highest levels were achieved at pH 2.0 followed by a marked decrease at pH 3.0–5.0 and a slight increase at pH 6.4 (Fig. 1A–D) for both PaU and PaS plants. In general, an inverse relationship was observed with total phenolic compounds and pH compared to other parameters (% seed germination, plant growth and biomass), with a general increase in total phenolic compounds occurring from pH 2.0 to 5.0 followed by a slight decrease at pH 6.4 for both PaU and PaS plants (Fig. 1B). In contrast, PaS plants exhibited statistically lower total phenolic compound levels than PaU plants but higher chlorophyll content at pH 2–5 (Fig. 1B).

The effects of varying temperature and CO_2 levels on PaU and PaS plant health was assessed (Fig. 2). The overall effect of the treatments (I–IV) on growth rates of PaU plants was III > I > IV > II, and PaS plants was I > III > IV > II, with PaU plants being larger ($P<0.001$) and maturing faster (onset of flowers and mature leaves; recorded observation; data not shown) than PaS plants. PaU and PaS plants were compared and analyzed for chlorophyll content, plant growth, and total phenolic levels when exposed to treatments I–IV; PaU plants were statistically higher for plant growth and total phenolics compared to PaS plants (Fig. 2A and C). In contrast, PaU plants exhibited statistically lower chlorophyll levels when compared to PaS plants (Fig. 2B). Although PaU and PaS plants were the same age, PaU plants were larger, and tissues were more mature when compared to PaS plants (personal observation, Yong Ok Kim). Leaves were broader and more mature and, as such, exhibited a lighter green color and subsequent decrease in chlorophyll content. The reverse was true for total phenolics with levels in PaU higher when compared to PaS plants.

Six PaU and PaS field plants and 100 seeds from both field sites were surface sterilized and plated on microbial

Table 2 Nutrient properties of plant tissues harvested from Ulsan And Suwon field sites

	Leaf		Root	
(ppm)	PaU	PaS	PaU	PaS
K	1405.06^b±12.38	**1758.5^a±20.83**	104.32^c±3.03	98.49^c±3.42
Na	2.07^c±0.05	**11.34^a±0.63**	5.27^b±0.27	1.96^c±0.03
Ca	1.01^d±0.10	2.73^b±0.11	2.33^c±0.13	**3.97^a±0.0.14**
Mg	128.72^b±0.85	**220.89^a±5.37**	7.08^c±0.10	4.33^c±0.16
Cl	**584.12^a±8.49**	49.48^c±0.87	111.35^b±0.63	12.49^d±0.2
NH_4	**68.25^a±0.97**	43.25^b±1.16	20.45^{c}0.55	19.15^c±1.42
N	169.20^b±2.37	0.20^d±0.03	**201.72^a±1.45**	54.45^c±0.31
P	**46.18^a±0.75**	36.53^b±0.33	1.24^c±0.13	0.21^c±0.09
S	**1092.1^a±11.17**	550.10^b±0.65	40.25^c±1.47	10.30^d±0.38

Values represent mean ±SE ($N=3$) and mean values wih the same letter are not significantly different (Duncan's multiple range test, $P<0.001$). Values in bold indicate significant differences between Ulsan and Suwon soils or *P. americana* plants (roots and leaves) harvested from Ulsan (PaU) and Suwon (PaS) field sites

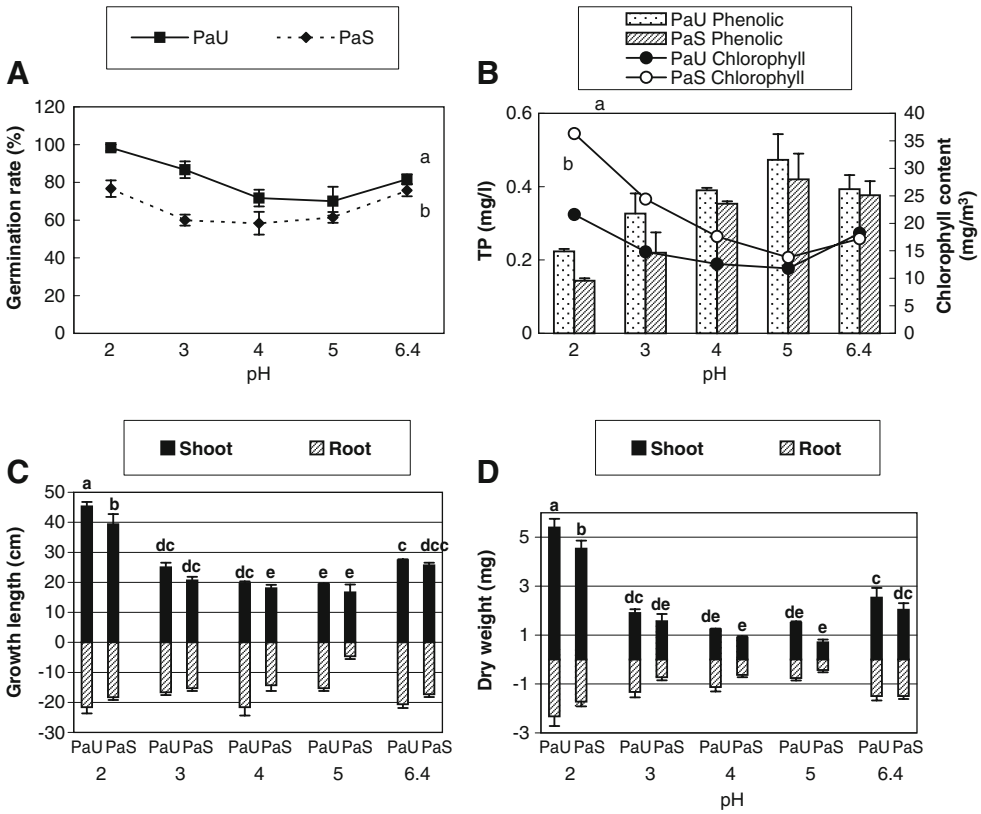

Fig. 1 Comparison of **A** germination rate; **B** phenolic compounds and chlorophyll levels; **C** growth and; **D** dry weight of plants grown from seeds harvested from Ulsan (*PaU*) contaminated soils and Suwon (*PaS*) noncontaminated soils field sites. PaS and PaU plants were exposed to simulated acid rain conditions (pH 2, 3, 4, 5) and the control pH of the potting soil was 6.4. *TP* Total phenolic compound levels; means with the *same letter* are not significantly different (Duncan's multiple range test, **A**, **B**, **C** and **D**=$P<0.001$), ±SE of $N=6$ measurements each are given

growth media to determine the plant and seed endophyte profiles. The surrounding rhizosphere soil was collected and analyzed to determine the composition of the soil microbial community. All six of the PaU plants had a fungal endophyte (*Glomeralla acutata*) associated with the leaf and root tissue, as did 97.25% of seeds. No such fungal associations were found in PaS plants and seeds. Analysis of the soils surrounding the plants indicated that the same fungal endophyte was present in Ulsan and absent in Suwon soils (Table 3, $P<0.01$).

Discussion

In 1979, *P. americana* was introduced into South Korea and became an important ecological problem by 1993 when it had spread throughout the country, displacing many native species (Park 1995). *P. americana* is widely distributed in contaminated areas of Korea, and a correlation between plant distribution and pollution absorption from soils has been reported (Park et al. 1999). Little is known about Phytolaccaceae ecology with the exception of a few papers that deal with the invasive threat of *P. americana* in Korea (Kim et al. 2005a, b). The extent of the effects of pollution on plants and the time frame for adaptation to stressful habitats in unknown but thought to involve a combination of plant genome changes and associations with microbes that can hasten the adaptation (within a growing season) in a non-Darwinian manner (Rodriguez et al. 2008). Our studies are the first to address the effects of pollution and global warming related stresses of *P. americana* via plant chemical analysis and the surrounding rhizosphere soils from two different habitats. Interestingly, PaU plants growing in the Ulsan contaminated site were larger (overall height, leaf size and number), more robust, and growing more densely than PaS plants growing in noncontaminated Suwon soils (personal observation, Yong Ok Kim).

It is not surprising that PaU plants had higher concentrations of phenolic compounds in the leaves and roots when compared to PaS plants, since plants exposed to metal or acid stress often produce secondary metabolites that are thought to play roles in plant health and survival (Inderjit 1996; Kasurinen et al. 2007). Terpenoids, flavonoids, alkaloids, and phenolics are the most common allelochemicals, with the latter allelochemical being abundant under field conditions (Seneviratne and Jayasinghearachchi 2003). Phenolic compounds are known to affect seed germination, seedling growth, cell division, fungal activity, protein synthesis, and enzyme activity (Callaway et al. 2004; Vivanco et al. 2004). It has been reported that phenolic-induced oxidative damage is mediated by heavy metals (Sakihama and Yamasaki 2002) and that accumulation of

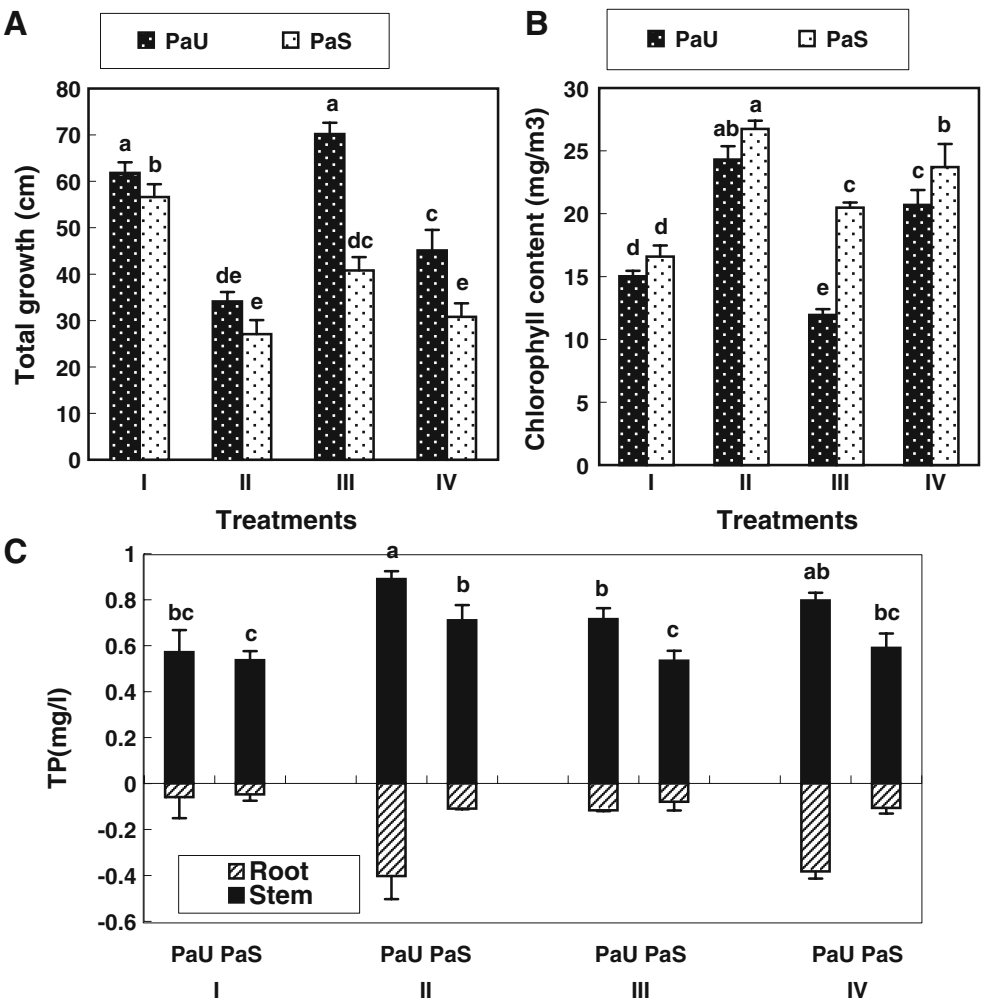

Fig. 2 Comparison of **A** Total growth; **B** Chlorophyll content; **C** Phenolic compound levels of plants grown from seed harvested from Ulsan (contaminated soils) and Suwon (noncontaminated soils) field sites. PaS and PaU plants were exposed to treatments *I* ambient CO_2 (360 µmol mol^{-1} CO_2) and temperature (25°C); *II* double ambient CO_2 (650 µmol mol^{-1} CO_2) and ambient temperature (25°C); *III* increased temperature (30°C) with ambient CO_2; and *IV* increased temperature (30°C) and double ambient CO_2. *TP* total phenolic compound levels; means with the *same letter* are not significantly different (Duncan's multiple range test, **A** and **B**=$P<0.001$, **C**=$P<0.01$), ±SE of $N=10$ measurements each are given

these metals occurs by conjugation with phenolics in various plant organs (Santiago et al. 2000; Lavid et al. 2001). Loponen et al. (2001) studied foliar concentrations of phenolics in contaminated and control areas to detect early symptoms of heavy-metal pollution in birch trees. They found that in heavily polluted areas, atmospheric stress factors appear to be correlated with the accumulation of phenolics in birch leaves (Andrea et al. 2006). In addition, exposure of plants to simulated low-pH acid rain (pH 3.0) conditions resulted in the appearance of necrotic spots on the leaf blades and subsequent accumulation of phenolics in necrotic areas (Sant'Anna-Santos et al. 2006).

Table 3 Comparison of Ulsan and Suwon soils and plant tissues for presence of *Glomerella acutata*

	Ulsan	Suwon
Seed	97.25^{a}±2.21	ND
Leaf	84.53^{a}±13.07	ND
Root	17.10^{b}±2.07	ND
Soil	85.42^{a}±5.77	ND

Values represent mean ±SE and mean values with the same letter are not significantly different (Duncan's multiple range test, $P<0.01$). Numbers denote percentage colonization of 100 seeds and colony forming units (CFU) equivalent to 0.17 g of plant tissues ($N=6$) and 10 mg of soil ($N=6$), respectively
ND not detected

Our studies showed that *P. americana* preferentially accumulates metals in leaf tissues compared to root tissues. This was not expected since several studies indicate that plants preferentially accumulate metals in root tissues (Rauser and Meuwly 1995). For example, Nishizono et al. (1987) reported the heavy metals, Cd, Cu, and Zn, accumulated in roots of *Athyrium yokoscense* compared with other organs. Lubben and Sauerbeck (1991) also suggested that roots are the first site of accumulation of heavy metals and subsequent tolerance to heavy metals (Wu and Bradshaw 1972).

Seed germination of both PaU and PaS plants decreased when exposed to simulated acid rain with the lowest germination rates occurring at pH 5.0 and 4.0, respectively. Although seed germination of PaU and PaS increased at pH 6.4, the highest percentage germination occurred at the lowest pH (2.0), and germination rates for PaU were higher

than PaS at all pH levels tested. The growth length, dry weight, and overall health of PaU and PaS were also highest at pH 2.0, with all values greater for PaU. These results are in contrast to those of others (Fan and Wang 2000) that showed simulated acid rain conditions of pH 2.0 caused foliar damage, reduced chlorophyll content, and reduced seedling growth; while seedling growth was simulated at pH levels between pH 3.5 and 5. Regardless, increased germination and growth of PaU plants at pH 2.0 suggests that PaU plants have more tolerance to acid rain than PaS plants.

The phenolic levels of both PaU and PaS plants significantly increased with pH under simulated acid rain conditions, and the phenolic content of PaU plants was higher than that of PaS plants (Fig. 1). Interestingly, in other plants species (*Mimosa artemisiana*, *Gallesia integrifolia*, and Norway spruce), enhanced accumulation of phenolics and development of necrotic spots on leaves occurred upon exposure to acidic pH (3.0; Sant'Anna-Santos et al. 2006). Although phenolic accumulation was directly proportional to pH in *P. americana*, plant growth was inversely proportional to pH, and lesions did not develop at low pH. Therefore, we surmise that the acidic nature of soils and enhanced bioavailability of compounds do not have a negative impact on growth or disease symptom development on PaU plants.

Exposure of plants to different environmental treatments (altered CO_2, temperature, and soil pH) resulted in altered growth rates (Fig. 2). PaU plants grew larger and matured faster in all treatments compared to PaS plants. In addition, the treatments affected plant growth differently with PaU plants having highest growth in treatment III (ambient CO_2 and increased temperature) while PaS plants had the greatest growth in treatment I (ambient CO_2 and temperature). Although the significance of this is not yet known, it demonstrates that PaU and PaS plants are physiologically different.

The chlorophyll content of leaves was greater for PaS plants than PaU plants in every treatment. A contributing factor to the observed overall decrease in plant chlorophyll content in PaU plants growing in contaminated soils is that they grew more quickly (both overall plant height and overall size and number of leaves), and the leaves measured were physiologically more mature than PaS plants. Chlorophyll levels in both plants were highest when the ambient CO_2 levels were doubled. However, measurements of the growth, chlorophyll content, and phenolic levels did not show a significant difference between treatment I of PaU plants when compared to PaS plants. Mature flowers and aging leaves in PaU plants appeared earlier than in PaS plants and, as such, is reflected as a decrease in chlorophyll content in PaU plants. Similarly, Loponen et al. (2001) and Peltonen et al. (2005) reported that elevated CO_2 increased phenolic compound levels by 25%, with phenolic compound levels 21% higher in samples from heavily contaminated smelter areas than from noncontaminated areas. We observed a similar phenomenon with higher levels of phenolics in PaU plants than PaS plants, especially in the roots of plants exposed to treatment II (25°C, 650 ppm CO_2) and IV (30°C, 650 ppm CO_2).

Total phenolic levels in the leaves and roots of treatment I were less than that of the other treatments, and the growth rates decreased, while chlorophyll content increased for treatments II and IV. This suggests that there may be a relationship between growth rates, total phenolic levels, and chlorophyll content.

Microbial analysis showed that a single dominant fungal endophyte (>97%) was present in the contaminated (PaU) and absent in the noncontaminated (PaS) site samples. The endophyte was identified as *G. acutata*, which was isolated from leaves, roots, and seeds, and was also abundant in the surrounding soils (Table 3). Interestingly, the endophyte was in highest abundance (84.53 CFU) in PaU leaves where the highest accumulation of metals was found. The abundance of the endophyte in Ulsan soils, and the variance in abundance levels in the different plant tissue types, suggest that this endophyte may have an ecologically significant role in PaU plants. Recent studies have shown that symbiotic fungi impart habitat-specific stress tolerances and are responsible for the survival of some plants in high-stress habitats (Redman et al. 2002a; Rodriguez et al. 2008). In addition, fungal endophytes are known to promote plant growth (Redman et al. 2002b; Varma et al. 2006). However, the ecological role of endophytes in contaminated habitats has not been well defined. Studies have shown that fungal symbionts have the ability to confer heavy metal tolerance to plants (Monnet et al. 2001). Some fungi produce siderophore and siderophore-like compounds and are able to sequester iron in the surrounding rhizosphere (Wilhite et al. 2001), while other fungi increase translocation of metals to leaf tissues (Al-Karaki et al. 2001).

Although the mechanism is yet unknown, collectively, these studies provide evidence to support the hypothesis that PaU plants have adapted to and are able to thrive in this industrially contaminated high stress habitat. The PaU plants represent acid-rain and heavy-metal-tolerant ecotypes, while PaS plants do not. Although additional work is required, the results presented here are promising and demonstrate the ability of PaU pokeweed plants to absorb and translocate metals to leaf tissues. As such, the results suggest that this system may be developed as a potential useful phyto-remediation tool to clean-up contaminated soils.

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References

ABER, J. D., HENDREY, G. R., BOTKIN, B., FRANCIS, A. J., and MELILLO, J. M. 1982. Potential effects of acid precipitation on soil nitrogen and productivity of forest ecosystems. *Water, Air and Soil Pollute* 18:405–412.

AGRICULTURAL IMPROVING INSTITUTE. 1988. Methods of Soil Chemical Analysis. p.450.

AL-KARAKI, G. N., HAMMAD, R., and RUSAN, M. 2001. Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. *Mycorrhiza* 11:43–47.

ANDREA, M., STEFFEN, A., BARBARA, K., GERHARD, H. B., UWE, S., and HANS-PETER, M. 2006. Growth at elevated CO_2 concentrations leads to modified profiles of secondary metabolites in tobacco cv. SamsunNN and to increased resistance against infection with potato virus Y. *Plant, Cell and Environ.* 29:126–137.

BAKER, A. J., REEVES, R. D., and HAJAR, A. S. M. 1994. Heavy metal accumulation and tolerance in British populations of the metallophyte *Thlaspi caerulescens* J. and C. Presi (Brassicaceae). *New Phytol.* 127:61–68.

BARNETT, H. L., and HUNTER, B. B. 1998. Illustrated genera of imperfect fungi, p. 240. American Phytopathology Society, St. Paul.

BERGHOLM, J., BERGGREN, D., and ALAI, G. 2003. Soil acidification induced by ammonium sulphate addition in a Norway spruce forest in southwest Sweden. *Water, Air, and Soil Pollution* 148:87–109.

BOHNERT, H. J., NELSON, D. E., and JENSEN, R. G. 1995. Adaptations to environmental stresses. *The Plant Cell* 7:1099–1111.

CALLAWAY, R. M., THELEN, G. G., RODRIGUEZ, A., and HOLVEN, W. E. 2004. Release from inhibitory soil biota in Europe may promote invasive plant invasion in North America. *Nature* 427:731–733.

DIGHTON, J., and SKEFFINGTON, R. A. 1987. Effects of artificial acid precipitation on the mycorrhizae of Scots seedlings. *New Phytol.* 107:191–202.

FAN, H. B., and WANG, Y. H. 2000. Effects of simulated acid rain on germination, foliar damage, chlorophyll contents and seedling growth of five hardwood species growing in China. *Forest Ecol. & Management* 126:321–329.

HALL, M. C., STILING, P., HUNGATE, B. A., DRAKE, B. G., and HUNTER, M. D. 2005. Effects of elevated CO_2 and herbivore damage on litter quality in a scrub oak ecosystem. *J. Chem. Ecol.* 31:2243–2356.

ILLMER, P., OBERTEGGER, U., and SCHINNER, F. 2003. Microbiological properties in acidic forest soils with special consideration of KCl extractable Al. *Water, Air, and Soil Pollution* 148:3–14.

INDERJIT, M. A. U. 1996. Plant phenolics in allelopathy. *Bot Rev.* 62:186–202.

KASURINEN, A., PELTONEN, P. A., JULKUNEN-TIITO, R., VAPAAVUORI, E., NUUTINEN, V., HOLOPAINEN, T., and HOLOPAINEN, J. K. 2007. Effects of elevated CO_2 and O_3 on leaf litter phenolics and subsequent performance of litter-feeding soil macrofauna. *Plant and Soil* 292:25–43.

KHAN, S., and KHAN, N. N. 1983. Influence of lead and cadmium on the growth and nutrient concentration of tomato (*Lycopersicon esculentum*) and egg-plant (*Solanum melongena*). *Plant and Soil* 74:387–394.

KIM, Y. O., JOHNSON, J. D., and LEE, E. J. 2005a. Allelochemical effects and chemical analysis of leaf extracts from three Phytolaccacese species. *J. Chem. Ecol.* 31:1175–1186.

KIM, Y. O., JOHNSON, J. D., and LEE, E. J. 2005b. Phytotoxicity of *Phytolacca americana* leaf extracts on the growth, and physiological response of *Cassia mimosoides*. *J. Chem. Ecol.* 31:2963–2973.

KORNER, C. 1996. The response of complex multispecies systems to elevated CO_2, pp. 20–42, in B. H. Walker, and W. L. Steffen (eds.). Global Change and Terrestrial Ecosystems. Cambridge University Press, Cambridge.

LAVID, N., SCHWARTZ, A., YARDEN, O., and TEL-OR, E. 2001. The involvement of polyphenols and peroxidase activities in heavy-metal accumulation by epidermal glands of the water lily (Nymphaeaceae). *Planta* 212:323–331.

LODHI, M. A. K. 1976. Role of allelopathy as expressed by dominating trees in a lowland forest in controlling the productivity and pattern of herbaceous growth. *Amer. J. Bot.* 63:1–8.

LOPONEN, J., LEMPA, K., OSSIPOV, V., KOZLOV, M. V., GIRS, A., HANGASMAA, K., HAUKIOJA, E., and PIHLAJA, K. 2001. Patterns in content of phenolic compounds in leaves of mountain birches along a strong pollution gradient. *Chemosphere.* 45:291–301.

LUBBEN, S., and SAUERBECK, D. 1991. The uptake and distribution of heavy metals by spring wheat. *Water Air and Soil Pollut.* 57–58: 239–247.

MACKINNEY, G. 1941. Absorption of light by chlorophyll solutions. *J. Biol. Chem.* 140:315–332.

MALINOWSKI, D., ALLOUSH, G., and BELESKY, D. 1998. Evidence for chemical changes on the root surface of tall fescue in response to infection with the fungal endophyte *Neotyphodium coenophialum*. *Plant and Soil* 205:1–12.

MONNET, F., VAILLANT, N., HITMI, A., COUDRET, A., and SALLANON, H. 2001. Endophytic *Neotyphodium lolii* induced tolerance to Zn stress in *Lolium perenne*. *Physiol. Plantarum* 113:557–563.

NISHIZONO, H., ICHIKAWA, J., SUZIKI, S., and ISHII, F. 1987. The role of the root cell wall in the heavy metal tolerance of *Athyrium yokoscense*. *Plant and Soil* 101:15–20.

PAGE, A. L., MILLER, R. H., and KEENEY, D. R. 1982. Methods of Soil Analysis, part 2, Chemical and Microbiological Properties, 2nd edn. American Society of Agronomy and Soil Science Society of America, Madison.

PAGEL BROWN, A. L., DAY, F. P., HUNGATE, B. A., DRAKE, B. G., and HINKLE, C. R. 2007. Root biomass and nutrient dynamics in a scrub-oak ecosystem under the influence of elevated atmospheric CO_2. *Plant and Soil.* 292:219–232.

PARK, S. H. 1995. Unrecorded naturalized species in Korea. *Korean J. of Species Taxo.* 25:123–130.

PARK, Y. M., PARK, B. J., and CHOI, K. R. 1999. pH changes in the Rhizosphere soil of *Phytolacca americana*. *Korean J. Ecol.* 22:7–11.

PELTONEN, P. A., VAPAAVUORI, E., and JULKUNEN-TIITO, R. 2005. Accumulation of phenolic compounds in birch leaves is changed by elevated carbon dioxide and ozone. *Global Change Biology* 11:1305–1324.

PETRINI, O. 1996. Ecological and physiological aspects of host-specificity in endophytic fungi, pp. 87–100, in S. C. Redlin, and L. M. Carris (eds.). Endophytic Fungi in Grasses and Woody Plants. APS, St. Paul.

PIPER, C. S. 1966. Soil and plant analysis, p. 1368. Hans, Bombay.

RAUSER, W. E., and MEUWLY, P. 1995. Retention of cadmium in roots of maize seedling. Role of complexation by phytochelatins and related thiol peptides. *Plant Physiol.* 109:195–202.

REDMAN, R. S., SHEEHAN, K. B., STOUT, R. G., RODRIGUEZ, R. J., and HENSON, J. M. 2002a. Thermotolerance conferred to plant host and fungal endophyte during mutualistic symbiosis. *Science.* 298:581.

REDMAN, R. S., ROOSSINCK, M. J., MAHER, S., ANDREWS, Q. C., SCHNEIDER, W. L., and RODRIGUEZ, R. J. 2002b. Field performance of cucurbit and tomato plants colonized with a nonpathogenic mutant of *Colletotrichum magna* (teleomorph: *Glomerella magna*; Jenkins and Winstead). *Symbiosis.* 32:55–70.

RO, H. M., KIM, P. G., LEE, I. B., YIEM, M. S., and WOO, S. Y. 2001. Photosynthetic characteristics and growth responses of dwarf

apple (*Malus domestica* Borkh. Cv. Fuji) saplings after 3 years of exposure to elevated atmospheric carbon dioxide concentration and temperature. *Trees.* 15:195–203. doi:10.1007/s004680100099.

ROBE, W. E., and GRIFFITHS, H. 2000. Physiological and photosynthetic plasticity in the amphibious, freshwater plant, *Littorella uniflora*, during the transition from aquatic to dry terrestrial environments. *Plant Cell Environ.* 23:1041–1054.

RODRIGUEZ, R., HENSON, J., VAN VOLENBURGH, E., HOY, M., WRIGHT, L., BECKWITH, F., KIM, Y. O., and REDMAN, R. S. 2008. Stress tolerance in plants via habitat-adapted symbiosis. *ISME-Nature.* 2:406–416. doi:10.1038/ismej.2007.106.

SAKIHAMA, Y., and YAMASAKI, H. 2002. Lipid peroxidation induced by phenolics in conjunction with aluminum ions. *Bio. Plant.* 45:249–254.

SANTIAGO, L. J. M., LOURO, R. P., and DE OLIVEIRA, D. E. 2000. Compartmentation of phenolic compounds and phenylalanine ammonia-lyase in leaves of (*Phyllanthus tenellus* Roxb.) and their induction by copper sulphate. *Ann. Bot.* 86:1023–1032.

SANT'ANNA-SANTOS, B., CAMPOS, F., DA SILVA, L., AZEVDGO, A. A., MARCOS DE ARAJO, J., ALVES, E. F., MONTEIRO DA SILVA, A., and AGUIAR, R. 2006. Effects of simulated acid rain on the foliar micromophology and anatomy of tree tropical species. *Environ. Exp. Botany.* 58:158–168.

SAS INSTITUTE 2000. SAS/STAT Guide for Personal Computers, Version 6.03 ed. SAS, Cary.

SCHURR, U., WALTER, A., and RASCHER, U. 2006. Functional dynamics of plant growth and photosynthesis - from steady-state to dynamics - from homogeneity to heterogeneity. *Plant Cell Environ.* 29:340–352.

SENEVIRATNE, G., and JAYASINGHEARACHCHI, H. S. 2003. Phenolic acids: Possible agents of modifying N_2-fixing symbiosis through rhizobial alteration. *Plant and Soil.* 252:385–395.

STAINFORTH, D. A., AINA, T., CHRISTENSEN, C., COLLINS, M., FAULL, N., FRAME, D. J., KETTLEBOROUGH, J. A., KNIGHT, S., MARTIN, A., MURPHY, J. M., PIANI, C., SEXTON, D., SMITH, L. A., SPICER, R. A., THORPE, A. J., and ALLEN, M. R. 2005. Uncertainty in predictions of the climate response to rising levels of greenhouse gases. *Nature.* 433:403–407.

SWAIN, T., and HILLIS, W. E. 1959. The phenolic constituents of *Prunus domestica* I. The quantitative analysis of the phenolic constitutents. *J. Sci. Food Agric.* 10:63–68.

US NATIONAL ACID PRECIPITATION ASSESSMENT PROGRAM. 1991. Acidic deposition: State of science and technology, Vol. I–IV, ed. P. M. Irving. Government Printing Office, Washington, DC 20402-9352, USA.

VARMA, A., VERMA, S., SUDHA, S. N., BUTEHORN, B., and FRANKEN, P. 2006. *Piriformospora indica*, a cultivable plant-growth-promoting root endophyte. *Appl. Environ. Microbiol.* 6:2741–2744.

VIVANCO, J. M., BAIS, H. P., STERMITZ, F. R., THELEN, G. C., and CALLAWAY, R. M. 2004. Biogeographical variation in community response to root allelochemistry: Novel weapons and exotic invasion. *Ecol. Lett.* 7:285–292.

WANG, F., LIN, X., and YIN, R. 2005. Heavy metal uptake by arbuscular mycorrhizas of *Elsholtzia splendens* and the potential for phytoremediation of contaminated soil. *Plant and Soil.* 269:225–232.

WANG, A. S., ANGLE, J. S., CHANEY, R. L., DELORME, T. A., and REEVES, R. D. 2006. Soil pH effects on uptake of Cd and Zn by *Thlaspi caerulescens*. *Plant and Soil.* 281:325–337.

WILHITE, S. E., LUMSDEN, R. D., and STRANEY, D. C. 2001. Peptide synthetase gene in *Trichoderman virens*. *Appl. Environ. Microbiol.* 11:5055–5062.

WU, L., and BRADSHAW, A. D. 1972. Aerial pollution and the rapid evolution of copper tolerance. *Nature.* 238:167–169.

XIN, L. H., HAN, S. J., LI, L., ZHOU, Y. M., and ZHENG, J. Q. 2007. Responses of soil enzymes to long-term CO_2 enrichment in forest ecosystems of Changbai Mountains. *Northeast Forestry University and Ecological Society of China.* 18:119–122.

via lolines as "exogenous resistance" since it originates from the symbiont rather than the grass itself.

Plants, particularly dicotyledonous ones, often can respond directly to damage by producing their own defensive chemicals (Karban and Myers 1989). We refer to this as "endogenous resistance." The response involves signaling pathways within the plant that are activated and which lead to the synthesis of many different secondary metabolites (McConn et al. 1997). The induced response is thought to be a tactic that allows plants to produce defensive compounds only when they are needed.

One signaling molecule used by many plants is jasmonic acid (JA), a ubiquitous phytohormone, synthesized through the octadecanoid pathway (Gols et al. 2003). Following herbivore feeding, endogenous concentrations of JA rapidly increase and lead to activation of numerous genes (Farmer and Ryan 1992; Reinbothe et al. 1994). Methyl jasmonate (MJ) and JA are known to regulate a number of physiological processes in plants that include the induction of proteinase inhibitors, meristematic growth, senescence, vegetative storage proteins, and interplant signals (de Bruxelles and Roberts 2001). Jasmonates also are involved in signal transduction, especially in relation to defense gene activation (Gunlach et al. 1992; Wasternack 2007).

Interactions between the endogenous inducible defenses of grasses and those provided through symbiotic endophytes (exogenous) have not been studied previously. By examining two inducible systems of defense, loline synthesis by *Neotyphodium* and tall fescue defense gene activation by jasmonates, we can learn more about their interactions and possible antagonistic or synergistic relationships. Additionally, the study of these defense systems may lead to a better understanding of the signaling that takes place between fungus and its host plant. We formulated two competing hypotheses: (a) *Neotyphodium coenophialum* responds to MJ by upregulating loline synthesis (either by responding directly to MJ or to biochemical signals produced by the plant in response to MJ), and (b) the plant responds to MJ by upregulating its own defensive chemicals and this response compromises the ability of *N. coenophialum* to produce lolines.

Methods and Materials

Plant Growth Tall fescue K-31 (*Lolium arundinaceum)* was planted in April 2007 into Sungrow Sunshine LC1 professional potting soil topped with vermiculite in 15.3-cm-diameter pots. Seeds were provided by H. Fribourg at the University of Tennessee (Knoxville, TN, USA) in 2001 and stored at −10°C until use. Infected seeds were obtained from infected plants (*N. coenophialum* is naturally transmitted through host seed) while plants free of endophyte infection were produced by heat treatment of seeds (Nott and Latch 1993) in the 1980s followed by subsequent annual cultivation of plants for seed production. Our plants were grown in a greenhouse with natural light [supplemental light (14-h L:10-h D) was also used for the first 6 weeks of growth]. Temperature varied from 15°C to 40°C. Seeds containing the fungal endophyte *N. coenophialum* were planted into 72 pots, while endophyte-free seeds were planted into 96 pots. After plants emerged from the soil, they were thinned to one plant per pot. Plants were fertilized every 1–2 weeks for the first 5 weeks with1.5-g/l solution of 5% NH_4, 10% NO_3, 16% P_2O_5, and 17% K_2O (Pro Sol Ozark, AR, USA). Plants were subsequently fertilized consistently on a weekly basis.

Plant Treatment

Experiment I. Eight weeks after germination, 31 infected and 58 infection-free plants were placed randomly into a controlled environmental chamber (185 cm L×77 cm W×178 cm H; 1.7-m^2 growth capacity; 22°C) with continuous airflow (0.15 m^3/min) for MJ exposure. Five cotton swabs were first submerged in MJ (95%, Sigma-Aldrich) and then suspended in the headspace. Plants were exposed for 3 h. Similar exposures to gaseous MJ have been used (Farmer and Ryan 1990; Hall and Horton 1994; Matsui et al. 1999); yet, generally, these are done in relatively small sealed containers, whereas ours was conducted in a large chamber with mixing of air from the outside. More commonly, MJ is applied directly to leaves as a spray (e.g., Thaler 1999). We did not quantify the amount of MJ applied to swabs, and the level we used may have been higher than what might occur in the headspace of grasses in nature, yet, since our primary goal was to determine if and how the fungus responds to MJ exposure, we wanted to provide a sufficiently strong signal to elicit a possible fungal response. It is possible that the MJ concentration we used was toxic to *N. coenophialum*, but this seems unlikely since the fungus lives within the intercellular spaces within the plant. At the conclusion of the exposure, plants were removed from the chamber and returned to the greenhouse. Nonexposed plants (N=79) were not placed into controlled environmental chambers due to the short duration of the treatment (3 h) and because all controlled environment chambers are in the same room, thus leading to the possibility that aromatic MJ from the treatment cham-

bers would contaminate chambers in which control plants would have been placed. In sum, four treatment combinations were produced in the two-way design: infection free–MJ free, infection free–MJ-exposed, infected–MJ free, and infected–MJ-exposed.

Experiment II. A second experiment was conducted to determine if MJ exposure would compromise the ability of *Neotyphodium* to mount a wound-inducible response of loline biosynthesis (as had been shown by Bultman et al. 2004 and Sullivan et al. 2007). Ten weeks after germination, all 28 plants (14 endophyte-infected and 14 endophyte free) were cut with scissors to 15.3 cm above the soil surface to simulate vertebrate grazing. At 10.5 weeks of age, all 28 plants were placed in a controlled environment chamber for MJ exposure. Plants then were returned to the greenhouse. Hence, Experiment II had the same design as Experiment I except that plants in Experiment II had been mechanically damaged prior to MJ exposure.

Herbivore Resistance

Experiment I. Three days after exposure to MJ and 9 weeks after germination, a bioassay was performed with *R. padi* (bird cherry oat aphid). Aphids were obtained from S. Clement (ARS-USDA, Pullman, WA, USA) and were reared on barley at 20°C prior to use in the experiment. Four aphids (second- and third-instar nymphs) were placed in a mesh clip bag that was secured to the newest fully expanded leaf blade of the largest tiller. Plants (N=20–26 per treatment combination) were placed on lighted plant stands (14:10 h, L:D) for 7 days at 21°C. *R. padi* reproduces parthenogenetically while feeding on grass host plants (Leather et al. 1989) and has been used extensively in grass–endophyte research (Eichenseer et al. 1991; Siegel et al. 1990; Breen 1994; Bultman et al. 2004). After 7 days, clip bags were removed from the plants, and the apterous aphids were counted.

Experiment II. Three days after exposure to MJ and 1 week after damage, an aphid bioassay identical to that described for Experiment I was performed.

Loline Extraction and Analysis Samples were taken 10 days after MJ exposure for loline analysis from four endophyte-infected control (not exposed to MJ) plants and four infected plants exposed to MJ. Three to four tillers of fresh material were cut from each plant. Plant material was freeze-dried and finely ground with a Wiley mill and then stored at −20°C. Analysis of lolines followed that of Bush et al. (1982). Briefly, 100 mg of ground plant tissue were added to 95:5 dichloromethane–ethanol and 100 ml saturated sodium bicarbonate. The plant mixture was vortexed and centrifuged, and the liquid layer was pipetted into a test tube. Nitrogen gas was blown onto the surface of the liquid layer to concentrate the solution. The solution was filtered with a syringe and placed into a gas chromatograph (GC)–mass spectrometer (MS) vial for analysis. Samples were inspected for retention times at 5.68 min for *N*-formyl loline and 5.94 min for *N*-acetyl loline with mass peaks at 82. Standards for comparison were obtained from L. Bush (University of Kentucky). Samples that were prepared and not immediately run were refrigerated for no more than 24 h before analysis on GC–MS.

RNA Extraction and Quantification of LolC Samples for analysis with real-time reverse transcriptase (RT)-polymerase chain reaction (PCR) were obtained at both 0.5 and 1.5 weeks after MJ exposure (we sampled at two dates to determine if fungal response varied over that time frame). Fresh tissue (150 mg) was harvested from the base of the youngest fully expanded leaves from two tillers per plant (N=4 plants per treatment combination per date), as in Sullivan et al. (2007). The tissue was stored at −80°C. Samples were prepared by grinding them in liquid nitrogen into a powder with mortar and pestle. RNA was extracted by using the RNAeasy minikit (Qiagen). Purified RNA was quantified by using a Hitachi U-2000 spectrophotometer (Sullivan et al. 2007). Probes, primers, and conditions described by Spiering et al. (2005) were used for the quantitative real-time RT-PCR. Primers specific for one of the loline biosynthesis genes, *LolC*, were used in the assay (Spiering et al. 2005). The amount of β-tubulin mRNA was used as a reference for the amount of fungal RNA in the sample. The reaction was performed by using the Brilliant II QRT-PCR Master Mix Kit (Stratagene) on a Smart Cycler II (Chepheid; Sullivan et al. 2007). One-hundred nanograms of total RNA were used in each reaction, and all reactions were run in triplicate. All runs included a negative control (H_2O) to ensure that no contamination was present, and a no-reverse-transcriptase control was run for every sample to ensure that there was no contaminating genomic DNA. RT-PCR can have significant variability among individual reactions as a result of stochastic variation (Keilholz et al. 1998); for this reason, replicate samples were not considered valid if the coefficient of variation in cycle threshold values varied by >5%. Plants used in the

RNA samplings were not used in any subsequent experiments to ensure that damage caused by the sampling was not causing a defense response (Sullivan et al. 2007).

Statistical Analysis Statistical analysis was performed with Systat (Systat 2004). Analysis of bioassay data (number of aphids per plant) was carried out with a two-way analysis of variance (ANOVA) to compare the effects of MJ and infection on the performance of *R. padi.* Aphid number was ranked prior to analysis to correct for departures from normality (Conover and Iman 1981). Loline data and mRNA data were analyzed by using a two-sample *T* test or its nonparametric equivalent where appropriate.

Results

Effects of Methyl Jasmonate Exposure on Herbivore Resistance

Experiment I. We tested whether or not the alkaloid-mediated insect resistance that *N. coenophialum* provides its host plant is influenced by MJ exposure. We found that fungal infection reduced aphid numbers (Fig. 1; $F_{1,\ 96}$=199.4, P<0.001). In contrast, there was no difference in aphid numbers between plants treated with MJ and those left untreated ($F_{1,\ 96}$=0.04, P=0.84). Interestingly, there was an interaction between endophyte infection and MJ exposure ($F_{1,\ 96}$=13.15, P<0.001). Endophyte-free plants treated with MJ showed increased resistance to aphids compared to endophyte-free unexposed (control) plants, while endophyte-infected plants exposed to MJ were less resistant to aphids than unexposed (control) plants (Fig. 1).

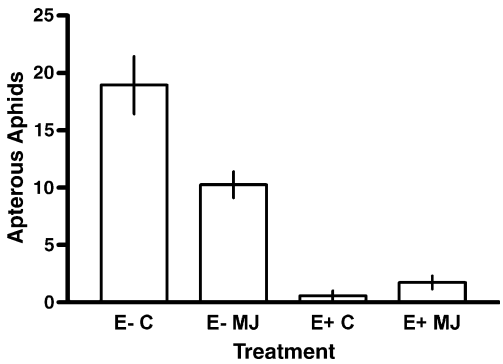

Fig. 1 Effects of methyl jasmonate (*MJ*) exposure to tall fescue plants in Experiment I on *R. padi* aphid performance. The experiment was a two-way ANOVA design with main effects of MJ exposure and endophyte infection. The two main effects interacted to affect aphid numbers ($F_{1,\ 96}$ =13.15, P<0.001). Sample sizes varied from 20 to 26 plants for each treatment combination. *Error bars* are mean+1 SEM throughout. Labeling: *E+* = endophyte-infected plants; *E−* = endophyte-free plants; *C* = control (no MJ exposure); *MJ*=methyl jasmonate exposure

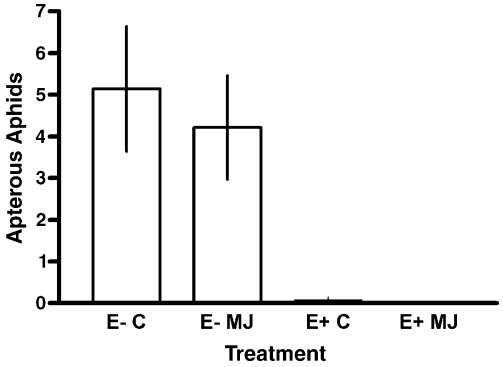

Fig. 2 Effect of main effects methyl jasmonate (*MJ*) exposure and endophyte infection on aphid performance in a two-way ANOVA design experiment. In contrast to Experiment I, all plants in Experiment II were mechanically damaged prior to MJ exposure. Samples sizes were 14 plants per treatment combination. The main effect of endophyte infection reduced aphid numbers ($F_{1,\ 52}$=80.4, P< 0.001), while MJ exposure did not (see text). Labeling as in Fig. 1

Experiment II. After mechanical wounding and exposure to MJ, a bioassay for herbivore performance was performed at 11 weeks postgermination. Analysis of variance showed that aphid numbers were lower on infected plants (Fig. 2; $F_{1,\ 52}$=80.4, P<0.001). There was no effect of MJ on aphid numbers ($F_{1,\ 52}$= 0.8, P=0.37) nor was there an interaction between endophyte infection and treatment ($F_{1,\ 52}$=0.13, P=0.72).

Loline Analysis

Experiment I. The quantity of total lolines (*N*-acetyl loline and *N*-formyl loline) produced by *N. coenophialum* in plants treated with MJ appeared to be lower than in control plants at 10 weeks postgermination, yet the trend was not significant (Fig. 3, Mann Whitney U=12.0, df=1, P=0.25).

Experiment II. Having found in Experiment I that MJ compromised endophyte-mediated constitutive resistance to aphids, we tested whether or not exposure also compromises inducible resistance provided by *N. coenophialum*. Endophyte-infected plants that had been mechanically damaged were subsequently exposed to MJ to determine if exposure would compromise the normal wound-induced response of elevated loline

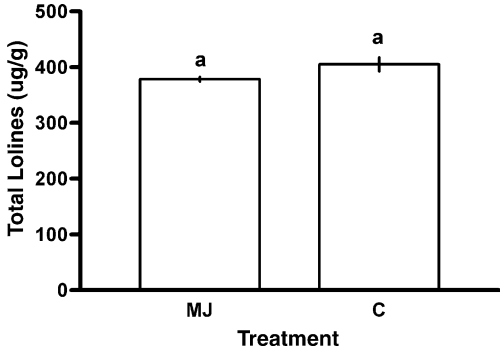

Fig. 3 Effect of methyl jasmonate exposure on total loline production by endophyte-infected tall fescue in Experiment I (Mann Whitney U=12.0, df=1, P=0.25). Labeling: C=control (no MJ exposure); MJ=methyl jasmonate exposure. *Common letters* over histograms represent lack of significant difference between means

production (Bultman et al. 2004). We found that total loline concentration at 2 weeks after mechanical wounding was lower in MJ-exposed compared to unexposed plants (Fig. 4; T=4.47, df=5, P<0.01).

Quantification of Loline Biosynthesis Gene

Experiment I. Samples for real-time RT-PCR analysis were taken at 8.5 and 9.5 weeks after germination to determine if MJ exposure influenced the regulation of the loline biosynthesis gene. The trends in the data were the same from both sampling times, and as such they were combined. We found that infected plants exposed to MJ had significantly lower levels of mRNA for the *LolC* gene compared to unexposed plants (Fig. 5; T=3.2, df=5, P<0.05).

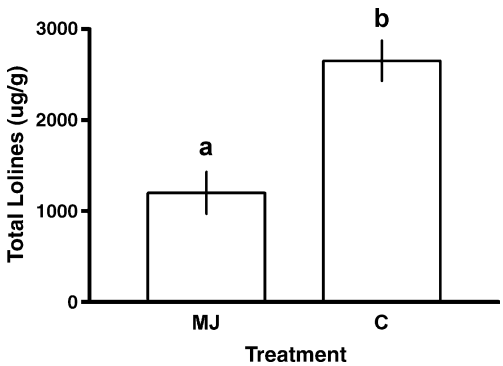

Fig. 4 Effect of methyl jasmonate (MJ) exposure on total loline production by endophyte-infected tall fescue following cutting. Significant differences between means denoted by histograms with *different letters*. Labeling as in Fig. 3

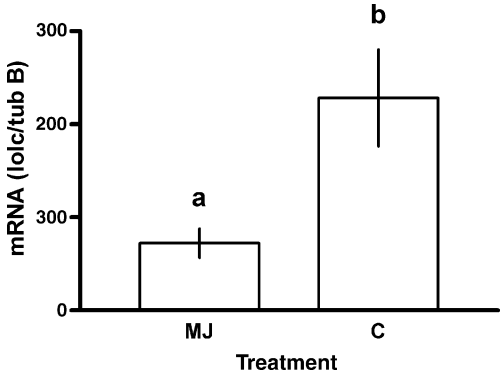

Fig. 5 Effects of methyl jasmonate (MJ) exposure on *LolC* mRNA production by *N. coenophialum* growing within tall fescue plants in Experiment I (T=3.2, df=5, P<0.05). Sample size was four plants per treatment combination. Significant differences between means denoted by histograms with *different letters*. Labeling as in Fig. 4

Discussion

Both exposure to MJ and infection by *N. coenophialum* heightened resistance to aphids in tall fescue plants, although the latter provided substantially stronger protection. While grasses are known to produce some antiherbivore compounds, like DIMBOA (Klun et al. 1967; Russel et al. 1975), which may even be wound inducible (Gutierrez et al. 1988), compared to other angiosperms, grasses are generally thought to be poorly defended. Instead of investing in defense, they appear to opt for herbivore tolerance. They have meristematic tissues close to the ground, which makes them more tolerant of herbivore damage, and they rapidly grow back tissue lost to herbivores (Barnard and Frankel 1964; Stebbins 1981). The discovery that fungal endophytes produce toxins suggests that many grasses utilize these symbionts, thus providing defense against herbivores that they would otherwise lack (Cheplick and Clay 1988; Bultman et al. 2004). Our study shows that exposure to MJ stimulates tall fescue to mount its own defense (in the absence of the endophyte) against *R. padi* aphids (Fig. 1). The defensive compounds responsible for this result are unknown but apparently are produced by the plant through an MJ-initiated biochemical cascade. While the role of jasmonates in monocotyledonous plants has not received a great deal of attention, they have been shown to play an important role in activation of pathogen defense genes (Mei et al. 2006) and to induce volatile emission that enhances parasitism of planthopper pests in rice (Lou et al. 2006). Interestingly, naturally uninfected tall fescue is more resistant to aphids than manipulatively endophyte-free plants (Lehtonen et al. 2005), suggesting that plants lacking a history of endophyte

infection are more able to mount an endogenous defense than those that have been recently disinfected.

Our results suggest that, even though tall fescue can mount an endogenous defensive response, plants that are infected with *N. coenophialum* should be at a selective advantage due to the heightened herbivore resistance that the fungus provides (Fig. 1). Indeed, this expectation has been repeatedly demonstrated by investigators (Clay 1991; Latch 1993; Breen 1994; Clement et al. 1994; Bush et al. 1997; Saikkonen et al. 1998; Faeth and Bultman 2002; Lehtonen et al. 2005). Most populations (whether managed or not) of tall fescue in the USA are highly infected with *N. coenophialum*, suggesting a selective advantage (Clay 1990).

We found that MJ exposure hindered the ability of *Neotyphodium* to produce lolines, alkaloids that reduce aphid performance (Fig. 4), and that MJ-treated infected plants were more susceptible to aphids than infected plants not exposed to MJ (Fig. 1). This is a noteworthy finding as it tells us about the signaling between fungus and plant. The fungus does not use MJ [which normally would be produced by the plant in response to stresses, like herbivory (see Creelman and Mullet 1997)] as a signal for production of lolines. In fact, just the opposite occurs. The presence of MJ compromises the fungus' ability to provide exogenous resistance to its host through the synthesis of lolines. Thus, we can rule out our hypothesis that the fungus uses MJ as a signal for loline synthesis. Rather, the competing hypothesis that MJ stimulates biochemical pathways within tall fescue that are antagonistic to loline production is supported. Moreover, the biochemical cascade that MJ initiates also inhibits the transcription of a loline biosynthesis gene in *N. coenophialum* (Fig. 5). These molecular data reveal that the effect of MJ on fungal loline production is not a result of drawing away resources that the fungus needs to produce lolines but rather an active inhibition of fungal gene transcription. The details of these biochemical signals between fungus and plant await further study.

In light of the finding that MJ suppresses the *LolC* gene, we conducted the second experiment to determine if MJ exposure could compromise the previously documented wound-induced response of endophyte-infected tall fescue (Bultman et al. 2004). Based on the results of Experiment I, we predicted that the exposure of plants to MJ 3 days after inflicting mechanical damage would reduce the accumulation of lolines and the aphid resistance that normally occurs following wounding (Bultman et al. 2004; Sullivan et al. 2007). Our results did not match our predictions. While loline concentration was lower in MJ-treated plants (Fig. 4), aphid performance did not differ among MJ-treated and untreated plants (Fig. 2). We think this is best explained by the high levels of lolines found in the infected plants after they were cut. Even with some reduction in loline concentration in MJ-treated plants, the levels were still high (>3× higher than in undamaged infected plants—compare Figs. 3 and 4), high enough to kill most of the aphids in the clip bags (Fig. 2). It may be that, once the plant is cut, upregulation of the fungal *LolC* gene occurs and application of MJ 3 days later can depress this response only slightly. Simultaneous exposure to MJ and plant damage would reveal if the timing of the two events affects the outcome.

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References

BARNARD, C., and FRANKEL, O. H. 1964. Grass, grazing animals, and man in historic perspective, pp. 1–12, in C. Barnard (ed.). Grasses and GrasslandsMacMillan, New York.

BREEN, J. 1994. *Acremonium* endophyte interactions with enhanced plant resistance to insects. *Annu. Rev. Entomol.* 39:401–423.

BULTMAN, T. L., BELL, G., and MARTIN, W. D. 2004. A fungal endophyte mediates reversal of wound-induced resistance and constrains tolerance in a grass. *Ecology* 85:679–685.

BUSH, L. P., CORNELIUS, P. L., BUCKNER, R. C., VARNEY, D. R., CHAPMAN, R. A., BURRISS, P. B. II, KENNEDY, C. W., JONES, T. A., and SAUNDERS, M. J. 1982. Association of *N*-acetyl loline and *N*-formyl loline with *Epichloe typhina* in tall fescue. *Crop Science.* 22:941–943.

BUSH, L. P., FANNIN, F. F., SIEGAL, M. R., DAHLMAN, D. L., and BURTON, H. R. 1993. Chemistry, occurrence and biological effects of saturated pyrrolizidine alkaloids associated with endophyte–grass interactions. *Agric. Ecosyst. Environ.* 44:81–102.

BUSH, L. P., WILKINSON, H. H., and SCHARDL, C. L. 1997. Bioprotective alkaloids of grass-fungal endophyte symbioses. *Plant Physiol.* 114:1–7.

CHEPLICK, G. P., and CLAY, K. 1988. Acquired chemical defences in grasses: the role of fungal endophytes. *Oikos* 52:309–318.

CLAY, K. 1988. Fungal endophytes of grasses: a defensive mutualism between plants and fungi. *Ecology.* 69:10–16.

CLAY, K. 1990. Fungal endophytes of grasses. *Annu. Rev. Ecol. Syst.* 21:275–297.

CLAY, K. 1991. Fungal endophytes, grasses, and herbivores., pp. 199–226, in P. Barbosa, V. A. Krischik, and C. G. Jones (eds.). Microbial Mediation of Plant–Herbivore Interactions-Wiley, New York.

CLEMENT, S. L., KAISER, W. J., and EICHENSEER, H. 1994. *Acremonium* endophytes in germplasms of major grasses and their utilization for insect resistance, pp. 185–199, in C. W. Bacon, and J.F. White Jr. (eds.). Biotechnology of endophytic fungi of grassesCRC, Boca Raton.

CONOVER, W. J., and IMAN, R. L. 1981. Rank transformations as a bridge between parametric and nonparametric statistics. *Am. Statist.* 35:124–129.

CREELMAN, R. A., and MULLET, J. E. 1997. Biosynthesis and action of jasmonates in plants. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* 48:355–381.

de Bruxelles, G. L., and Roberts, M. R. 2001. Signals regulating multiple responses to wounding and herbivores. *Critical Rev. Plant Sci.* 20:487–521.

Eichenseer, H., Dahlman, D. L., and Bush, L. P. 1991. Influence of endophyte infection, plant age and harvest interval on *Rhopalosiphum padi* survival and its relation to quantity of *N*-formyl and *N*-acetyl loline in tall fescue. *Entomol. Exp. Appl.* 60:29–38.

Faeth, S. H., and Bultman, T. L. 2002. Endophytic fungi and interactions among host plants, herbivores, and natural enemies, pp. 89–123, in T. Tscharntke, and B.A. Hawkins (eds.). Multitrophic Level InteractionsCambridge University Press, Cambridge.

Farmer, E. E., and Ryan, C. A. 1990. Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *PNAS USA* 87:7713–7716.

Farmer, E. E., and Ryan, C. A. 1992. Octadecanoid precursors of jasmonic acid activate the synthesis of wound-inducible proteinase inhibitors. *Plant Cell* 4:129–134.

Gols, R., Roosjen, M., Dijkman, H., and Dicke, M. 2003. Induction of direct and indirect plant responses by jasmonic acid, low spider mite densities, or a combination of jasmonic acid treatment and spider mite infestation. *J. Chem. Ecol.* 29:2651–2666.

Gunlach, H., Muller, M. J., Kutchan, T. M., and Zenk, M. H. 1992. Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. *Proc. Nat. Acad. Sci. USA* 89:2389–2393.

Gutierrez, C., Castanera, P., and Torres, V. 1988. Wound-induced changes in DIMBOA (2,4 dihydroxy-7-methoxy-2H-1, 4 benzoxazin-3(4H)-one) concentration in maize plants caused by *Sesamia nonagriodes* (Lepidoptera: Noctuidae). *Annals Appl. Biol.* 113:447–454.

Hall, S. S., and Horton, R. F. 1994. Methyl jasmonate and bean leaf abscission. *Plant Growth Reg.* 14:187–192.

Karban, R., and Myers, J. H. 1989. Induced plant responses to herbivory. *Annu. Rev. Ecol. System.* 20:331–348.

Keilholz, U., Willhauck, M., Rimoldi, D., Brasseur, F., Dummer, W., Rass, K., De Vries, T., Blaheta, J., Voit, C., Lethé, B., and Burchill, S. 1998. Reliability of reverse transcription-polymerase chain reaction (RT-PCR)-based assays for the detection of circulating tumor cells: a quality-assurance initiative of the EORTC Melanoma Cooperative Group. *Eur. J. Cancer* 34:750–753.

Klun, J. A., Tipton, C. L., and Brindley, T. A. 1967. 2,4-Dihydroxy-7-methoxy-1, 4-benzoxazin-3-one (DIMBOA), an active agent in the resistance of maize to the European corn borer. *J. Econom. Entomol.* 60:1529–1533.

Latch, G. C. M. 1993. Physiological interactions of endophytic fungi and their hosts: biotic stress tolerance imparted to grasses by endophytes. *Agri. Ecosys. Environ.* 44:143–156.

Leather, S. R., Walters, K. F. A., and Dixon, A. F. G. 1989. Factors determining the pest status of the bird cherry-oat aphid *Rhopalosiphum padi* (L.) (Homoptera: Aphididae), in Europe: a study and review. *Bull. Entomol. Res.* 79:345–360.

Lehtonen, P., Helander, M., and Saikkonen, K. 2005. Are endophyte-mediated effects on herbivores conditional on soil nutrients? *Oecologia* 142:38–45.

Lou, Y., Hua, X., Turlings, T., Cheng, J., Chen, X., and Ye, G. 2006. Differences in induced volatile emissions among rice varieties result in differential attraction and parasitism of *Nilaparvata lugens* eggs by the parasitoid *Anagrus nilaparvatae* in the field. *J. Chem. Ecol.* 32:2375–2387.

Matsui, K., Wilkinson, J., Hiatt, B., Knauf, V., and Kajiwara, T. 1999. Molecular cloning and expression of *Arabidopsis* fatty acid hydroperoxide lyase. *Plant Cell Physiol.* 40:477–481.

Mcconn, M., Creelman, R. A., Bell, E., Mullet, J. E., and Browse, J. 1997. Jasmonate is essential for insect defense in *Arabidopsis*. *Plant Biol.* 94:5473–5477.

Mei, C., Qi, M., Sheng, G., and Yang, Y. 2006. Inducible overexpression of a rice allene oxide synthetase gene increases the endogenous jasmonic acid level, *PR* gene expression, and host resistance to fungal infection. *Mol. Plant Microbe Interact.* 19:1127–1137.

Nott, H. M., and Latch, G. C. M. 1993. A simple method of killing endophyte in ryegrass seed, pp. 14–15, in D.E. Hume, G.C.M. Latch, and H.A. Easton (eds.). Proceedings of the 2nd International Symposium on *Acremonium*/Grass Interactions.AgResearch, Palmerston North.

Reinbothe, S., Mollenhauer, B., and Reinbothe, C. 1994. Jips and rips—the regulation of plant gene-expression by jasmonates in response to environmental cues and pathogens. *Plant Cell* 6:1197–1209.

Russell, W. A., Guthrie, W. D., Klun, J. A., and Grindeland, R. 1975. Selection for resistance in maize to first-brood European corn borer by using leaf-feeding damage of the insect and chemical analysis for DIMBOA in the plant. *J. Econ. Entomol.* 68:31–34.

Saikkonen, K., Faeth, S. H., Helander, M., and Sullivan, T. J. 1998. Fungal endophytes: a continuum of interactions with host plants. *Annu. Rev. Ecol. Syst.* 29:319–343.

Schardl, C. L., and Clay, K. 1997. Evolution of mutualistic endophytes from plant pathogens, pp. 221–238, in G. Carrol, and I.P. Tudzynski (eds.). The Mycota V: Plant Relationships, Part B Springer, Berlin.

Schardl, C. L., Grossman, R. B., Nagabhyru, P., Faulkner, J. R., and Mallik, U. P. 2007. Loline alkaloids: currencies of mutualism. *Phytochemistry* 68:980–996.

Siegel, M. R., Latch, G. C. M., Bush, L. P., Fannin, F. F., and Rowan, D. D. 1990. Fungal endophyte-infected grasses—alkaloid accumulation and aphid response. *J. Chem. Ecol.* 16:3301–3315.

Spiering, M. J., Moon, C. D., Wilkinson, H. H., and Schardl, C. L. 2005. Gene clusters for insecticidal loline alkaloids in the grass endophytic fungus *Neotyphodium uncinatum*. *Genetics* 169:1403–1414.

Stebbins, G. L. 1981. Coevolution of grasses and herbivores. *Annal. Missouri Bot. Gard.* 68:75–86.

Sullivan, T. J., Rodstom, J., Vandop, J., Librizzi, J., Grahm, C., Schardl, C. L., and Bultman, T. L. 2007. Symbiont-mediated changes in *Lolium arundinaceum* inducible defenses: evidence from changes in gene expression and leaf composition. *New Phytol.* 176:673–679.

SYSTAT 2004. The System for Statistics. SYSTAT, Evanston.

Thaler, J. A. 1999. Induced resistance in agricultural crops: effects of jasmonic acid on herbivory and yield in tomato plants. *Environ. Entomol.* 28:30–37.

Wasternack, C. 2007. Jasmonates: an update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Annal. Botany* 100:681–697.

Wilkinson, H. H., Siegel, M. R., Blankenship, J. D., Mallory, A. C., Bush, L. P., and Schardl, C. L. 2000. Contribution of fungal loline alkaloids to protection from aphids in grass–endophyte mutualism. *Am. Phytopathol. Soc.* 13:1027–1033.

'witches brooms' and tasteless dwarf fruits, for which no curative approaches are available (Seemüller et al. 2002).

The univoltine psyllid species *C. picta* (Foerster) (Hemiptera: Psyllidae) was discovered recently as the vector of this phytoplasma in Central Europe (Frisinghelli et al. 2000; Mayer et al. 2008b); the phytoplasma infects both its host plant and the vector insect. Our previous study provided evidence that apple plants infected by this phytoplasma released higher amounts of the sesquiterpene ß-caryophyllene than uninfected plants and that newly hatched adults of *C. picta* were attracted by the odor of infected apple trees (Mayer et al. 2008a). Hence, we established the hypothesis that *Ca.* P. mali may manipulate both the odor of infected plants and the behavior of its vector insect, thus promoting its propagation. In this follow-up study, we confirm this hypothesis by both laboratory and field bioassays, and we highlight the applied aspect of our findings.

Methods and materials

Maintaining Insects for Bioassays Adults of *C. picta* were collected from apple trees (*Malus domestica*) in early spring after having returned from their overwintering host plants. They were maintained in rearing cages (60×60×90 cm) either on uninfected or *Ca.* P. mali-infected apple plants on which they could feed and oviposit. The rearing cages were located in a greenhouse chamber under natural light conditions and a day–night temperature program (D:N= 20:15°C). For behavioral trials, test insects were collected from the boxes 1 day before testing in groups of ten in Eppendorf vials at 4°C. All insects were tested within 2 weeks after emergence.

Behavioral Bioassays Behavioral tests were carried out by using a dynamic Y-shaped olfactometer consisting of a glass tube (entrance arm 12.5 cm, test arms 21.0 cm, inner diameter 0.6 cm) mounted on an angular board. Charcoal-cleaned air (granulated 4–8 mm, Applichem GmbH, Darmstadt, Germany) was pumped (75 ml/min) through two glass jars (2 l) containing the volatile sources. For testing the behavior of uninfected and inexperienced females, a twig of uninfected apple plants (length 10–15 cm, phenology stage 69–71) was placed together with a water-filled glass vial in each of the jars. One microliter of ß-caryophyllene (80%, FEMA 2252, Sigma-Aldrich, Steinheim, Germany) was pipetted onto a filter paper disk (diameter 5 mm), which was set into the inflow of one olfactometer arm. In all other experiments (Fig. 1B–D), a filter paper disk treated with 1 µl ß-caryophyllene was offered against an untreated disk. According to Mayer et al. (2008a), for each test, ten females were placed into the entrance arm at one time. Every individual passing a final marking (10.0 cm after the branching) on one of the test arms after 15 min was recorded and transferred to a vial filled with ethanol (70%) for later analysis of their infection status (see below). All experiments were repeated 20 times. Data were analyzed statistically after log (x+0.5) transformation by *dependent paired t-test* using the Statistica 5.5 scientific software package.

DNA Extraction from Insect Material and PCR Analysis Psyllids used in biotests (see above) were subjected to DNA extraction by using the methods described in Mayer et al. (2008a). Insect DNA was purified by using the primer pair fAT/rAS specific for *Ca.* P. mali, amplifying a 400-bp sequence in the 16S-23S rRNA spacer region. Polymerase chain reactions (PCRs) were performed in a thermal cycler (Robocycler 96, Stratagene, La Jolla, CA, USA) according to Mayer et al. (2008a).

Field study ß-caryophyllene (purity >80%, SAFC Supply Solutions, USA; 2 ml) was added to a membrane dispenser (Langlock type Do 48, Wilhelm, Germany). The dispenser consisted of a 4-ml glass vial with an open lid (diameter 12 mm) containing four layers of permeable rubber membranes. The dispenser was mounted in the center of a transparent plastic cup (Polarcup 500 ml, Huhtamaki GmbH, Germany) that was sealed by a respective lid. ß-caryophyllene could evaporate through 20 openings (diameter 10 mm) evenly distributed over the sticky cup surface. The dispensers were refilled with ß-caryophyllene depending on weather conditions. Cups with empty dispensers were used as negative controls. Cups with empty dispensers, which were wrapped by collars of yellow plastic foil (32×5 cm), were used as positive controls. The traps were placed randomly in ten blocks, each consisting of one trap per treatment in an apple orchard close to Heidelberg (Germany) from April 24, 2008 to May 13, 2008. The traps were controlled every 2–5 days, and the captured psyllids determined. The data were analyzed by a nonparametric *Friedman-analysis of variance* followed by *Wilcoxon matched pairs tests* after sequential Bonferroni correction using the Statistica 5.5 scientific software package.

Results

Adult *C. picta* females reared on uninfected plants without any previous contact to *C.* P. mali during their ontogenesis (inexperienced) significantly preferred the olfactometer arm supplied with ß-caryophyllene both when offered simultaneously with uninfected apple odor (71.1%, Fig. 1A) and without apple odor (73.3%, Fig. 1B; *dependent paired t test*, $P<0.001$). Adult *C. picta* females, which were reared

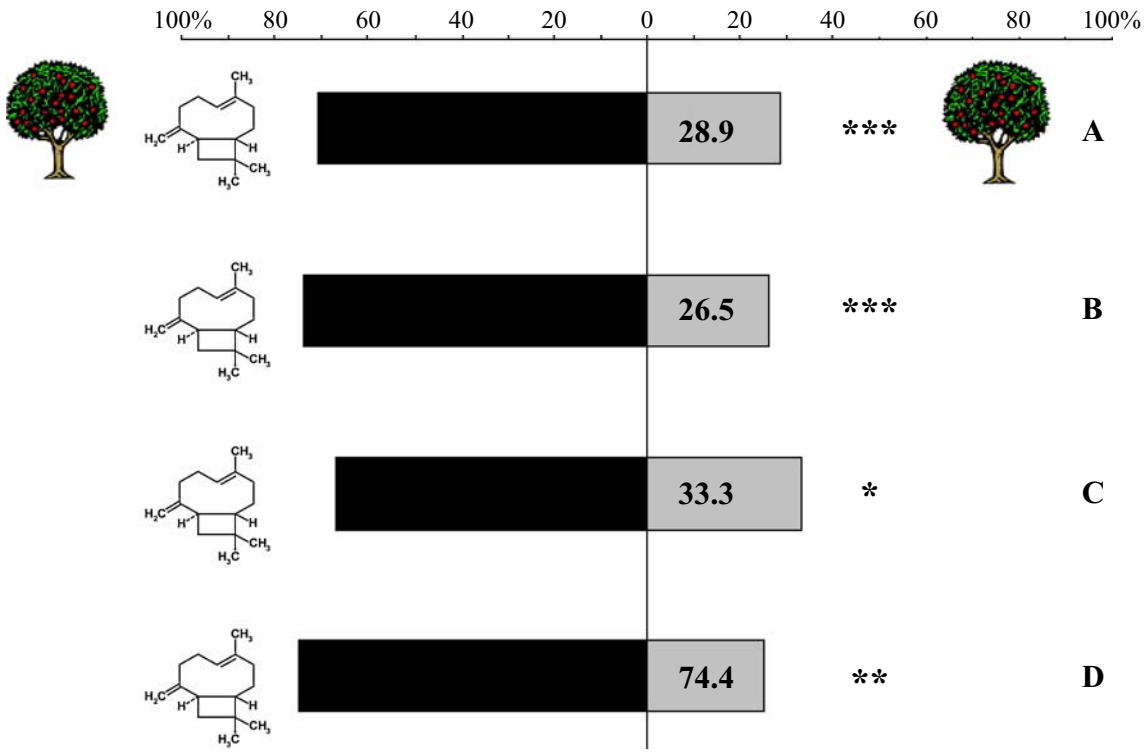

Fig. 1 Results of olfactometer-bioassays with newly hatched adults (emigrants) of *Cacopsylla picta*. *A* Apple odor + ß-caryophyllene (*left*) vs. apple odor (*right*): inexperienced and uninfected emigrants developed on healthy plants (N=149; motivation 74.5%). *B–D* ß-caryophyllene (*left*) vs. empty control (*right*) *B* inexperienced and uninfected emigrants developed on healthy plants (N=132; mot. 66.0%). *C* Experienced and uninfected emigrants developed on infected plants (N=42; mot. 42.5%). *D* Experienced and infected emigrants developed on infected plants (N=43; mot. 42.5%). *T test for dependent samples*: *P<0.05, **P<0.01, ***P<0.001

during their entire ontogenesis on infected plants (experienced), also significantly preferred the olfactometer arm supplied with ß-caryophyllene when offered against an empty control (70.6%). No differences were observed between the behavior of phytoplasma-infected (66.7%, *dependent paired t test*, P<0.05; Fig 1C) or uninfected psyllids (74.4%, *dependent paired t test*, P<0.01; Fig. 1D). Less than half (42.5%) of the experienced *C. picta* females were motivated to enter an olfactometer arm, whereas 74.5% of inexperienced individuals made a decision when ß-caryophyllene was offered together with apple odor and 66.0% when ß-caryophyllene was offered alone. Interestingly, rearing of *C. picta* on infected apple trees resulted only in 50.6% infection of the potential vectors by the phytoplasma, as was shown by PCR analysis of whole body extracts using specific primers for detection of *Ca.* P. mali.

Sticky traps equipped with a dispenser filled with ß-caryophyllene captured significantly more adults (both males and females) of *C. picta* than the negative control trap containing an empty dispenser (Table 1). Compared to the positive control equipped with yellow foil, no significant difference was detected. However, the captures of traps baited with ß-caryophyllene contained mainly *C. picta*

Table 1 Mean captures/trap and standard error of *Cacopsylla picta* remigrants with infochemical-baited (ß-caryophyllene) or yellow sticky traps

Dispenser content	Mean captures/trap	Statistical comparison	*N*
ß-Caryophyllene	0.38±0.09	A (**)	50
Empty	0.04±0.03	B	50
Yellow foil	0.34±0.08	A (*)	50

Sticky traps without visual or chemical baits (empty dispenser) were used as controls. *Wilcoxon matched pairs test* followed by *sequential Bonferroni correction*. Different capital letters indicate significant differences between captures in traps with different dispenser contents
n.s. not significant
*P<0.05
** P<0.01

(90.5% of the captures are *C. picta*), whereas yellow traps also captured many other psyllid species (69% *C. picta*).

Discussion

The interactions between the pathogen *Ca.* P. mali, the host plants, and the vector insect *C. picta* are complex and summarized in Fig. 2. After overwintering on an alternate host plant [conifers like *Picea abies* and others (unpublished results)], brown colored *C. picta* remigrate into apple orchards for reproduction. For only 2–3 months per year, they live exclusively on their reproduction host plant *M. domestica* for mating, oviposition, and juvenile development (Mayer et al. 2008a). Other plants are not attractive to this highly specialized herbivore. Soon after adult emergence (phenology stages 69–71), the young adults (colored green) are able to ingest *Ca.* P. mali from infected plant phloem (acquisition feeding). Attracted by ß-caryophyllene, which is only produced by infected apples during this time, both males and females are lured to infected plants, irrespective of whether they developed on healthy (Fig. 2, white dot) or infected plants (black dot) (Mayer et al. 2008a). Psyllids that developed from the egg to the adult stage on phytoplasma-infected plants (experienced psyllids) showed a lower inclination to move in the olfactometer bioassays than inexperienced individuals that developed on healthy apple plants (Fig. 1). As shown recently, the pathogen impaired at least indirectly the juvenile development of its vector (unpublished results). Hence, it is possible that these effects influence the activity of the psyllids, and as a consequence, the motivation of the experienced psyllids may decrease.

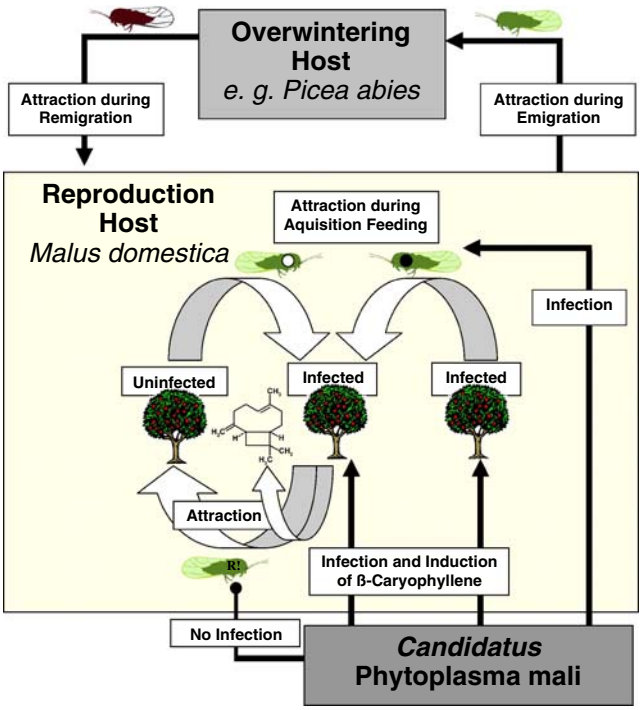

Fig. 2 Multitrophic interactions between *C. picta*, its reproduction, overwintering and nonhost plants, and the pathogen *Ca.* P. mali. Active processes (infection, attraction, induction) are indicated by an *arrow*, otherwise by a *dot*. The structure of ß-caryophyllene is given. The *black dot* indicates an infected psyllid, the white dot an uninfected one and "R!" potentially resistant individuals. For further description please refer to text

Witches' brooms produced exclusively by infected plants increase their leaf surface and may support the emission of volatile ß-caryophyllene. Psyllids that had developed on infected plants but did not get infected (R!) were attracted by ß-caryophyllene (Fig. 1D), but not by the odor of infected plants (Mayer et al. 2008a). Thus, either healthy plant odors may contain more attractive components, or infected plant odors may contain additional substances that mask the attractiveness of ß-caryophyllene to this psyllid phenotype. However, shortly after feeding on apple, the adults emigrate to conifers where they stay until spring (Fig. 2). During this time, the phytoplasma can replicate within its vector and invade its salivary glands.

The sesquiterpene ß-caryophyllene has multifaceted functions in different systems. It was detected in the odor of leaves, flowers, and fruits, and it is known to be attractive to several herbivore species (Khalilova et al. 1998). Furthermore, maize plants attacked by root-feeding larvae of the leaf beetle *Diabrotica virgifera virgifera*, emit ß-caryophyllene as a belowground signal, which attracts entomopathogenic nematodes (Rasmann et al. 2005). The production of ß-caryophyllene aboveground in the infected apple plant is induced by the phytoplasma just at the time of acquisition feeding, thus resulting in increased attractiveness of infected plants to their insect vector (Mayer et al. 2008a). Thus, this infochemical is detrimental for both its producer and its vector insect because attracted phloem-feeding insects cause more damage to the plant, and the psyllids themselves may get infected by the phytoplasma, respectively. At least during their ontogenesis, the infection of the vector insect has detrimental effects on its own fitness, resulting in, e.g., smaller body size and a higher mortality (unpublished results). Consequently, only the phytoplasma appears to have a benefit in this multitrophic system. According to the widely accepted terminology (after Dicke and Sabelis 1988), the costs and benefits for involved organisms are assessed to describe the ecological function of an infochemical without consideration of its origin. Thus, ß-caryophyllene emitted by infected apple plants is not a plant kairomone but a pathogen-induced allomone because it is beneficial for the phytoplasma and detrimental for the psyllid.

Our field studies that used sticky traps equipped with dispensers containing this infochemical implicate for the

first time that an allomone can represent a promising compound to develop new traps for monitoring or maybe even mass trapping of *C. picta*, the most important vector of apple proliferation disease!

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References

DICKE, M., and SABELIS, M. W. 1988. Infochemical terminology: based on cost-benefit analysis rather than origin of compounds? *Funct. Ecol.* 2:131–139.

FRISINGHELLI, C., DELAITI, L., GRANDO, M. S., FORTI, D., and VINDIMIAN, M. E. 2000. *Cacopsylla costalis* (Flor 1861), as a vector of apple proliferation in Trentino. *J. Phytopathol.* 148:425–431.

KHALILOVA, A. Z., PARAMONOV, E. A., ODINOKOV, V. N., and KHALILOV, L. M. 1998. Identification and biological activity of volatile organic substances emitted by plants and insects. 1. Components of the native scents of *Leptinotarsa decemlineata* and *Solanum tuberosum*. *Chem. Nat. Comp.* 34:647–649.

MAYER, C. J., VILCINSKAS, A., and GROSS, J. 2008a. Phytopathogen lures its insect vector by altering host plant odor. *J. Chem. Ecol.* 34:1045–1049.

MAYER, C. J., JARAUSCH, B., JARAUSCH, W., VILCINSKAS, A., and GROSS, J. 2008b. *Cacopsylla melanoneura* has no relevance as vector of apple proliferation in Germany. *Phytopathology* (in press)

MUMM, R., POSTHUMUS, M. A., and DICKE, M. 2008. Significance of terpenoids in induced indirect plant defence against herbivorous arthropods. *Plant Cell Environ.* 31:575–585.

RASMANN, S., KÖLLNER, T. G., DEGENHARD, J., HILTPOLD, I., TOEPFER, S., KUHLMANN, U., GERSHENZON, J., and TURLINGS, T. 2005. Recruitment of entomopathogenic nematodes by insect-damaged maize roots. *Nature* 434:732–737.

ROSTÁS, M., SIMON, M., and HILKER , M. 2003. Ecological cross effects of induced plant responses towards herbivores and phytopathogenic fungi. *Basic Appl. Ecol.* 4:43–62.

SCHNEE, C., KOLLNER, T. G., HELD, M., TURLINGS, T. C. J., GERSHENZON, J., and DEGENHARDT, J. 2006. The products of a single maize sesquiterpene synthase form a volatile defense signal that attracts natural enemies of maize herbivores. *Proc. Natl. Acad. Sci. USA* 103:1129–1134.

SEEMÜLLER, E., and SCHNEIDER, B. 2004. '*Candidatus* Phytoplasma mali', '*Candidatus* Phytoplasma pyri' and '*Candidatus* Phytoplasma prunorum', the causal agents of apple proliferation, pear decline and European stone fruit yellows, respectively. *Int. J. Syst. Evol. Microbiol.* 53:1217–1226.

SEEMÜLLER, E., GARNIER, M., and SCHNEIDER, B. 2002. Mycoplasmas of plants and insects, pp. 91–116, in S. Razin, and R. Herrmann (eds.). Molecular Biology and Pathology of Mycoplasmas. Kluwer Acad. Publ., London.

STOUT, M. J., THALER, J. S., and THOMMA, B. P. H. J. 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annu. Rev. Entomol.* 51:663–689.

permanent production and storage of defense metabolites, and resources required for their biosynthesis are invested only in times of increased herbivore or pathogen pressure (Karban and Baldwin 1997).

Despite several observations of induced defense in brown algae (Cronin and Hay 1996; Pavia and Toth 2000; Hemmi et al. 2004; Macaya et al. 2005; Molis et al. 2006), there is an ongoing debate about how the induction of the de novo biosynthesis of defensive metabolites is regulated (Tarakhovskaya et al. 2007). In higher plants, hormones such as jasmonic acid (JA) and methyl jasmonate (MeJA), as well as external elicitors and continuous mechanical tissue disruption, can orchestrate the induction of chemical defenses after mechanical wounding, herbivore damage, or pathogen attack (Karban and Baldwin 1997; Baldwin et al. 2002; Wittstock and Gershenzon 2002; Mithöfer et al. 2005; Garcia-Brugger et al. 2006; Wasternack 2007). JA and MeJA have been identified in red algae (Rhodophyta) as well (Krupina and Dathe 1991; Bouarab et al. 2004; Tarakhovskaya et al. 2007). JA influences growth and development in the green microalga *Chlorella vulgaris* (Czerpak et al. 2006). Moreover, JA and MeJA have a positive effect on the tolerance of the green microalga *Scenedesmus incrassulatus* to stress temperatures and infections (Christov et al. 2001), but the occurrence of these metabolites in both algae has not been verified. The question whether JA and its derivatives play a role in the induced defense reactions of the red macroalga *Chondrus crispus* was addressed by Bouarab et al. (2004). They suggested a role of this metabolite as a hormone comparable to that found in higher plants. However, JA and MeJA seem not to be ubiquitous in red algae since *Gracilaria chilensis* does not contain detectable amounts even when heavily challenged with pathogens (Wiesemeier and Pohnert, personal observation).

While several reports on the potential hormonal role of JA and MeJA in red and green algae are available, the occurrence and significance in brown algae has been poorly addressed. Arnold et al. (2001) reported that exposure of the common rockweed *Fucus vesiculosus* to MeJA during periods of tidal emergence causes induction of polyphenolic chemical defenses. Since timing and magnitude of this induced increase in phlorotannin concentration was similar to that caused by herbivory, the question arose whether jasmonates or their oxylipin relatives are natural elements of antiherbivore responses in *Fucus*.

In this study, we report a series of experiments on the occurrence and potential influence of JA and its derivative MeJA on the formation of medium- and non-polar secondary metabolites in different brown alga species. In addition, the JA mimic coronalone (Schüler et al. 2004) was investigated to exclude artefacts due to metabolization of JA in the medium. Special attention was given to the brown alga *Dictyota dichotoma* for two reasons: (a) this alga is known to exhibit induced defense (Cronin and Hay, 1996), and (b) it is rich in bioactive terpenoids (Fattorusso et al. 1976; Teixeira et al. 2001; Barbosa et al. 2004; Kim et al. 2006), a compound class often involved in induced defense of higher plants. The chemical profiling was paired with bioassays that used herbivores of algae; these assays were conducted to study the role of JA and MeJA as hormones involved in the production of medium- and non-polar antiherbivore metabolites of brown algae.

Methods and Materials

Test Organisms The brown algae *D. dichotoma*, *Colpomenia peregrina*, *Ectocarpus fasciculatus*, *F. vesiculosus*, *Himanthalia elongata*, *Saccharina latissima* (formerly *Laminaria saccharina*), and *Sargassum muticum* were collected on the Atlantic coast in Roscoff, France and transferred to the laboratory within 2 days. *D. dichotoma* was collected from June until August 2004 as well as in March 2006. All other brown algae were collected exclusively on the later date. Algae were kept at 16°C under a 14:10 h L/D regime in artificial seawater. Artificial seawater was prepared by dissolving 33 g l^{-1} Instant Ocean (Aquarium Systems, Sarrebourg, France) in distilled water.

The amphipod *Ampithoe longimana* and the isopod *Paracerceis caudata* are widely distributed along the Atlantic coast of North America. Test animals for these assays were collected in Morehead City, NC (USA) and air-shipped to the laboratory, where they were kept in artificial seawater under identical culture conditions as the brown algae.

Induction Experiments Racemic MeJA was provided by R. Kaiser, Givaudan-Roure, Dübendorf, Switzerland; racemic JA was synthesized by base-mediated saponification of MeJA and subsequently purified by column chromatography. Coronalone (Schüler et al. 2004) was provided by W. Boland, Max-Planck-Institute for Chemical Ecology, Jena, Germany. Linolenic acid (LEA) was obtained from Sigma/Aldrich, Düren, Germany. For induction experiments, defined pieces of freshly collected algae (*D. dichotoma*, *C. peregrina*, *E. fasciculatus*, *F. vesiculosus*, *H. elongata*, *S. latissima*, and *S. muticum*, 80–400 mg) were kept in 20 ml filtered seawater that contained different amounts of potential elicitors (added from 100 mg ml^{-1} stock solutions in methanol). Solvent controls were run in parallel. *D. dichotoma* was treated with 0.1, 0.5, and 1 mg ml^{-1} JA and LEA, respectively, and with 0.1 and 0.5 mg ml^{-1} MeJA, where solubility problems occurred at higher concentration. Incubations were performed for 2, 24, and 48 h. The other algae were treated with 0.5 mg JA and MeJA, respectively,

for 12 and 48 h. All treatments were performed at least in triplicate. After the induction period, algal pieces were rinsed carefully with seawater, blotted dry with a paper towel, weighed, wrapped in aluminum foil, shock-frozen in liquid nitrogen, and kept at −20°C until further workup.

For bioassays, *D. dichotoma* was induced with 0.5 mg ml^{-1} JA, MeJA, or LEA (added from 100 mg ml^{-1} stock solutions in methanol) in seawater for 30 h. A solvent control was run in parallel. After the incubation period, the algae were rinsed carefully with seawater, blotted dry with a paper towel, and used for the preparation of artificial food as described below.

Profiling of Acidic Oxylipins Bearing Aldehyde or Ketone Structural Elements and Screening for Jasmonic Acid Profiling of oxylipins in *D. dichotoma* was accomplished by using a solid phase extraction protocol optimized by Schulze et al. (2006). Briefly, alga pieces (1–3 g) were covered with 5 ml MeOH that contained 0.05% 2,6-di-*tert*-butyl-4-methylphenol, followed by the addition of *O*-(2,3,4,5,6-pentafluorobenzyl)hydroxylamine hydrochloride (3 ml, 0.05 M in methanol). To monitor extraction efficiency, [9,10-$^2H_2$]-dihydrojasmonic acid (150 ng) was added. The mixture was homogenized for 5 min under an inert argon atmosphere with a high-performance dispenser at 24,000 rpm (Ultra-Turrax T 25, IKA-Werk, Staufen, Germany). Subsequent acidification with diluted HCl (pH 3) was followed by extraction of the MeOH/water phase with hexane (3×10 ml). The remaining polar phase was acidified again by the addition of 4 ml of diluted HCl (pH 3) to enhance phase separation and extracted with dichloromethane (3×10 ml). The organic fractions were passed separately through preconditioned Chromabond NH_2 cartridges (0.5 g, aminopropyl, Macherey-Nagel, Düren, Germany). After sample loading, the cartridges were washed with 2-propanol/CH_2Cl_2 (5 ml, 2:1, *v*/*v*) and eluted with diethyl ether/formic acid (10 ml, 98:2, *v*/*v*). The solvent was removed at RT by a stream of argon. For derivatization of the carboxyl groups, samples were treated with an ethereal solution of diazomethane (1 ml). After 2 min, excess of diazomethane and the solvent was removed by a stream of argon, and the residue was dissolved in dichloromethane (30 µl). Samples were analyzed by using a Finnigan TraceMS coupled to a Finnigan GCQ gas chromatograph/mass spectrometer (Thermo-Finnigan, Waltham, MA, USA) equipped with an Alltech EC5 capillary column (15 m×0.25 mm, 0.25 µm, Alltech, Hamburg, Germany). The ions at m/z=399 were used to monitor JA and m/z=481 to monitor 12-oxophytodienoic acid (OPDA).

Profiling of Terpenoids and Other (Semi)volatiles For metabolic profiling of terpenoids and other (semi)volatiles, frozen samples of the seven brown algal species were ground in a mortar and extracted directly with dichloromethane (3×0.5–1 ml). The combined organic phases were dried over Na_2SO_4, and the solvent was removed under a stream of nitrogen. Samples were taken up with dichloromethane containing 20 µg ml^{-1} laurylaldehyde as standard to give a final volume corresponding to 2 ml solvent per gram alga. These samples were submitted directly in split mode to a Finnigan TraceMS coupled to a Finnigan ITS gas chromatograph equipped with an Alltech EC5 capillary column (15 m×0.25 mm, 0.25 µm, Alltech, Hamburg, Germany). The temperature program was 50°C (2 min) then ramped at 10°C min^{-1} to 280°C (2 min). Peaks were assigned by retention time and verified by mass spectra. In cases of poor chromatographic resolution, characteristic ion traces were used for the relative quantification.

Metabolic Profiling of Methylated Extracts Frozen samples were ground and extracted as described above. [$^2H_{27}$]-myristic acid (0.1 µl of a 10 mg ml^{-1} stock solution in MeOH per milligram alga) was added as standard prior to extraction. After removal of the solvents under a stream of nitrogen, an excess of trimethylsilyldiazomethane solution (1 ml of a 2M solution in diethylether, Sigma/Aldrich, Buchs, Switzerland) and 10 µl MeOH was used for derivatization. After 1 h, the excess derivatization reagent was removed under a stream of nitrogen, and 500 µl dichloromethane was added. These samples were submitted directly in split mode for gas chromatography (GC)/mass spectroscopy (MS) analysis. Profiling of derivatized samples was performed as described above (temperature program of the GC, 60°C (2 min), then ramped at 20°C min^{-1} to 140°C, then at 5°C min^{-1} to 250°C and at 20°C min^{-1} to 300°C (2 min)). Fatty acid methyl esters were identified by comparison of retention time and mass spectra with commercially available standards.

LC/MS Profiling For metabolic profiling of more polar metabolites, frozen samples were ground in a mortar and extracted directly with methanol or a 1:1 mixture of methanol and water (3 ml per 400 mg alga). Samples were centrifuged and the supernatant was submitted directly to liquid chromatography (LC)/MS analysis. HPLC/MS profiling was done on a Finnigan LCQ connected to an Agilent 1100 System equipped with a Grom-Sil 120 ODS-3 CP column (125×2 mm, particle size 3 µm, Grom, Rottenburg-Hailfingen, Germany). Samples were run twice with APCI and ESI ionization, respectively. A gradient of water/0.5% acetic acid (solvent A) and acetonitrile/0.5% acetic acid (solvent B) was used at a total flow rate of 0.2 ml min^{-1}: solvent B 10% (7 min), 27–37 min 100% B, and 38.50 min 10% B (9.5 min).

Peaks were assigned by retention time in the base peak monitoring mode and verified by mass spectra. In cases of

poor chromatographic resolution, characteristic ion traces were used for relative quantification.

Evaluation of Profiling Experiments Each treatment was replicated at least in triplicate. For profiling, all peaks >5% were quantified and normalized to the internal standard. Differences between induced and uninduced samples were evaluated after calculating the standard deviation using Microsoft Excel. If ambiguous results were observed, additional control/induction pairs were generated and consistency was verified. Minor peaks were evaluated by visual pattern comparison. Quantification was done by using peak areas of characteristic ion traces (LC/MS) or TIC (GC/MS). Peaks were considered different in control and treatment when >10% difference of the respective integrals was observed in all three measurements of the control and of the treated sample. For major peaks, differences were evaluated additionally based on standard deviation.

Bioassays Agar (Aldrich, Seelze, Germany)-based food was prepared by using the methods described in detail by Wiesemeier et al. (2007). Freshly collected *D. dichotoma* were put into 200 ml culture containers and either treated with 500 μg ml^{-1} of JA, MeJA, or LEA for 30 h, or kept as solvent control. Subsequently, the algae were washed carefully with excess seawater to avoid contamination of the food with inducing reagents. These algae were freeze-dried, finely powdered, and subsequently incorporated into an agar matrix prepared by mixing 0.72 g of agar with 36 ml distilled water and dissolving the mixture by heating in a microwave. Subsequently, 2 g of powdered alga was added, and the mixture was shaken vigorously to homogenize. This mixture was poured into 25 ml plastic centrifuge tubes (Falcon, Franklin Lakes, NY, USA). The tubes were inverted and cooled to room temperature. After agar solidification, the falcon tube was opened and the agar was cut to give pieces of a quarter of a ca. 2-mm-thick agar disc.

We tested responses of *A. longimana* and *P. caudata* to agar-based food with induced and uninduced *D. dichotoma*. Feeding assays were performed in 12-well ice cube containers, each well filled with 20 ml of sea water (18°C). One amphipod per vessel could choose between one control and one induced food pellet that were arranged on specified sides of the assay container. During 25 min, we noted every 5 min on which type of food each test animal was present. This behavioral association assay is a valid surrogate for feeding assays because these test animals tend to live on, and thus associate spatially with the foods that they consume (Cruz-Rivera and Hay 2003). To statistically evaluate food choice, we used the Wilcoxon test for paired samples.

Results

Oxylipin Profiling The oxylipin profiling method used allows the detection of JA in concentrations down to 20 ng g^{-1} fresh material. Nevertheless, JA was not found in the brown alga *D. dichotoma* (Fig. 1). Moreover, the

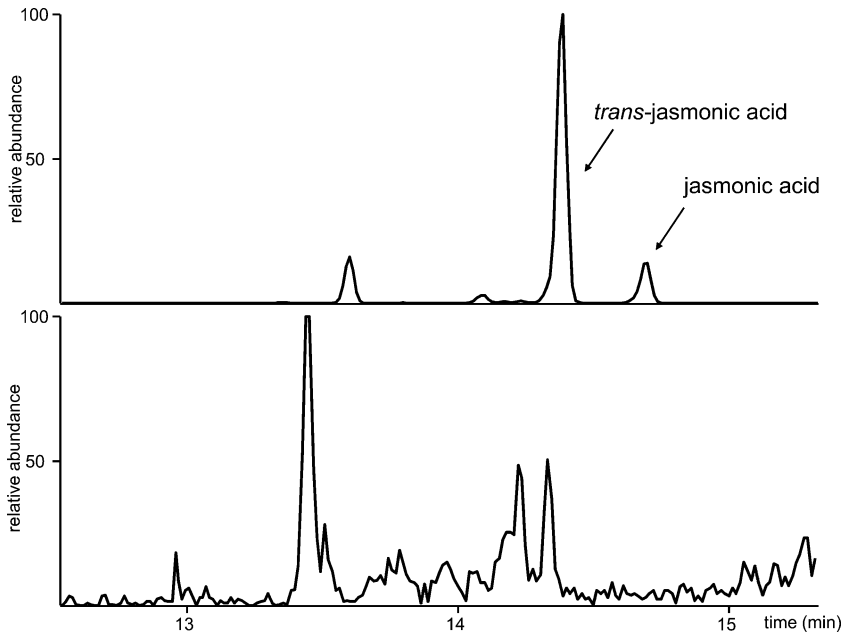

Fig. 1 GC/MS (extracted single ion chromatogram *m*/*z*=399 corresponding to M-20 due to a loss of HF) separation of derivatized jasmonic acid (*upper trace*) and the oxylipin extract of *Dictyota dichotoma* (*lower trace*). Spectra were recorded by using chemical ionization with methane as reagent gas in the negative mode

biosynthetic precursor 12-oxophytodienoic acid also could not be detected (data not shown). In parallel experiments, samples from higher plants (*Phaseolus lunatus*) were run as positive control. As expected, JA and OPDA were detected in these controls (Schulze et al., 2006).

Metabolic Profiling Induction experiments with JA, MeJA, and LEA (control) were run with freshly collected samples to verify whether significant changes of the metabolic profile of the algae occur in response to the putative hormones. Metabolites with a broad biosynthetic spectrum that included terpenes, phenolics, lipids, and fatty acids were covered by using general methods of metabolic profiling (Weckwerth et al. 2005).

No significant metabolic response was observed when the putative hormones were administered in the seawater in concentrations below 500 μg ml^{-1} to the algae *D. dichotoma*, *C. peregrina*, *E. fasciculatus*, *F. vesiculosus*, *H. elongata*, *S. latissima*, or *S. muticum*. In similar experiments with higher plants, significant up-regulation of secondary metabolites can be detected (Boland et al. 1995). In Fig. 2, a GC/MS comparison of trimethylsilyl-diazomethane-methylated organic extracts of *D. dichotoma* is shown. Similar chromatograms were used for the evaluation of metabolic differences of terpenoids and other (semi)volatiles. Peak lists were generated from these chromatograms. If inconsistent results were obtained, re-evaluation of the peaks was performed by using single ion monitoring, or a second set of replicates was generated. None of the carefully evaluated peak lists of all seven algal species showed significant differences between treatment and control. LC/MS profiling of JA-treated samples was evaluated by comparison of extracts from JA-treated and untreated algae. Peaks were evaluated in base peak monitoring mode and verified with single ion monitoring. In no evaluated case (peaks with more than 5% intensity) could a statistical significant up- or down-regulation of signals be observed.

Only if high amounts ≥500 μg ml^{-1} of potential elicitors added over a prolonged period of more than 24 h was a metabolic response observed (Fig. 2). With the exception of *S. muticum*, all investigated algae released fatty acids, but their nature and amount varied strongly between experiments. In the experiments with *D. dichotoma*, up to 500-fold up-regulation of free palmitic acid, palmitoleic acid, and oleic acid was observed. Besides these metabolites, strongly varying amounts of saturated fatty acids, such as lauric (C12:0), myristic (C14:0), stearic (C18:0), arachidic (C20:0), and behenic acid (C22:0), as well as unsaturated fatty acids, such as stearidonic (C18:4), α-linolenic (C18:3), arachidonic (C20:4), and eicosapentaenoic acid (C20:5) were released by all investigated algae with the exception of *S. muticum*. This metabolic response was unspecific and could be triggered with high concentrations of JA, MeJA, and LEA. During these treatments, algae were heavily stressed and depigmentation was observed after more than 48 h. The solvent control did not lead to comparable metabolic and stress reactions.

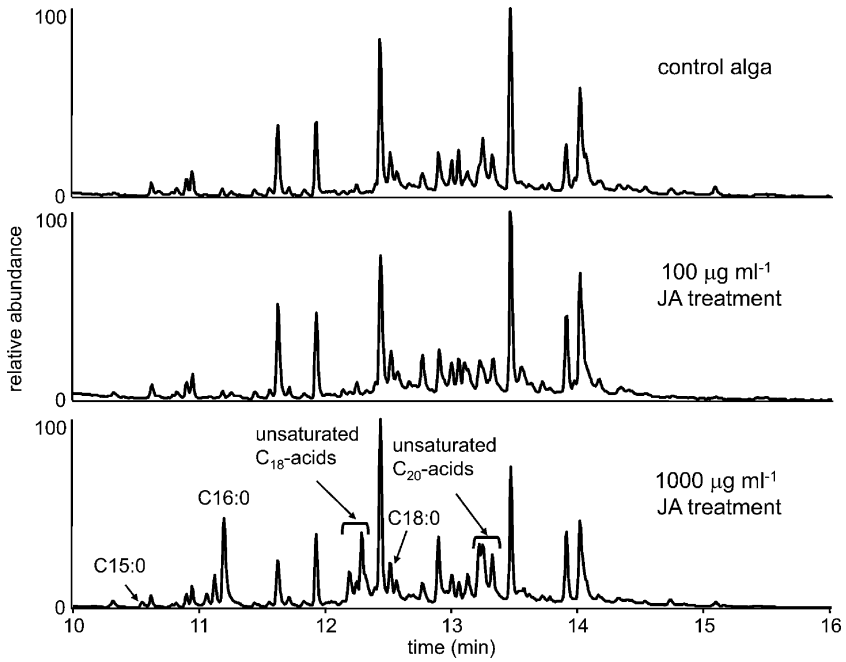

Fig. 2 GC/MS profiling (total ion current) of methylated *Dictyota dichotoma* extracts. *Upper trace*: untreated control; *middle trace*: after incubation with 100 μg ml^{-1} JA; *lower trace*: after incubation with 1,000 μg mL^{-1} JA. The respective up-regulated fatty acids are indicated. C16:0 palmitic acid, C18:0 stearic acid

Additional profiling of terpenoids and other semivolatiles of *D. dichotoma* after challenging the algae with coronalone (10, 100, and 1,000 μg ml^{-1}) for 24 h also did not reveal any specific induction of secondary metabolites (data not shown).

Bioassays on the Induction of Chemical Defense Bioassays were performed with two model herbivores, the amphipod *A. longimana* and the isopod *P. caudata* (Wiesemeier et al. 2007). Food from control algae was compared with food from algae treated with 500 μg ml^{-1} of the inducing reagents, which corresponds to the lowest concentrations at which a metabolic response was observed. *P. caudata* associated unselectively with both types of food offered; no preference was detectable for control food nor for JA-, MeJA-, or LEA-treated *D. dichotoma*. In contrast, *A. longimana* selected preferentially (P=0.01) food from algae pre-treated with 500 μg ml^{-1} JA, MeJA, or LEA for 30 h (Fig. 3).

Discussion

The brown alga *D. dichotoma* was submitted to a robust metabolic profiling method of oxylipins based on derivatization and detection with gas chromatography/mass spectrometry (Wichard et al. 2005; Schulze et al. 2006). Despite a detection limit well below 20 ng g^{-1} plant material (Schulze et al. 2006), no traces of JA were found in this algal species (Fig. 1). Given the resting level of JA in plant tissue (around 50 ng g^{-1}) and the amounts above 1,300 ng g^{-1} of JA that can be reached during active induction (Schulze et al. 2006; Adra et al. 2006), it can be excluded that JA concentrations in the investigated brown algae were high enough to induce defense responses triggered by mechanisms that are comparable to those in higher plants. Moreover, 12-oxophytodienoic acid, a biosynthetic precursor of JA that is present in considerable amounts in higher plants, could not be detected in algal extracts (Feussner and Wasternack 2002). Samples from higher plants were run in parallel as positive controls. In these samples, JA and OPDA were detected, thereby providing proof of suitability of the method employed (data on higher plants shown in Schulze et al. 2006). This finding suggests that brown algae lack a higher plant-like pathway towards JA. Nevertheless, it cannot be excluded that some algal species may contain JA or related metabolites.

Because we studied only freshly collected material with unknown grazing history, it is possible that only non-stressed algae with low resting levels of JA were surveyed. These resting levels might have been below the detection limit of our analytical method. Therefore, we performed a series of experiments where metabolic profiles of JA-,

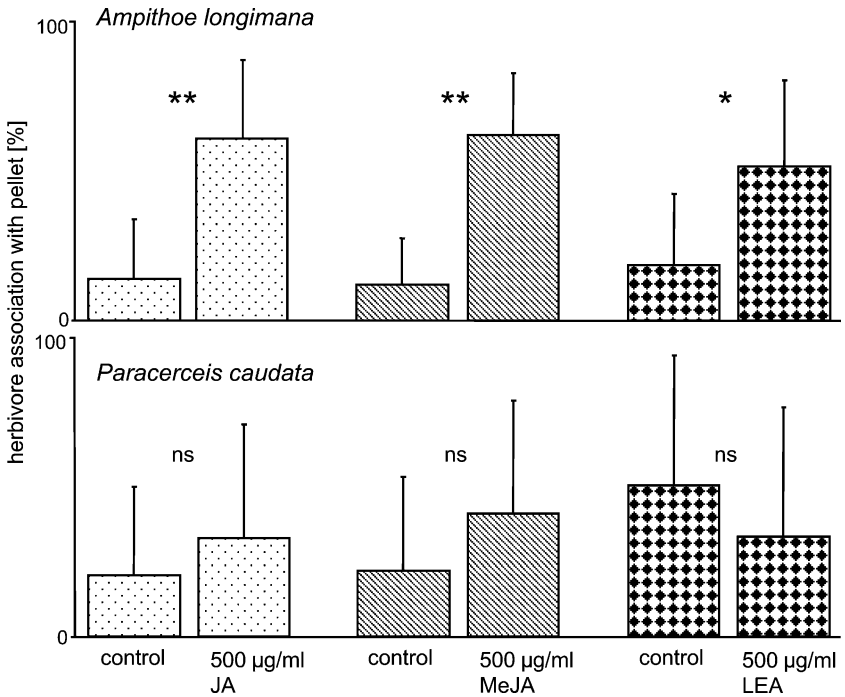

Fig. 3 Food selection by the amphipod *Ampithoe longimana* (*top*) and the isopod *Paracerceis caudata* (*bottom*). Mean percentage (+SD) of individuals present on food items. Choice assays were performed with artificial food derived from control alga (*Dictyota dichotoma*) and *Dictyota dichotoma* after jasmonic acid (*JA*) incubation, methyl jasmonate (*MeJA*) incubation, and linolenic acid (*LEA*) incubation. Number of replicates N=21 *Ampithoe longimana*/JA, N=22 *Ampithoe longimana*/MeJA, N=24 *Ampithoe longimana*/LEA, N=24 for all *Paracerceis caudata* experiments. **P=0.01, *P=0.05, *ns* not significant

MeJA-, and LEA-treated algae were compared to control samples. We selected a concentration range of these potential inducing reagents starting with amounts similar to those used for the induction of higher plants (1,000 μg ml^{-1}; Boland et al. 1995). This comprehensive metabolic profiling enabled us to monitor changes of most volatiles, semivolatiles, acids, labile aldehydes, lipids, and other compound classes detectable by GC/MS and LC/MS. We did not aim to characterize all detected metabolites but rather we followed protocols established for the metabolic profiling of plant tissues that reveal global changes in metabolic profiles (Weckwerth et al. 2005; Shulaev et al. 2008). Accordingly, we did not bias towards specific compound classes but rather surveyed all accessible metabolites of the algae. Due to methodological limitations, we did not focus on phlorotannins, a dominant compound class in brown algae, often associated with chemical defense (e.g., Pavia and Toth 2000; Koivikko et al. 2008). In cases where a regulation of metabolite production was observed in response to the treatment, we verified the structures. In other cases, we relied on MS libraries to characterize compound classes but not exact structures.

This procedure gave no evidence for an induced up- or down-regulation of biosynthetic pathways with levels of inducing agents below 500 μg ml^{-1} of the potential hormones. This lack of secondary metabolite induction was found consistently in all investigated brown algal species. In similar induction experiments with higher plants as a positive control, identical analytical methods can be routinely used to monitor metabolic changes that manifest as dramatic change of metabolic profiles (see, e.g., Boland et al. 1995; Schüler et al. 2004). Since we did not observe any increase of amounts of JA in the tissue of brown algae, this value of 500 μg ml^{-1} medium is high and should be sufficient for induction if hormone-mediated processes were involved.

The employed hormone concentrations definitely exceed those required to trigger plant responses (Heil 2004), but we selected them to overcome dilution effects, since in the case of algae it is impossible to apply the inducing agents via vascular transport as is often the practice with higher plants. To avoid potential interferences of artificially elevated concentrations of putative hormones, a series of experiments also was run with 10 μg $ml^{-1}$of JA, MeJA, and LEA, respectively. These treatments did also not result in any change of the metabolic profile (data not shown).

The role of JA in the induction of terpene biosynthesis is well established for higher plants (Hopke et al. 1994; Keinanen et al. 2001), but similar processes were not observed in *D. dichotoma* as no change in the terpene profile was observed after treatments with the potential elicitors. The group of *D. dichotoma* metabolites that belong to diterpenes was identified by comparison with characteristic EI mass spectra from the literature, but no exact structures were assigned (Fattorusso et al. 1976; Teixeira et al. 2001; Barbosa et al. 2004; Kim et al. 2006). Additional experiments were performed with this alga by using coronalone as a JA analogue (Schüler et al. 2004). Coronalone mimics the action of coronatine, which is known to induce JA-like up-regulation of the production of terpenes in higher plants (Boland et al. 1995). We used coronalone in an additional bioassay to elucidate whether JA-responsive induction of secondary metabolites can be observed with a non-natural elicitor. It might be possible that coronalone is taken up more readily by the algae than JA. In addition, coronalone may not be subjected to the same (bacterial) transformation as JA if administered in the culture medium (Schüler et al. 2004). This JA analogue, which proved to be even more active than the hormone in experiments with higher plants, also had no effect on the absolute and relative concentration of *D. dichotoma* terpenoids.

Unspecific stress on the algae due to exposure to higher concentrations of the elicitors (500–1,000 μg elicitor ml^{-1} seawater) over prolonged time spans led to the release of free fatty acids (Fig. 2). This was found for all investigated species with the exception of *S. muticum*. The metabolic responses were unspecific and were observed with the JA and MeJA treatments as well as with the LEA control. It is likely that increased stress causes activation of lipases, thus releasing fatty acids from (storage) lipids. Whereas saturated fatty acids, such as lauric (C12:0), myristic (C14:0), stearic (C18:0), arachidic (C20:0), and behenic acid (C22:0), and unsaturated fatty acids, such as stearidonic (C18:4), linolenic (C18:3), arachidonic (C20:4), and eicosapentaenoic acid (C20:5), were up-regulated occasionally, and only palmitic (C16:0), palmitoleic (C16:1), and oleic acid (C18:1) were found in all induction experiments with elevated amounts of phytohormones (Fig. 2). Because release of fatty acids was observed after all treatments (JA, MeJA, and LEA), we exclude this result as a specific hormonal action.

Comparative bioassays with induced and non-induced (control) *D. dichotoma* were performed to verify whether—despite the lack of a detectable metabolic reaction—a more subtle up-regulation of chemical defense can be triggered by JA, MeJA, or the control treatment with LEA. The amphipod *A. longimana* and the isopod *P. caudata* served as model herbivores that are widely distributed along the Atlantic coast and that can be monitored closely under controlled conditions in the lab. Bioassay procedures to monitor defense reactions of brown algae that are due to medium- and non-polar metabolites as well as to volatiles are suitable, but they have not been verified for phlorotannins (results in Wiesemeier et al. 2007). No significant food preference was detected in assays where *P. caudata* had the choice between artificial foods derived from an

induced or uninduced algae. In contrast, *A. longimana* preferred food from JA-, MeJA-, and LEA-treated algae, thereby contradicting the concept of induced defense. Interestingly, all three treatments (JA, MeJA, and LEA) led to weakly significant but comparable responses (Fig. 3). The lack of a specific induction of JA or MeJA and the fact that algae were highly stressed due to the elevated concentrations of the inducing reagents suggests that an unspecific stress response that weakens the algae might be detected by the herbivores. Since feeding by *A. longimana* is known to induce chemical defense in the closely related algal species *Dictyota menstrualis* (Cronin and Hay 1996), it thus has been documented that this amphipod is susceptible to induced brown algal chemical defense. Our results show that treatment with test compounds did not trigger the same chemical defense induction as feeding by the herbivore itself. Hormones or regulative principles other than JA and MeJA must, therefore, be responsible for the observed reaction (Cronin and Hay 1996).

The preference of treated, heavily stressed food by *A. longimana* might be due to the amphipod's ability to detect chemical cues of stressed algae that do not invest in chemical defense. Interestingly, the up-regulation of free fatty acids did not result in reduced food palatability, as has been observed in the interactions of grazers on epilithic biofilms (Jüttner 2001), thus indicating a resistance of these herbivores against free fatty acids that are lipolytic or deterrent in other alga–herbivore interactions (Fu et al. 2004).

In summary, we could not detect any indication for a role of JA and related metabolites in induced chemical defense of brown algae. The study covered a broad range of possible metabolic responses, but did not focus on the regulation of polyphenolics, which do play documented roles in induced chemical defense of some brown algae (Pavia and Toth 2000). We suggest that for the classes of metabolites investigated in our study, fundamentally different mechanisms for induction may have developed in higher plants and brown algae. Whether these mechanisms rely on structurally different hormones or on other regulative principles can currently not be ascertained.

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References

ADRA, C., BORGOGNI, A., and MARUCCHINI, C. 2006. Quantification of jasmonic acid by SPME in tomato plants stressed by ozone. *J. Agric. Food Chem.* 54:9317–9321.

ARNOLD, T. M., TARGETT, N. M., TANNER, C. E., HATCH, W. I., and FERRARI, K. E. 2001. Evidence for methyl jasmonate-induced phlorotannin production in *Fucus vesiculosus* (Phaeophyceae). *J. Phycol.* 37:1026–1029.

BALDWIN, I. T., KESSLER, A., and HALITSCHKE, R. 2002. Volatile signaling in plant–plant–herbivore interactions: what is real? *Curr. Opin. Plant Biol.* 5:351–354.

BARBOSA, J. P., TEIXEIRA, V. L., and PEREIRA, R. C. 2004. A dolabellane diterpene from the brown alga *Dictyota pfaffii* as chemical defense against herbivores. *Bot. Mar.* 47:147–151.

BOLAND, W., HOPKE, J., DONATH, J., NUSKE, J., and BUBLITZ, F. 1995. Jasmonic acid and coronatin induce odor production in plants. *Angew. Chem. Int. Ed.* 34:1600–1602.

BOUARAB, K., ADAS, F., GAQUEREL, E., KLOAREG, B., SALAUN, J. P., and POTIN, P. 2004. The innate immunity of a marine red alga involves oxylipins from both the eicosanoid and octadecanoid pathways. *Plant Physiol.* 135:1838–1848.

CHRISTOV, C., POUNEVA, I., BOZHKOVA, M., TONCHEVA, T., FOURNADZIEVA, S., and ZAFIROVA, T. 2001. Influence of temperature and methyl jasmonate on *Scenedesmus incrassulatus*. *Biol. Plant.* 44:367–371.

CRONIN, G., and HAY, M. E. 1996. Induction of seaweed chemical defenses by amphipod grazing. *Ecology* 77:2287–2301.

CRUZ-RIVERA, E., and HAY, M. E. 2003. Prey nutritional quality interacts with chemical defenses to affect consumer feeding and fitness. *Ecol. Monogr.* 73:483–506.

CZERPAK, R., PIOTROWSKA, A., and SZULECKA, K. 2006. Jasmonic acid affects changes in the growth and some components content in alga *Chlorella vulgaris*. *Acta Physiol. Plant* 28:195–203.

FATTORUSSO, E., MAGNO, S., MAYOL, L., SANTACROCE, C., SICA, D., AMICO, V., ORIENTE, G., PIATTELLI, M., and TRINGALI, C. 1976. Dictyol A and B, two novel diterpene alcohols from brown alga *Dictyota dichotoma*. *J. Chem. Soc. Chem. Comm.* 2:575–576.

FEUSSNER, I., and WASTERNACK, C. 2002. The lipoxygenase pathway. *Annu. Rev. Plant Biol.* 53:275–297.

FU, M., KOULMAN, A., VAN RIJSSEL, M., LÜTZEN, A., BOER, M. K., TYL, M. R., and LIEBEZEIT, G. 2004. Chemical characterisation of three haemolytic compounds from the microalgal species *Fibrocapsa japonica* (Raphidophyceae). *Toxicon* 43:355–363.

GARCIA-BRUGGER, A., LAMOTTE, O., VANDELLE, E., BOURQUE, S., LECOURIEUX, D., POINSSOT, B., WENDEHENNE, D., and PUGIN, A. 2006. Early signaling events induced by elicitors of plant defenses. *Mol. Plant–Microb. Interact.* 19:711–724.

HEIL, M. 2004. Induction of two indirect defences benefits Lima bean (*Phaseolus lunatus*, Fabaceae) in nature. *J. Ecol.* 92:527–536.

HEMMI, A., HONKANEN, T., and JORMALAINEN, V. 2004. Inducible resistance to herbivory in *Fucus vesiculosus*—duration, spreading and variation with nutrient availability. *Mar. Ecol. Prog. Ser.* 273:109–120.

HOPKE, J., DONATH, J., BLECHERT, S., and BOLAND, W. 1994. Herbivore-induced volatiles—the emission of acyclic homoterpenes from leaves of *Phaseolus lunatus* and *Zea mays* can be triggered by a beta-glucosidase and jasmonic acid. *FEBS Lett.* 352:146–150.

JÜTTNER, F. 2001. Liberation of 5,8,11,14,17-eicosapentaenoic acid and other polyunsaturated fatty acids from lipids as a grazer defense reaction in epilithic diatom biofilms. *J. Phycol.* 37:744–755.

KARBAN, R., and BALDWIN, I. T. 1997. Induced Responses to Herbivory. University of Chicago Press, Chicago, IL.

KEINANEN, M., OLDHAM, N. J., and BALDWIN, I. T. 2001. Rapid HPLC screening of jasmonate-induced increases in tobacco alkaloids, phenolics, and diterpene glycosides in *Nicotiana attenuata*. *J. Agric. Food Chem.* 49:3553–3558.

KIM, J. Y., ALAMSJAH, M. A., HAMADA, A., FUJITA, Y., and ISHIBASHI, F. 2006. Algicidal diterpenes from the brown alga *Dictyota dichotoma*. *Biosci. Biotech. Biochem.* 70:2571–2574.

KOIVIKKO, R., ERANEN, J. K., and LOPONEN, J. 2008. Variation of phlorotannins among three populations of *Fucus vesiculosus* as revealed by HPLC and colorimetric quantification. *J. Chem. Ecol.* 34:57–64.

KRUPINA, M. V., and DATHE, W. 1991. Occurrence of jasmonic acid in the red alga *Gelidium latifolium*. *Z. Naturforsch. C* 46:1127–1129.

KUPPER, F. C., MULLER, D. G., and PETERS, A. F. 2002. Oligoalginate recognition and oxidative burst play a key role in natural and induced resistance of sporophytes of laminariales. *J. Chem. Ecol.* 28:2057–2081.

MACAYA, E. C., ROTHAUSLER, E., THIEL, M., MOLIS, M., and WAHL, M. 2005. Induction of defenses and within-alga variation of palatability in two brown algae from the northern–central coast of Chile: effects of mesograzers and UV radiation. *J. Exp. Mar. Biol. Ecol.* 325:214–227.

MITHÖFER, A., WANNER, G., and BOLAND, W. 2005. Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* 137:1160–1168.

MOLIS, M., KORNER, J., KO, Y. W., KIM, J. H., and WAHL, M. 2006. Inducible responses in the brown seaweed *Ecklonia cava*: the role of grazer identity and season. *J. Ecol.* 94:243–249.

PAVIA, H., and TOTH, G. B. 2000. Inducible chemical resistance to herbivory in the brown seaweed *Ascophyllum nodosum*. *Ecology* 81:3212–3225.

PAVIA, H., TOTH, G. B., and ABERG, P. 2002. Optimal defense theory: elasticity analysis as a tool to predict intraplant variation in defenses. *Ecology* 83:891–897.

POHNERT, G. 2004. Chemical defense strategies of marine organisms. *Top. Curr. Chem.* 239:179–219.

SCHULZE, B., LAUCHLI, R., SONWA, M. M., SCHMIDT, A., and BOLAND, W. 2006. Profiling of structurally labile oxylipins in plants by in situ derivatization with pentafluorobenzyl hydroxylamine. *Anal. Biochem.* 348:269–283.

SCHÜLER, G., MITHÖFER, A., BALDWIN, I. T., BERGER, S., EBEL, J., SANTOS, J. G., HERRMANN, G., HÖLSCHER, D., KRAMELL, R., KUTCHAN, T. M., MAUCHER, H., SCHNEIDER, B., STENZEL, I., WASTERNACK, C., and BOLAND, W. 2004. Coronalon: a powerful tool in plant stress physiology. *FEBS Lett.* 563:17–22.

SHULAEV, V., CORTES, D., MILLER, G., and MITTLER, R. 2008. Metabolomics for plant stress response. *Physiol. Plant.* 132:199–208.

TARAKHOVSKAYA, E. R., MASLOV, Y. I., and SHISHOVA, M. F. 2007. Phytohormones in algae. *Russ. J. Plant Physiol.* 54:163–170.

TEIXEIRA, V. L., CAVALCANTI, D. N., and PEREIRA, R. C. 2001. Chemotaxonomic study of the diterpenes from the brown alga *Dictyota menstrualis*. *Biochem. Sys. Ecol.* 29:313–316.

WASTERNACK, C. 2007. Jasmonates: An update on biosynthesis, signal transduction and action in plant stress response, growth and development. *Ann. Bot.* 100:681–697.

WECKWERTH, W., and MORGENTHAL, K. 2005. Metabolomics: from pattern recognition to biological interpretation. *Drug Discov. Today* 10:1551–1558.

WICHARD, T., POULET, S. A., and POHNERT, G. 2005. Determination and quantification of alpha, beta, gamma, delta-unsaturated aldehydes as pentafluorobenzyl-oxime derivates in diatom cultures and natural phytoplankton populations: application in marine field studies. *J. Chrom. B.* 814:155–161.

WIESEMEIER, T., HAY, M. E., and POHNERT, G. 2007. The potential role of wound-activated volatile release in the chemical defence of the brown alga *Dictyota dichotoma*: blend recognition by marine herbivores. *Aquat. Sci.* 69:403–412.

WITTSTOCK, U., and GERSHENZON, J. 2002. Constitutive plant toxins and their role in defense against herbivores and pathogens. *Curr. Opin. Plant Biol.* 5:300–307.

tiveness. We report here on the attractiveness of blends and hybrid molecules of isophorone/isophorol and α-ionone/α-ionol to evaluate their potency for practical use as lure compounds in an eradication program against *B. latifrons*.

Materials and Methods

Chemicals (Fig. 1) (±)-α-Ionone (**1**), isophorone (**3**), and (±)-isophorol (**4**) were obtained from Sigma–Aldrich (Fluka) (St. Louis, MO, USA). (±)-α-Ionol (**2**, a mixture of racemic diastereomeric isomers) was obtained from Sankei Chemical Co., Ltd. (Kagoshima, Japan). (±)-3-Oxo-α-ionone (**5**), (±)-3-oxo-α-ionol (**6**) (a diastereomeric mixture) and (±)-3-hydroxy-α-ionol (**8**) (a diastereomeric mixture) were obtained from NARD Institute, Ltd. (Hyogo, Japan). (−)-*trans*-3-Hydroxy-α-ionone (**7**, $[\alpha]_D=-46.0°$, $c=0.30$, methanol) was prepared by microbial oxidation of racemic α-ionone (Wako Pure Chemical Industries, Ltd., Japan) using *Aspergillus niger* (Yamazaki et al. 1988) (Fig. 1). The identities of compounds **5**–**8** were confirmed by their ^{1}H NMR spectra (400 MHz) and mass spectra.

Insects A culture of *B. latifrons*, obtained from the Okinawa Prefectural Agricultural Research Center (originally collected in Yonaguni Island), was reared at 26–27°C and 60–70% RH under a photoperiod of 14 L:10D at the Naha Plant Protection Station in Okinawa, Japan. Adults were provided with water and a diet of four parts sucrose and one part dry yeast AY-65 (Asahi Food & Healthcare, Ltd., Tokyo, Japan). Sexually mature, mated flies (21–27-day-old) were used for the behavioral tests in a bioassay chamber under the same environmental conditions.

Laboratory Bioassays Fifty sexually mature adults of each sex were transferred into a meshed cage (30×30×45 cm) in the morning (09:00–11:00) and acclimatized with food and water until the bioassay was started 1 h after the lights came on at 8:45 the following morning. A piece of filter paper (a diameter of 90 mm, Advantec No. 2), impregnated with 100 μg of one of the test compounds in ethanol applied to the center (approximately 10 mm diam) and dried at ambient temperature, was placed on a plastic cup (45 mm high) at the center of the cage. Filter paper treated with ethanol alone served as an experimental control. The number of males and females (1) "attracted" (on the filter paper) and (2) licking the sample area ("feeding") were recorded during 10 s every 10 min for 60 min (i.e., six times). We tested every compound (100 μg) [**1**–**8**], and some two compound blends [**1**+**3**; **1**+**4**; **2**+**3**; **2**+**4**] at a 1:1 ratio (*w*/*w*, total 100 μg). A minimum of 15 replicates were carried out for each compound/blend tested.

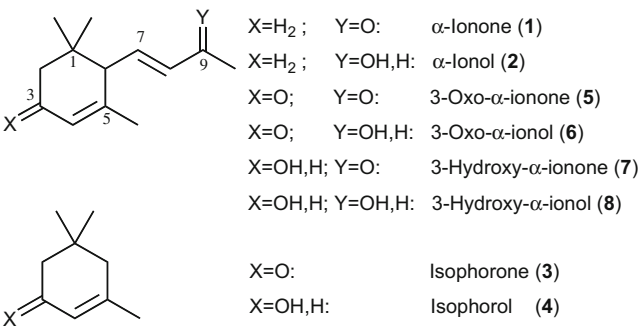

Fig. 1 Structures of the compounds tested as attractants and phagostimulants for *Bactrocera latifrons*

Fig. 2 Males of *Bactrocera latifrons* attracted to, and voraciously feeding on, filter paper treated with 3-oxo-α-ionone (**5**) (100 μg applied to the center as an approximately 10 mm diameter spot)

Statistical Analysis Within a given replicate, the data collected at 10-min intervals were summed to yield a grand total, and these were square root transformed [sq rt (x+0.5)] to normalize the distribution before analysis. Means of the transformed values were compared by Tukey–Kramer HSD multiple comparison test (α=0.05) with the statistical package JMP (ver. 5.0.1J, SAS Institute Inc. 2001).

Results

There were two distinct male behavioral responses: attraction and feeding (directly licking the chemical source) (Fig. 2). 3-Oxo-α-ionone (**5**) and 3-oxo-α-ionol (**6**) elicited similar levels of both attraction and feeding, and were significantly greater than those recorded for any other compound or mixture (Table 1). Once attracted to compounds **5** and **6**, most males persistently licked the chemical source for >10 min, leaving clear salivation marks on the filter paper. The corresponding 3-hydroxy analogs (**7** and **8**) were not as attractive as the 3-oxo derivatives (**5** and **6**),

Table 1 Mean total number (±SD) of males and females of *Bactrocera latifrons* attracted to and feeding on the chemical source

Test chemicals	Number of males attracted		Number of males feeding		Number of females attracted		Number of females feeding		*N*
3-Oxo-α-ionone (**5**)	105.1±26.0	a	91.6±23.9	a	1.7±1.5	a	0.2±0.6	ab	40
3-Oxo-α-ionol (**6**)	100.7±19.2	a	86.0±17.6	a	1.6±2.1	ab	0.4±0.7	a	31
3-Hydroxy-α-ionone (**7**)	35.0±22.1	de	28.0±18.0	cd	0.6±0.8	bc	0.0±0.0	b	21
3-Hydroxy-α-ionol (**8**)	32.6±12.7	de	27.0±11.4	cd	0.4±0.7	c	0.2±0.7	ab	20
α-Ionone (**1**)	26.5±12.9	e	12.8±6.9	e	0.3±0.6	c	0.0±0.0	b	21
α-Ionol (**2**)	42.2±17.4	def	24.9±10.5	d	0.1±0.2	c	0.0±0.0	b	19
Isophorone (**3**)	45.4±20.0	cd	22.5±9.8	d	0.3±0.6	c	0.0±0.2	b	23
Isophorol (**4**)	46.0±14.9	cde	24.9±8.5	d	0.2±0.4	c	0.0±0.0	b	22
α-Ionone+isophorone (**1**+**3**)	51.1±23.1	bcd	24.3±13.2	de	0.3±0.6	c	0.1±0.3	ab	15
α-Ionone+isophorol (**1**+**4**)	43.3±25.1	cde	19.3±13.9	de	0.1±0.4	c	0.0±0.0	b	15
α-Ionol+isophorone (**2**+**3**)	66.1±18.8	b	41.9±12.9	b	0.4±0.9	c	0.0±0.0	b	21
α-Ionol+isophorol (**2**+**4**)	60.7±22.5	bc	38.9±14.5	bc	0.3±0.6	c	0.0±0.0	b	24
Control	0.7±0.9	g	0.0±0.0	f	0.4±0.6	c	0.0±0.0	b	20

The values with different letters indicate significant differences within the same column by Tukey–Kramer HSD test of the square-root transformed data ($P<0.05$)

eliciting responses similar to α-ionol (**2**), isophorone (**3**), and isophorol (**4**) (Table 1). However, some of the blends of α-ionone/α-ionol and isophorone/isophorol elicited higher responses than any of the individual components (**1**, **2**, **3**, or **4**) within the subsets (Table 1).

Females did not display a strong response towards any of the test compounds, although the 3-oxo analogs **5** and **6** elicited weak but significantly higher responses (both as attractants and feeding stimulants) than the other compounds or mixtures (Table 1).

Discussion

Within the series of 3-oxygenated α-ionone/α-ionol analogs tested, 3-oxo-α-ionone (**5**) and 3-oxo-α-ionol (**6**) were the most effective both as attractants and phagostimulants for *B. latifrons*, and significantly better than the corresponding 3-hydroxy compounds **7** and **8** or the known attractants α-ionone (**1**) and α-ionol (**2**) (Flath et al. 1994). However, there was a higher proportion of males that fed on all four 3-oxygenated analogs [number of feeding males/number of attracted males: **5** (87%), **6** (85%), **7** (80%), and **8** (84%)] than on α-ionone and α-ionol [**1** (48%) and **2** (59%)].

3-Oxygenated α-ionone analogs have been reported from many plants, including tomato, grape, and blackberry, although the compounds are found mostly as glycosidically bound aroma in various fruit tissues (Strauss et al. 1987; Humpf and Schreier 1991; Marlatt et al. 1992). Interestingly, a small quantity of 3-hydroxy-α-ionone was detected in the tissues of eggplant fruit, one of the favorite hosts of *B. latifrons* (Nishida et al., unpublished). Furthermore, males that fed voraciously on eggplant incorporated a series of 3-oxygenated α-ionone derivatives in the rectal gland, which is presumed to be a pheromone reservoir organ in *B. latifrons* (Little 1992). This suggests that these male attractants may act as sex pheromones, or their precursors, to attract females during the crepuscular courtship period, in a manner similar to the case of methyl eugenol for the oriental fruit fly, *B. dorsalis* (Tan and Nishida 1998).

Isophorone (**3**) and isophorol (**4**), which conform to the partial structures of compounds **5**–**8**, also elicited responses in males, although not significantly different from those of compounds **7** and **8**. However, when either **3** or **4** were combined with α-ionol (**2**), the resulting mixtures gave significantly higher attractancy than any of the three compounds individually. While we do not understand how this synergism actually works, these artificial blends may be used as effective long-range attractants for *B. latifrons* males, as **3** and **4** are much more volatile than compounds **5**–**8**. Clearly, under laboratory conditions in small cages, 3-oxygenated α-ionones/α-ionols are attractants for *B. latifrons* males, but further research is required to determine over what range they and/or blends that incorporate **3** and **4** would be effective under field conditions.

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References

FLATH, R. A., CUNNINGHAM, R. T., LIQUIDO, N. J., and MCGOVERN, T. P. 1994. Alpha-ionol as attractant for trapping *Bactrocera latifrons* (Diptera: Tephritidae). *J. Econ. Entomol.* 87:1470–1476.

HUMPF, H. U., and SCHREIER, P. 1991. Bound aroma compounds from the fruit and the leaves of blackberry (*Rubus laciniata* L.). *J. Agric. Food Chem.* 39:1830–1832.

JMP 2001. User's manual, version 5.0.1j. SAS Institute, Cary.

KANEDA, M., SUGIMOTO, S., and ISHIHARA, H. 1985. Discovery of *Dacus latifrons* in Yonaguni Island. *Naha Plant Protection News (Naha Shokubutsu-Bôeki Jôhô)* 59:294, (in Japanese).

KUBA, H., MATSUYAMA, T., and MOUGI, N. 2006. Current status of the solanaceous fruit fly control project in Yonaguni Island. *FFTC–OPARC Joint International Symposium on Area-Wide Management of Insects Pests. October 2–3, 2006. Okinawa Prefectural Agricultural Research Center, Okinawa, Japan. Proceeding of the Symposium*: 41–51.

LIQUIDO, N. J., HARRIS, E. J., and DEKKER, L. A. 1994. Ecology of *Bactrocera latifrons* (Diptera: Tephritidae) populations: host plants, natural enemies, distribution, and abundance. *Ann. Entomol. Soc. Am.* 87:71–84.

LITTLE, H. F. 1992. Fine structure of presumed pheromone glands of *Dacus cucurbitae* and *D. latifrons* (Diptera: Tephritidae). *Ann. Entomol. Soc. Am.* 85:326–330.

MARLATT, C., HO, C. T., and CHIEN, M. 1992. Studies of aroma constituents bound as glycosides in tomato. *J. Agric. Food Chem.* 40:249–252.

MCQUATE, G. T., and PECK, S. L. 2001. Enhancement of attraction of alpha-ionol to male *Bactrocera latifrons* (Diptera: Tephritidae) by addition of a synergist, cade-oil. *J. Econ. Entomol.* 94:39–46.

MCQUATE, G. T., KEUN, Y. S., SYLVA, C. D., LI, Q. X., and JANG, E. B. 2004. Active ingredients in cade oil that synergize attractiveness of α-ionol to male *Bactrocera latifrons* (Diptera: Tephritidae). *J. Econ. Entomol.* 97:862–870.

MWATAWALA, M. W., De MEYER, M., WHITE, I. M., MAERERE, A., and MAKUNDI, R. H. 2007. Detection of the solanum fruit fly, *Bactrocera latifrons* (Hendel) in Tanzania (Dipt., Tephritidae). *J. Appl. Entomol.* 131:501–503.

STRAUSS, C. R., WILSON, B., and WILLIAMS, P. J. 1987. 3-Oxo-α-ionol, vomifoliol and roseoside in *Vitis vinifera* fruit. *Phytochemistry* 26:1995–1997.

SHIMIZU, Y., KOHAMA, T., UESATO, T., MATSUYAMA, T., and YAMAGISHI, M. 2007. Invasion of solanum fruit fly *Bactrocera latifrons* (Diptera: Tephritidae) to Yonaguni Island, Okinawa Prefecture, Japan. *Appl. Entomol. Zool.* 42:269–275.

TAN, K. H., and NISHIDA, R. 1998. Ecological significance of male attractant in the defence and mating strategies of the fruit fly pest, *Bactrocera papayae*. *Entomol. Exp. Appl.* 89:155–158.

VARGAS, R. I., and NISHIDA, T. 1985. Survey for *Dacus latifrons* (Diptera: Tephritidae). *J. Econ. Entomol.* 78:1311–1314.

WHITE, I. M., and ELSON-HARRIS, M. M. 1992. Fruit flies of economic significance: their identification and bionomics. CABI, Wallingford.

YAMAZAKI, Y., HAYASHI, Y., ARITA, M., HIEDA, T., and MIKAMI, Y. 1988. Microbial conversion of α-ionone, α-methylionone, and α-isomethylionone. *Appl. Environ. Microbiol.* 54:2354–2360.

locate inflorescences for efficient pollination. Worldwide, there are about 750 fig species (*Ficus*, Moraceae; Berg 1989), and figs generally are pollinated by female fig wasps (Agaonidae: Hymenoptera) that are specific for each *Ficus* species, and they oviposit in the fig syconium (Wiebes 1979; Ware and Compton 1992). Although visual factors may be involved (Ramirez 1974; Janzen 1979; Verkerke 1989), several studies have shown that the pollinating wasps are attracted by olfactory cues (Bronstein 1987; Van Noort 1989; Ware et al. 1993; Hossaert-McKey et al. 1994; Gibernau et al. 1998; Grison et al. 1999). However, in the five stages of fig development [i.e., prefemale floral stage, female floral stage, interfloral stage (time period between female floral stage and male floral stage), male floral stage, and post-floral stage], only the female stage is receptive to pollinators (Galil and Eisikowitch 1968; Bronstein 1987). Ware and Compton (1994) concluded that figs should either cease to produce attractive signals after being pollinated or that new compounds should be produced (or specific components increased) that repel pollinators. This provides an alternate explanation for the pollinators' indifference to post-pollination flowers (Schiestl and Ayasse 2001; Song et al. 2001). Recent studies of *Ficus hispida* L. volatiles did not detect new compounds after pollination, but the quantity of volatile emission decreased quickly after 24 h (Proffit et al. 2008). Some compounds present only in small amounts disappeared when checked by gas chromatography-mass spectrometry, but the abundant compounds β-ocimene, (*E*)-β-farnesene, and linalool still existed 2 weeks after pollination (Proffit et al. 2008).

It is clear that pollinators can distinguish between receptive and non-receptive figs (Van Noort et al. 1989; Ware and Compton 1994; Song et al. 2001). However, few bioassays have been conducted to test the pollinators' responses to figs at different developmental stages (Grision-Pigé et al. 2002a). Because floral odors are complex mixtures of volatiles (Knudsen et al. 1993, 2006), it is difficult to determine how alterations in the composition, proportion, and concentration of floral odor components influence pollinator behavior (Patt et al. 1995). Nonetheless, tests of individual volatile compounds and combinations may shed light on the chemical mechanism involved in post-pollination volatile changes.

In this study, we used a Y-tube olfactometer to test fig wasp, *Ceratosolen solmsi marchali* Mayr, behavior to volatile compounds from *F. hispida* and preferences of pollinators to receptive and interfloral figs from their dioecious host. Based on previous studies (Song et al. 2001; Proffit et al. 2008), we also chose three monoterpene compounds from receptive figs, one abundant compound (linalool) and two minor ones (limonene and β-pinene), to test the response of the pollinator. All of these volatile compounds are found in the odor of receptive figs of *F. hispida*, and their quantities have decreased by the interfloral fig stage. The specific purpose of our study was to (1) determine experimentally whether the pollinator was sensitive to floral scent changes between receptive and interfloral figs of *F. hispida*; (2) investigate whether quantity or quality of fig volatiles play an important role in attracting fig pollinators; (3) examine whether linalool, the main constituent of the receptive fig odor of *F. hispida*, plays an important role in attracting pollinators; and (4) whether the minor constituents of fig odor enhance attractiveness.

Methods and Materials

Plants and Insects F. hispida, growing naturally at Xishuangbanna Tropical Botanical Garden (101°15′ E, 21°55′ N) in southwest China, was used to obtain both plant and insect materials. *C. solmsi marchali*, the pollinator of *F. hispida*, develops in syconia of this species. Both the fig and its pollinator have been studied and described in earlier publications (Hill 1967; Abdurahiman and Joseph 1976; Patel et al. 1995; Patel 1996; Patel and Hossaert-McKey 2000; Moore et al. 2003; Peng et al. 2005; Proffit et al. 2008). Pre-receptive figs were isolated from pollinators by using mesh bags. Fresh figs with their branches were cut in the morning, placed into a vase containing water, and used for bioassays of volatiles. When a fig is nearly mature, fertilized female wasps will leave via a channel bored by males (Galil and Eisikowitch 1968). Pre-mature male figs without holes were collected at dusk and put into mesh bags. The next morning, wasps came out of those figs and were selected for olfactometer studies. Fig floral stages were estimated by their physical characteristics: loose obstacle, heavy-sweet scent, and pollinators being attracted outside the bag are signals of a receptive fig; interfloral figs have a hard, solid feel and a deeply colored surface, while a mature fig has a soft, white and smooth surface.

Chemicals Previous studies indicate that linalool is the most abundant compound in the odor of receptive *F. hispida* (Song et al. 2001; Proffit et al. 2008). For this reason, the response of *C. solmsi marchali* was tested against (*R*)-(+)-linalool (Linke Reagent Co., Ltd., China, ≥93%), (*S*)-(−)-linalool (Juhua Reagent Co., Ltd., China, ≥98%) and a 1:1 mixture of these two enantiomers. Limonene and β-pinene are minor volatiles in the odor of receptive *F. hispida* and numerous other receptive *Ficus* species (Grison-Pigé et al. 2002b). Enantiomers of limonene were tested against *C. somisi marchali* by using (*R*)-(+)-limonene (Fluka, ≥98%), (*S*)-(−)-limonene (Fluka, ≥97%) and a 1:1 mixture of these two enantiomers. The levoisomer of β-pinene [(−)-β-pinene (Cole-Parmer, ≥98%)] was also tested against *C. somisi marchali*.

Olfactometer Methodology All experiments were conducted in a Y-tube olfactometer (stem, 8 cm; arms, 9 cm; at 55° angle; ID 1.5 cm) with each of the two arms connected to a glass container or a polyethylene terephtalate (Nalophan) bag (Kalle Nalo GmbH, Wursthüllen, Germany) that contained an odor source. This small dimension olfactometer was adopted to test small wasps that respond to attractants by walking. It is similar to the one described by Tooker et al. (2005). Air is drawn through Teflon tubing by an air pump and passed through a charcoal filter and distilled water. The cleaned and humidified air stream, regulated to a flow rate of 0.2 L/min with two flowmeters, was split via a "Y" hose junction to create two equal air streams. To limit visual distractions for the insects, the olfactometer was placed in the center of a flat, white table illuminated with three 40-W cool white fluorescent tubes placed above the arms of the Y tube. The windows of the bioassay room were covered by black fabric curtains, and the air temperature was monitored and maintained at ~26°C.

Each wasp was allowed 5 min to respond to odors, and a choice of the left or right arm of the olfactometer was noted when the insect went 1 cm (decision line) past the Y junction and stayed for at least 1 min. Wasps not reaching the decision line within 5 min were removed and recorded as "no choice". Every four to five bioassays, the treatment arm was switched between the two arms of Y-tube to avoid any influence of unforeseen asymmetries in the setup. The olfactometer was rinsed with absolute alcohol and then dried by an air blower after every bioassay.

Bioassays of Odors of Receptive and Interfloral Fig Sources (Experiment A) In order to compare responses of *C. solmsi marchali* to receptive and non-receptive floral scents, experiments (Table 1, Exp. A1, A2, A3, and A4) were conducted with four different types of *F. hispida* scents vs. clean air.

Table 1 Response of the pollinator wasp *Ceratosolen solmsi marchali* to odors of receptive and interfloral fig(s) of *Ficus hispida* (Experiment A)

Experiment	Type and (number of figs) in the arms of the Y tube	
	Arm 1	Arm 2
A1	Male receptive fig (1)	Clean air
A2	Male interfloral fig (1)	Clean air
A3	Female receptive fig (1)	Clean air
A4	Female interfloral fig (1)	Clean air
A5	Male receptive fig (1)	Male interfloral fig (1)
A6	Male receptive fig (1)	Male interfloral fig (9)
A7	Male receptive fig (1)	Male interfloral fig (25)
A8	Female receptive fig (1)	Female interfloral fig (1)
A9	Female receptive fig (1)	Female interfloral fig (9)
A10	Female receptive fig (1)	Female interfloral fig (25)

Grison-pigé et al. (2001) reported that figs have a higher quantity of volatile emission in their receptive stage. The ratios of scent quantity emitted by one fig between receptive fig vs. interfloral fig were about 13:1 for male *F. hispida* and 17:1 for female *F. hispida* (Proffit et al. 2008). In six subsequent experiments (Table 1, Exp. A5, A6, A7, A8, A9, and A10), we set 1:1, 1:9, and 1:25 ratios for the number of receptive figs vs. interfloral figs to balance the higher quantity of odor emission from receptive figs.

Fresh figs were packed into a polyethylene terephtalate bag and used as a sample odor source. A similar sized bag was used as a control for supplying clean air in the first four experiments. Fig sources were changed every 1–2 h. Trials were replicated until there were at least 24 wasps responding to one of the odor sources in the Y-tube. In each experiment, all replicates were tested over 3 days. All bioassays of fresh fig sources were conducted between 08:30 to 11:30 during November and December 2005.

Bioassay of Fig Volatiles (Experiment B) The olfactory responses of *C. solmsi marchali* to three fig volatiles emitted from receptive *F. hipida* (e.g., linalool, limonene, β-pinene, and blends of linalool and limonene and β-pinene). This experiment examined whether a major or a minor constituent of volatiles attracts the pollinator at different quantities and determined whether these compounds could enhance the attraction of pollinators.

The concentrations of volatiles in the blends were set according to the amount emitted by the receptive figs. In the case of *F. hispida*, linalool, limonene, and β-pinene comprise 13.57%, 1.23%, and 0.24% of the total volatiles emitted by the receptive figs of male trees, and 15.74%, 2.90%, and 1.21% of the volatiles emitted by the receptive figs of female trees, respectively (Proffit et al. 2008). The compounds were diluted separately in dichloromethane (10^{-6} μl ~ 1 μl/100 μl). Cellulose sponge pieces (1×1×0.2 cm) were impregnated with 100 μl of diluted compounds. In the same way, a piece of cellulose sponge with pure solvent (100 μl of dichloromethane) was prepared as the control. After 20 min of solvent evaporation (to get rid of dichloromethane completely), each piece of sponge was stored in a pipette tube, the tube was put into a 30 ml glass container, and the container was connected with one arm of the Y-tube. The compound put into the pipette tube was used as a sample odor source and tested individually on one wasp. Each pipette tube was made from a 7 cm long, 1 ml polypropylene pipette tip, from which 3 cm of the bottom and 1.5 cm of the tip were removed. Trials were replicated until there were at least 40 wasps responding to either of the odor sources in the Y-tube.

Seven experiments (Table 2, Exp. B1-1, B1-2, B1-3, B2-1, B2-2, B2-3, and B3) were conducted to test single compounds. Based on the results of single compound tests,

the attractive enantiomers of linalool were mixed with the other two compounds (Table 2, Exp. B4 and B5). Each blend was tested respectively in four doses.

All the bioassays on compounds were conducted between 07:30 to 11:30 from February to May 2006 and from May to July 2007.

Statistical Analyses The results from each bioassay experiment were subjected to a chi-square (χ^2) test. The null hypothesis was that wasps had a 50:50 distribution between the two odor sources in two arms, respectively. Given the sample sizes in the experiments, statistical analyses were not needed when all the responding wasps entered one of the arms.

Results

Before testing all odor sources, a series of control tests were performed with arms of the olfactometer permeated with clean air. There was no observed difference in the responses of wasps to the two arms of the Y-tube: 20 directed to the right and 20 to the left when the arms were switched by turning the Y-tube over every five tests ($\chi^2=0$, $df=1$, $P=1.000$).

Bioassays of Odors from Receptive and Interfloral Fig Sources (Experiment A) In the treatment of fig odor vs. clean air, wasps chose the arm of the olefactometer with fig odor over the control, even when odors were emitted from interfloral figs (Fig. 1a). Subsequent experiments investigated the choices of *C. solmsi marchali* made between receptive and interfloral fig odors. Pollinators always showed a strong preference to the receptive fig odor, regardless of increases in interfloral fig numbers (Fig. 1b).

Bioassay of Fig Volatile Compounds (Experiment B) In the assays with single volatile compounds, all were tested with less than 1 μl. Among them, the highest doses of enantiomers of linalool were presented as 0.1 μl because the pollinators reacted in a confused, disordered way to it at a dose of 0.25 μl. At all tested doses, (*R*)-(+)-linalool, (*R*)-(+)-and (*S*)-(−)-limonene were never active (Fig. 2a,d,e). However, the other four compounds all evoked dose-dependent responses from *C. solmsi marchali*, although the threshold doses at which each first induced a significant response were markedly different. The wasp pollinators showed a strong preference for (*S*)-(−)-linalool when offered at amounts of 0.01 or 0.1 μl (Fig. 2b). The mixture of (*R*)-(+)- and (*S*)-(−)-linalool was attractive to wasps at doses of 0.001, 0.01, and 0.1 μl (Fig. 2c). No response was observed at lower doses for (*S*)-(−)-linalool or (±)-linalool. At the higher doses, (±)-limonene and (−)-β-pinene both evoked a significant wasp avoidance response, while they were neutral at the lower doses (Fig. 2f,g). Compared with either enantiomer of linalool and limonene, the mixture of *(R)*-(+)- and *(S)*-(-)-linalool enhanced pollinator attraction, while *(R)*-(+)-linalool and (*S*)-(−)-limonene enhanced wasp avoidance.

Subsequent experiments with volatile blends utilized a 10:1 ratio of (*S*)-(−)-linalool or (±)-linalool vs. (±)-limonene, or a 20:1 ratio of (*S*)-(−)-linalool or (±)-linalool vs. (−)-β-pinene. The (±)-limonene and (*S*)-(−)-linalool

Table 2 Response of the pollinator wasp *Ceratosolen solmsi marchali* to fig volatile compounds from *Ficus hispida* compared with control (Experiment B)

Experiment	Compound	Dose (μl)				
		1	2	3	4	5
Single compound						
B1-1	(*R*)-(+)-linalool	0.00001	0.0001	0.001	0.01	0.1
B1-2	(*S*)-(−)-linalool	0.00001	0.0001	0.001	0.01	0.1
B1-3	(±)-linalool	0.00001	0.0001	0.001	0.01	0.1
B2-1	(*R*)-(+)-limonene	0.0001	0.001	0.01	0.1	1
B2-2	(*S*)-(−)-limonene	0.001	0.01	0.1	0.25	1
B2-3	(±)-limonene	0.0001	0.001	0.01	0.1	1
B3	(−)-β-pinene	0.001	0.01	0.1	0.25	1
Blends						
B4	Linalool + (*R*)-(+)-limonene + (*S*)-(−)-limonene=10:0.5:0.5					
B4-1	(*R*)-(+)-linalool + (±)limonene	0.0001	0.001	0.01	0.1	
B4-2	(*S*)-(−)-linalool + (±)limonene	0.0001	0.001	0.01	0.1	
B4-3	(±)-linalool + (±)limonene	0.0001	0.001	0.01	0.1	
B5	linalool + (−)-β-pinene=20:1					
B5-1	(*R*)-(+)-linalool + (−)-β-pinene	0.0001	0.001	0.01	0.1	
B5-2	*(S)*-(−)-linalool + (−)-β-pinene	0.0001	0.001	0.01	0.1	
B5-3	(±)-linalool + (−)-β-pinene	0.0001	0.001	0.01	0.1	

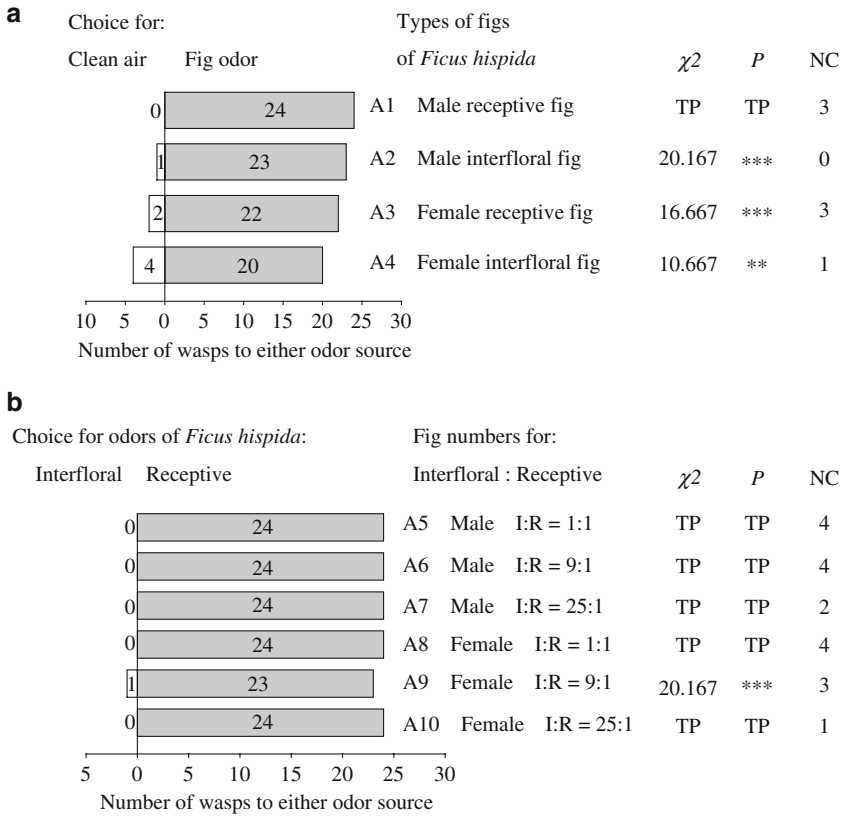


Fig. 1 Responses of females of *Ceratosolen solmsi marchali* to (**a**) volatiles of male or female figs of *Ficus hispida* at receptive or interfloral stages compared with clean air. The volatiles tested were emitted by one fig; (**b**) volatiles of male or female figs of *F. hispida* compared between receptive and interfloral stages. The ratios of number of interfloral vs. receptive figs (I:R) were set as 1:1, 9:1 and 25:1. Numbers in the bars are the number of wasps responded to the arms of the Y-tube. χ^2 test was used to evaluate whether the number of responding wasps differed from a 50:50 distribution between the two olfactometer arms (TP: there was no χ^2 test performed because one of the odor sources in either arm had a total preference (TP) of pollinators; ***$P<0.001$; **$P<0.01$). Wasps that did not response within 5 min [no choice (NC)] were excluded from statistical analysis

mixture was attractive to pollinators at the two higher doses (Fig. 2h). (±)-Limonene plus (±)-linalool was attractive at the three higher doses (Fig. 2i); however, at lower doses, pollinators did not respond to either of the two blends (Fig. 2h,i). The 20:1 ratio mixtures of both (*S*)-(−)-linalool and (±)-linalool to (−)-β-pinene were attractive to pollinators at the three higher doses but were neutral at the lower doses (Fig. 2j,k).

At higher doses, (*S*)-(−)-linalool and (±)-linalool actively attracted *C. solmsi marchali*, but a blend of (−)-β-pinene and (±)-limonene was repellent. (*R*)-(+)-Linalool, (*R*)-(+)-limonene, and (*S*)-(−)-limonene did not induce any response from the wasps. The non-active compounds (*R*)-(+)-linalool and (−)-β-pinene enhanced the attractiveness of (*S*)-(−)-linalool, while enantiomers of limonene did not.

Discussion

Post-pollination changes in floral characteristics, which include shape and color changes, cessation of nectar production, and scent alteration, can be detected by pollinators (Arditti 1979). The structure of inflorescences and specificity of the association make pollinator attraction by *Ficus* species unique. Both tactile and olfactory cues have been suggested to be involved in fig wasp's localization (Ramírez 1974; Janzen 1979; Ware and Compton 1992). Long-distance olfactory cues are used by wasps to find host trees that bear receptive figs. Tactile cues are used once the insect has landed on the fig surface to determine whether it is suitable (Gibernau et al. 1998). However, in this study, without any visual or tactile cues, fig wasps were attracted to host fig volatiles in the Y-tube. Thus, at short distances, tactile cues may not be indispensable for the pollinator to locate a receptive fig.

Few studies have tested directly pollinator responses to pollination-induced floral changes. (Gori 1983; Primack 1985; van Doorn 1997). The behavior of pollinating wasps at the receptive stage compared with that at the interfloral stage has not been studied, probably because the wasps are rarely observed on figs at the interfloral stage (van Noort et al. 1989; Ware and Compton 1994). Our findings showed

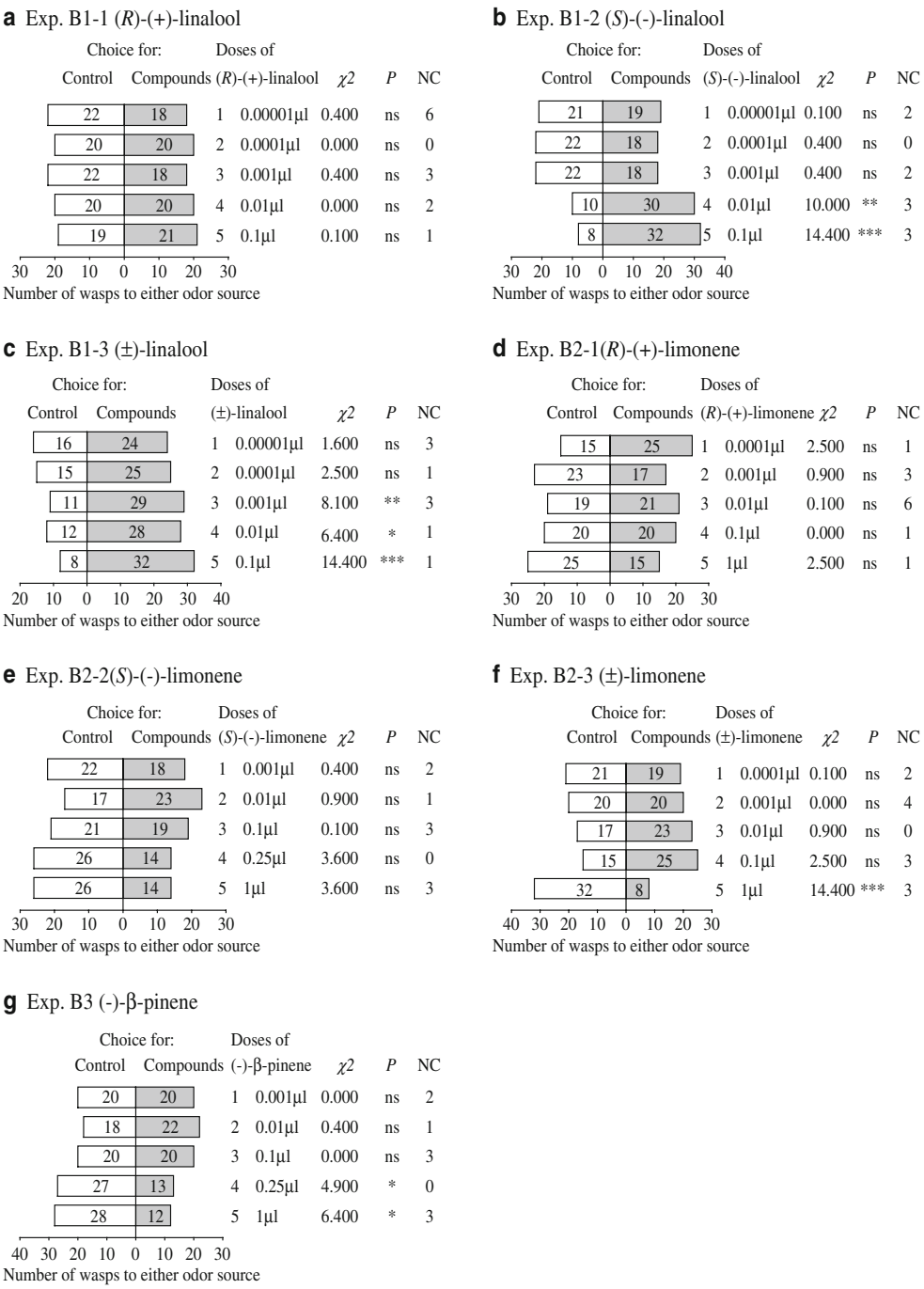
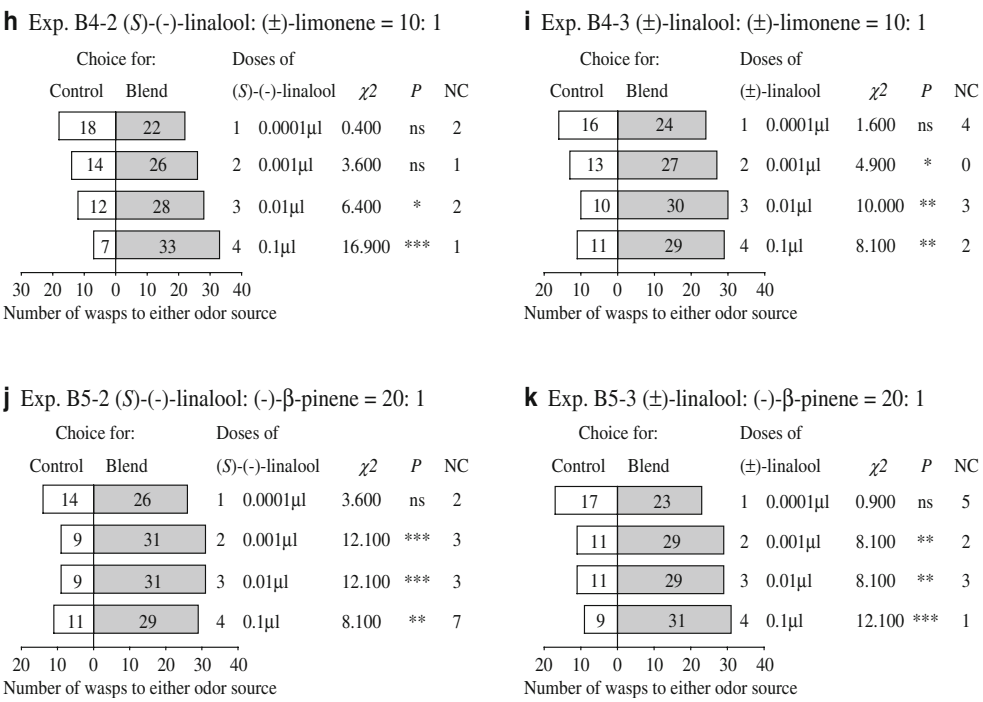


Fig. 2 Responses of females of *Ceratosolen solmsi marchali* to: (**a**–**g**) single volatile component mainly found in receptive figs of *F. hispida*, (**h**–**k**) blends mixed by a 10:1 ratio of *(S)*-(−)-linalool or (±)-linalool to (±)-limonene, and a 20:1 ratio of (*S*)-(−)-linalool or (±)-linalool to (−)-β-pinene. Each blend was tested respectively in four doses. The doses labeled in the figure belong to (*S*)-(−)-linalool or (±)-linalool in blends. Numbers in the bars are the number of wasps responded to the arms of the Y-tube. χ^2-test was used to evaluate whether the results differed from a 50:50 distribution between the two olfactometer arms (***$P<0.001$; **$P<0.01$; *$P<0.05$; ns: $P\geq0.05$). Wasps that did not response within 5 min [no choice (NC)] were excluded from the statistic analysis

that two odor sources emitted from receptive figs and interfloral figs of *F. hispida* caused different behavioral responses of *C. solmsi marchali*. These results parallel previous studies in which pollinators were found to be attracted only to receptive figs (Ware and Compton 1994; Gibernau et al. 1998; Song et al. 2001). All previous studies were carried out under conditions when non-receptive figs coexisted with receptive figs. In the present study, we found unexpectedly that pollinators were attracted also to interfloral figs in the absence of receptive figs.

Raguso (2001) has summarized the three roles of post-pollination volatile emission in pollinator attraction as follows: distance attraction, futile visits prevention, and promotion of learned avoidance of reward-depleted flowers. It seems that the attractive effect of *F. hispida* interfloral figs cannot be explained by the above. We suggest that the similarity of odor composition in the two stages may be responsible for the wasps' tendency for futile attraction to interfloral inflorescences in the Y-tube olfactometer. Nearly all volatiles found in *F. hispida* interfloral figs occur also in

Fig. 2 (continued)



receptive figs. Linalool, the major volatile, continued to be emitted from the interfloral figs and was a strong attraction to pollinators in our tests. There might be an adaptive advantage to having pollinated figs that still draw wasps to a tree. In situations where pollinators are limiting, any extra attraction to trees could be advantageous—i.e., as long as wasps are drawn to figs that need to be pollinated, in preference to pollinated ones. In particular, this might be valuable for female trees where relatively few figs are attractive at any one time. Although pollinated figs are still attractive, wasps cannot get into them because the figs tightened obstacles after pollination. However, not all interfloral figs of *Ficus* species are attractive to their respective pollinators. In other studies, the interfloral figs of dioecious *Ficus semicordata* were not attractive to their pollinator *C. gravelyi*, (Chen and Song, unpublished data), probably because of differences in the volatile composition compared with receptive figs). The fig–fig wasp mutualism is a complex and diverse system, so conclusions deduced from one species should be carefully applied to another species.

Numerous studies have focused on whether quantity or quality plays a more important role in plant–insect chemical attraction (e.g., Visser 1986; Hoballah et al. 2002). Since *F. hispida* has lower volatile emission following the receptive stage, another aim of our study was to elucidate whether the quantity of odor in this fig species influenced the cognitive behavior of its pollinator. Our results indicate that higher quantities of interfloral fig odor were less attractive to pollinators than receptive fig odor. Although receptive figs may emit higher levels of volatiles, the stage-dependent host localization behavior of wasps is regulated by the composition and proportion of the compounds (i.e., quality), not just the quantity of volatiles. Moreover, all of the volatiles studied induced a behavioral response in wasps that was dose-dependent. All compounds tested at the lower concentrations were neutral, even those that were attractive to pollinators at higher concentrations. There is little doubt that compounds termed "attractants" can also act as neutrals or repellents at high concentrations. Single compound tests indicated that some compounds are more important than others in attracting wasps. Attractive compounds that were identified in this study, e.g., linalool, are common in many floral fragrances (Gibernau et al. 1997). In simulating changes in fig odors from the receptive to interfloral stage, we set four concentration degrees in the blend tests. Just as the interfloral figs with smaller volatile quantities were less attractive to pollinators, the attractive compounds were not attractive to the pollinator when their concentrations were comparatively low. At high concentrations, all blends of (*S*)-(−)-linalool or (±)-linalool with one of the minor components attracted pollinator wasps. However, at low concentrations, all blends were neutral. In addition to host quality odor, i.e., volatile composition, the quantity of active volatiles is also important.

Our mixture experiments confirmed synergistic effects of volatiles in plant odors (Visser 1986). In several cases, blends of active and non-active compounds are more attractive to parasites or pollinators than single compounds (Visser and Avé 1978; Dariusz and Stephen 1999). In this study, neither (±)-limonene nor (−)-β-pinene were attractive to *C. solmsi marchali* when applied singly; however, blends

of either of those compounds with linalool were more attractive. The attractiveness of (*S*)-(−)-linalool to *C. solmsi marchali* was enhanced by decreasing the active threshold dose from 0.01 to 0.001 μl when mixed with non-active (*R*)-(+)-linalool, (±)-limonene, or (−)-β-pinene.

The fig–fig wasp mutualism is a complex system, with variation among *Ficus* species and fig wasp species, volatile compounds, and volatile emission rhythms (Wiebes 1979; Berg 1989; Grison et al. 1999; Grison-Pigé 2001, 2002b). Our experiment is an initial exploration of pollinating fig wasp responses to pollination-induced changes. In the future, comprehensive studies should be undertaken to investigate further the chemical interaction in this specialized insect–plant mutualism.

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References

ABDURAHIMAN, U. C., and JOSEPH, K. J. 1976. Observations on the biology and behaviour of *Ceratosolen marchali* Mayr (Agaonidae, Chalcidoidea, Hymenoptera). *Entomon* 1:115–121.

ARDITTI, J. 1979. Aspects of the physiology of orchids, pp. 422–697, in H . W. Woolhouse (ed.). Advances in Botanical Research Academic, London.

BERG, C. C. 1989. Classification and distribution of *Ficus*. *Experientia* 45:605–611.

BRONSTEIN, J. L. 1987. Maintenance of species-specificity in Neotropical fig–pollinator wasp mutualism. *Oikos* 48:39–46.

DARIUSZ, C., and STEPHEN, A. T. 1999. Synergistic effect of ethanol to *a*-pinene in primart attraction of the larger pine shoot beetle, *Tomicus piniperda*. *J. Chem. Ecol.* 25:1121–1130.

DUDAREVA, N., and PICHERSKY, E. 2000. Biochemical and molecular genetic aspects of floral scents. *Plant Physiol.* 122:627–633.

GALIL, J., and EISIKOWITCH, D. 1968. Flowering cycles and fruit types of *Ficus sycomorus* in Israel. *New Phytol.* 67:745–758.

GIBERNAU, M., BUSER, H. R., FREY, J. E., and HOSSAERT-MCKEY, M. 1997. Volatile compounds from extracts of figs of *Ficus carica*. *Phytochemistry* 46:241–244.

GIBERNAU, M., HOSSAERT-MCKEY, M., FREY, J. E., and KJELLBERG, F. 1998. Are olfactory signals sufficient to attract fig pollinators? *Ecoscience* 5:306–311.

GORI, D. 1983. Post-pollination phenomena and adaptive floral changes, pp. 31–49, in C. E. Jones, and R. J. Little (eds.). Handbook of Experimental Pollination BiologyScientific and Academic Editions, New York.

GRISON, L., EDWARDS, A. A., and HOSSAERT-MCKEY, M. 1999. Interspecies variation in floral fragrances emitted by tropical *Ficus* species. *Phytochemistry* 52:1293–1299.

GRISON-PIGÉ, L., BESSIÈRE, J. -M., TURLINGS, T. C. J., KJELLBERG, F., ROY, J., and HOSSAERT-MCKEY, M. 2001. Limited intersex mimicry of floral odour in *Ficus carica*. *Funct. Ecol.* 15:551–558.

GRISON, -PIGÉ, L., BESSIÈRE, J. -M., and HOSSAERT-MCKEY, M. 2002a. Specific attraction of fig-pollinating wasps: role of volatile compounds released by tropical figs. *J. Chem. Ecol.* 28:283–295.

GRISON-PIGÉ, L., HOSSAERT-MCKEY, M., GREEFF, J. M., and BESSIÈRE, J. -M. 2002b. Fig volatile compounds—a first comparative study. *Phytochemistry* 61:61–71.

HILL, D. S. 1967. Fig (*Ficus* spp.) and fig-wasps (Chalcidoidea). *J. Nat. Hist.* 1:413–434.

HOBALLAH, M. E. F., TAMO, C., and TURLINGS, T. C. J. 2002. Different attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important? *J. Chem. Ecol.* 28:951–968.

HOSSAERT-MCKEY, M., GIBERAU, M., and FREY, J. E. 1994. Chemosensory attraction of fig-wasps to substances produced by receptive figs. *Entomol. Exp. Appl.* 70:185–191.

JANZEN, D. H. 1979. How to be a fig. *Annu. Rev. Ecol. Syst.* 10:13–51.

KNUDSEN, J. T., TOLLSTEN, L., and BERGSTRÖM, G. 1993. Floral scents—a checklist of volatile compounds isolated by head-space thechniques. *Phytochemistry* 33:253–280.

KNUDSEN, J. T., ERIKSSON, R., GERSHENZON, J., and STÅHL, B. 2006. Diversity and distribution of floral scent. *Bot. Rev.* 72:1–120.

MOORE, J. C., HATCHER, M. J., DUNN, A. M., and COMPTON, S. G. 2003. Fig choice by the pollinator of a gynodioecious fig: selection to rush, or intersexual mimicry? *Oikos* 101:180–186.

PATEL, A. 1996. Variation in a mutualism: phenology and the maintenance of gynodioecy in two Indian fig species. *J. Ecol.* 84:667–680.

PATEL, A., and HOSSAERT-MCKEY, M. 2000. Components of reproductive success in two dioecious fig species, *Ficus exasperate* and *Ficus hispida*. *Ecology* 81:2850–2866.

PATEL, A., ANSTETT, M. -C., HOSSAERT-MCKEY, M., and KJELLBERG, F. 1995. Pollinators entering female dioecious figs: why commit suicide? *J. Evol. Biol.* 8:301–313.

PATT, J. M., FRENCH, J. C., SCHAL, C., LECH, J., and HARTMAN, T. G. 1995. The pollination biology of Tuckahoe, *Peltandra virginica* (Araceae). *Am. J. Bot.* 82:1230–1240.

PELLMYR, O., and THIEN, L. B. 1986. Insect reproduction and floral fragrances: keys to the evolution of the angiosperms? *Taxon* 35:76–85.

PENG, Y. Q., YANG, D. R., and WANG Q. Y. 2005. Quantitative tests of interaction between pollinating and non-pollinating fig wasps on dioecious *Ficus hispida*. *Ecol. Entomol.* 30:70–77.

PRIMACK, R. B. 1985. Longevity of individual flowers. *Annu. Rev. Ecol. Syst.* 16:15–38.

PROFFIT, M., SCHATZ, B., BESSIÈRE, J. -M., CHEN, C., SOLER, C., and HOSSAERT-MCKEY, M. 2008. Signalling receptivity: comparison of the emission of volatile compounds by fig of *Ficus hispida* before, during and after the phase of receptivity to pollinators. *Symbiosis* 45:15–24.

RAGUSO, R. A. 2001. Floral scent, olfaction, and scent-driven foraging behavior, pp. 83–105, in J. D. Thomson, and L. Chittka (eds.). Cognitive Ecology of Pollination: Animal Behaviour and Floral EvolutionCambridge University Press, Cambridge.

RAMÍREZ, B. W. 1974. Coevolution of *Ficus* and Agaonidae. *Ann. Mo. Bot. Gard.* 61:770–780.

SCHIESTL, F. P., and AYASSE, M. 2001. Post-pollination emission of a repellent compound in a sexually deceptive orchid: a new mechanism for maximizing reproductive success? *Oecologia* 126:531–534.

SCHIESTL, F. P., AYASSE, M., PAULUS, H. F., ERDMANN, D., and FRANCKE, W. 1997. Variation of floral scent emission and postpollination changes in individual flowers of *Ophrys sphegodes* subsp. *Sphegodes*. *J. Chem. Ecol.* 23:2881–2895.

SONG, Q. S., YANG, D. R., ZHANG, G. M., and YANG, C. R. 2001. Volatiles from *Ficus hispida* and their attractiveness to fig wasps. *J. Chem. Ecol.* 27:1929–1942.

THEIS, N., and RAGUSO, R. A. 2005. The effect of pollination on floral fragrance in thistles. *J. Chem. Ecol.* 31:2581–2600.

TOOKER, J. F., CRUMRIN, A. L., and HANKS, L. M. 2005. Plant volatiles are behavioral cues for adult females of the gall wasp *Antistrophus rufus*. *Chemoecology* 15:85–88.

VAN DER PIJL, L. 1961. Ecological aspects of flower evolution. II. Zoophilous flower classes. *Evolution* 15:44–59.

VAN DOORN, W. G. 1997. Effects of pollination on floral attraction and longevity. *J. Exp. Bot.* 48:1615–1622.

VAN NOORT, S., WARE, A. B., and COMPTON, S. G. 1989. Pollinator specific volatile attractants released from the figs of *Ficus burtt-davyi*. *S. Afr. J. Sci C.* 85:323–324.

VERKERKE, W. 1989. Structure and function of the fig. *Experientia* 45:612–622.

VISSER, J. H. 1986. Host odor perception in phytophagous insects. *Annu. Rev. Entomol.* 31:121–144.

VISSER, J., and AVÉ, D. 1978. General green leaf volatiles in the olfactory orientation of the Colorado beetle, *Leptinotarsa decemlineata*. *Entomol. Exp. Appl.* 24:538–549.

WARE, A. B., and COMPTON, S. G. 1992. Breakdown of pollinator specificity in an African fig tree. *Biotropica* 24:544–549.

WARE, A. B., and COMPTON, S. G. 1994. Responses of fig wasps to host plant volatile cues. *J. Chem. Ecol.* 20:785–802.

WARE, A. B., COMPTON, S. G., KAYE, P. T., and VAN NOORT, S. 1993. Fig volatiles: their role in attracting pollinators and maintaining pollinator specificity. *Plant Syst. Evol.* 186:147–156.

WEISS, M. R. 1991. Floral colour changes as cues for pollinators. *Nature* 354:227–229.

WIEBES, J. T. 1979. Co-evolution of figs and their insect pollinators. *Annu. Rev. Ecol. Syst.* 10:1–12.

The impact of herbivory is dependent on the relative density and species composition of browsers (Mysterud 2006). Both herbivore density and plant damage can be either site-specific or change seasonally, and even spatially. Larger-bodied mammals mostly affect the vegetation around farms and corrals where overgrazing is common (Wezel and Bender 2004). Large-scale variation in plant damage is correlated with herbivore density (Tsujino and Yumoto 2004). Herbivores that live in certain patch types may cause extensive damage in nearby plant associations as rabbits do in grasslands that surround junipers (Villafuerte and Moreno 1997), thus resulting in short-scale patchiness.

In previous studies, we focused on the juniper–poplar forests of a sandy region of Hungary. The differences in vegetation was attributed partly to local and seasonal changes in browsing pressure (Kertesz et al. 1993) by either the European rabbit (*Oryctolagus cuniculus*) or domestic sheep (*Ovis aries*), as the density of other browsers was negligible in the juniper forests (Katona and Altbacker 2002). Earlier studies had shown that browsing habits of the main herbivores differed by sites, as junipers (*Juniperus communis*) were browsed by rabbits at the Bugac site during the winter period exclusively (Matrai et al. 1998) and by sheep but not rabbits at the Orgovány site. Junipers were seemingly intact at the Bócsa site, even though both rabbits and sheep were present in the area. Traditional sheep grazing, mostly occurring from pens surrounding the forest, was restricted to the spring and autumn periods, and sheep grazing was discontinued by the authorities of Kiskunság National Park in 2001. This change in the management and recent collapse of the rabbit population (Katona and Altbacker 2002) resulted in reduced browsing pressure. As juniper shrubs regenerate slowly, comparison of the herbivory signs in local juniper stands has enabled us to evaluate the distribution of PSMs without current confounding impacts, such as induced responses in damaged branches (Nagy et al. 2000; Miller et al. 2005). Assuming that present plant damage still reflects previous browsing pressure, we describe the spatial and temporal pattern of the morphological and chemical constituents. The following hypotheses were tested: (1) the level of juniper damage should correlate negatively with essential oil yield; (2) oil yield should change by season in accordance with browsing pressure; (3) heavily browsed and nonbrowsed junipers should differ in their chemical composition; (4) browsing damage will reveal the main browser: it should be lowest in the middle of the forest if junipers were eaten by sheep or highest if rabbits were the main agents shaping the shrubs; and (5) long-term differences in local browsing pressure will be reflected in site-specific age distributions of juniper populations.

Methods and Materials

Study Area

This study was conducted in the sand dunes of the Kiskunság National Park in central Hungary (Fig. 1). Mosaics of juniper patches and intermittent sandy grasslands form the semidesert vegetation were categorized as juniper–poplar forests (Kertesz et al. 1993). The focus area of Orgovány (46°47′30″ N; 19°27′34″ E) was chosen for analyzing herbivore–juniper interactions. For a broader-scale analysis, we selected two additional shrubland areas: Bugac (46°38′58″ N; 19°36′38″ E) and Bócsa (46°38′56″ N; 19°27′31″ E) where the age distribution of juniper stands was analyzed for a comparison (see below).

Classification of Browsing Damage Level

The shape of damaged junipers was studied at the area of Orgovány (see Fig. 1). Browsing damage was classified from the ground to a height of 100 cm on a scale of 1–5 from nonbrowsed to heavily browsed (Fig. 2). Junipers were labeled as nonbrowsed when damage (shape distortion) was between 0% and 20%; mildly browsed when damaged ranged between 21% and 40%; medium-browsed when damage was between 41% and 60%; well-browsed when damage was between 61% and 80%; and heavily browsed

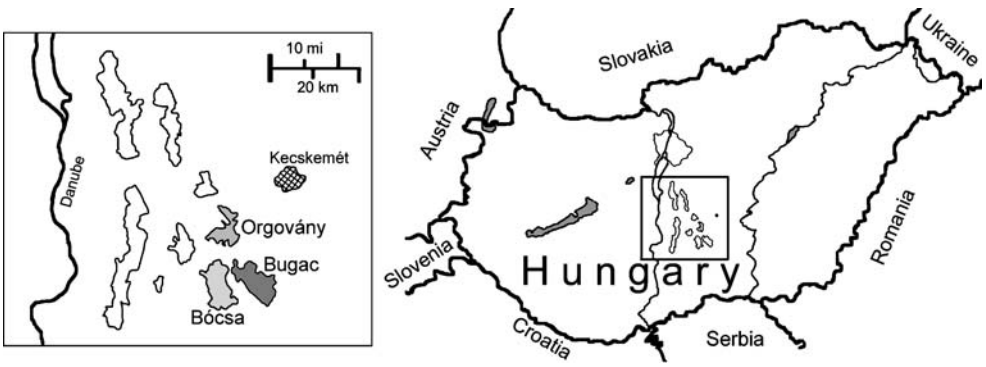

Fig. 1 Studies were conducted in the Orgovány, Bugac, and Bócsa areas of Kiskunság National Park, Hungary

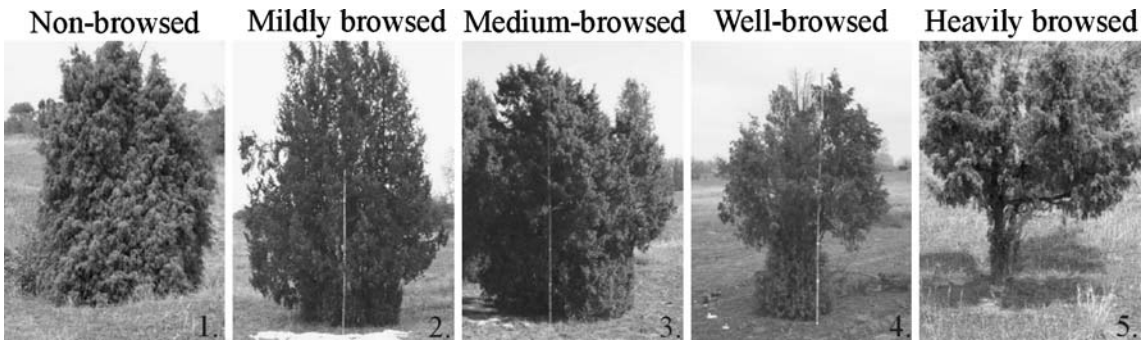

Fig. 2 Classification of browsing damage level of junipers. Pictures show *1* nonbrowsed (damage between 0% and 20%); *2* mildly browsed (damage between 21% and 40%); *3* medium-browsed (damage between 41% and 60%); *4* well-browsed (damage between 61% and 80%); *5* heavily browsed (damage between 81% and 100%) shrubs

if damage were between 81% and 100% of the bottom part of the shrub.

Seasonal Pattern of Essential Oil Yield

Ten randomly chosen, independent juniper shrubs were chosen in July and October of 2003 and February and May of 2004. Two nonadjacent shrubs were selected per damage level category. The height of these shrubs (2–2.5 m) and their gender (female) were similar. Samples consisting of 10 cm long tips of new branches were collected from the same height (about 1 m) from each shrub (100–120 g of foliage after removal of berries). Plant material was placed into plastic bags, stored in a cooler (5°C) for transport, and then oven dried at 37±3°C (Memmert U-type oven). The method described by Pharmacopoea Hungarica (Vegh 1986) was used to extract the essential oil except that the steam distillation time was reduced to 1.5 h because of the negligible (less than 5% of the total) additional yield afterwards (Bernath, unpublished data). Dried plant material (40–50 g), chopped into 2–3 cm pieces, was distilled with a Clevenger apparatus constructed according to Vegh (1986). Statistical analysis consisted of two-way repeated-measures analysis of variance (ANOVA) with damage level as a fixed factor and season as a repeated factor.

Essential Oil Composition

Plant material was collected during February 2004 from the Orgovány area. Heavily browsed and nonbrowsed shrubs (N=10 and 10, respectively) were selected randomly from the most browsed part of the forest. The terminal 10–15 cm of foliage was clipped from several branches between ground level and 2 m height. Sample preparation and extraction of essential oil was as described above. The chemical composition of essential oils extracted from the above 10 heavily browsed and 10 nonbrowsed shrubs was analyzed with a Shimadzu 14B gas chromatograph equipped with a FID detector and SE-30 capillary column (30 m×0.25 mm and 0.25 mm film thickness). The carrier gas was nitrogen (1 mL min^{-1}), and the split ratio was 75/1. The oven temperature program was as follows: 110°C for 3 min, up to 220°C at 8°C min^{-1} (5 min), 21.75 min analysis time, IB 220°C, and detection 250°C. Data were analyzed using the Shimadzu Class VP Chromatography Data System (Shimadzu, Japan) software. The area normalization method (Novak et al. 2001, 2003) was used to determine the relative percentage of oil constituents. Identification of compounds was performed by peak enrichment with gas chromatography (GC) standards by (Carl Roth; Karlsruhe, Germany). Student's t tests were used to test the statistical differences in essential oil components of heavily browsed and nonbrowsed samples.

Effect of Grazing Pressure

The spatial distribution of grazing damage was analyzed at the Orgovány site (see Fig. 1) where rabbits disappeared in 1995 and traditional sheep grazing was discontinued in 2001. Three plots (approximately 100 m in diameter) were defined along a transect running between the last active sheep pen found at the border of the juniper forest (N=100) and the middle of the juniper forest (N=100). Within each plot, the shape of junipers was classified by using a spiral sampling from the center point. We expected a decreasing trend if sheep were the most important agents shaping the shrubs, an opposite trend if rabbits browsed most intensively, or an inverse U-shaped spatial pattern if both species contributed to browsing damage. The Mantel–Haenszel χ^2 test was used to analyze data.

Long-Term Consequences of Juniper Browsing

The age distribution of juniper shrubs was evaluated in three separate forests (Bugac, Bócsa, and Orgovány) in May 2003. The basal diameter of the stems from plants at the border of the juniper forest (N=100) and the middle of

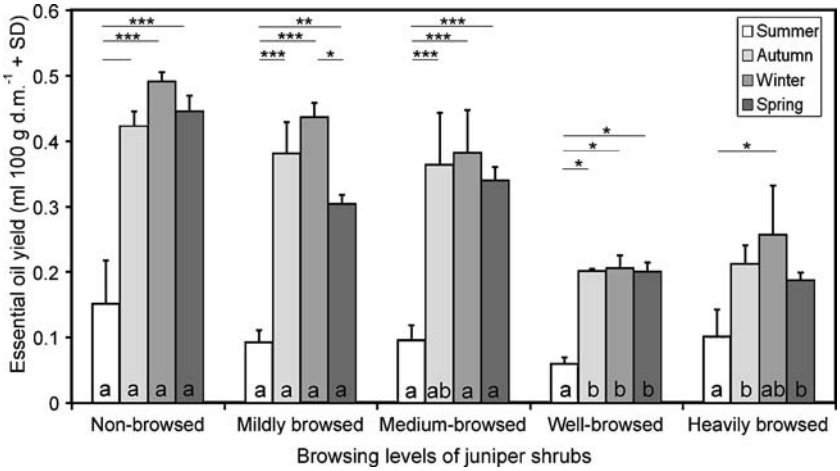

Fig. 3 Seasonal pattern of juniper essential oil yield (N=2–2) belonging to different browsing damage levels. Mean values were tested for significant differences (P<0.05) by two-way repeated-measures ANOVA followed by Duncan's post hoc tests for all pairwise comparisons. *Different letters* indicate significant differences among damage level categories, while *P<0.05; **P<0.01; ***P<0.001 are significant differences among seasons

the juniper forest (N=100) was measured at each site around 5–5 randomly chosen points by using spiral sampling (altogether 500 individuals per area). Stem diameter was measured with a slide caliper (MEBA, Croatia) at a height of 10 cm. Polished cross-sections of dead junipers (N=13) of different diameters were prepared to count annual rings for age determination. Data were compared by site as a fixed factor and age categories as a repeated factor with two-way repeated-measures ANOVA followed by Duncan's post hoc tests.

Results

Seasonal Pattern of Essential Oil Yield of Differently Browsed Junipers

The relationship between oil yield, damage level, and seasonal pattern is shown in Fig. 3. Damage level was inversely proportional to essential oil yield. Analysis of oil yield, done with a two-way repeated-measures ANOVA and Duncan's post hoc test, indicated a significant effect of browsing level (F(4,5)=5.64, P=0.027) and season (F(3,15)=48.24, P<0.001) without indication of a significant interaction (F(12,15)=1.70, P=0.164). This suggests a similar seasonal change in the different browsing level categories. Oil yield was lower in the summer period than in the other seasons.

Essential Oil Components of Heavily Browsed and Nonbrowsed Juniper Shrubs

Essential oil components of heavily browsed and non-browsed juniper shrubs (Fig. 4) were tested with Student's t tests using the Welch correction). Significant differences were found between the following chemical constituents of the essential oil: α-pinene (t(10)=2.187, P=0.05); δ-3-carene (t(9)=3.061, P=0.01); β-myrcene (t(17)=2.111, P=0.04); and other unidentified components designated by their retention time 11.14 (t(12)=2.488, P=0.02) and 12.22 (t(12)=2.405, P=0.03). The concentration of some chemicals was lower (δ-3-carene and β-myrcene) in the browsed shrubs than in the undamaged junipers. In some cases, they were higher (α-pinene).

Browsed Shrubs and Herbivore Pressure

The frequency of browsed shrubs was compared among three plots (Fig. 5). There was a decrease in the ratio of heavily browsed shrubs toward the center of the shrubland (Mantel–Haenszel χ^2 test for linear trend: $\chi^2(2)=46.143$; P<0.001). The majority of shrubs was browsed on the plot closest to the formerly active sheep pen, indicating a high browsing pressure by sheep. Deep in the forest, damage was less evident, suggesting that it was mainly sheep browsing rather than rabbits that contributed to the shaping of the junipers at Orgovány.

Long-Term Consequences of Juniper Browsing

We estimated the age of junipers by measuring their stem diameter. The linear regression equation (y=0.386x+4.97) describes a significant stem–age function (R^2=0.87, P<0.001) and was used to estimate age in Fig. 6. We compared the frequency distributions by using a two-way repeated-measures ANOVA followed by Duncan's post hoc tests for all pairwise comparisons and found that age distributions were different among the areas (F(2,12)=0.42, P<0.001), age classes (F(7,84)=0.28, P<0.001), and areas×age classes (F(14,84)=0.11, P<0.001). Further analyses of site differences within age classes showed that the juniper stand at Bugac is different from the other two populations. There was a smaller proportion of age classes of juniper less than 20 years, indicating that Bugac lacks young individuals.

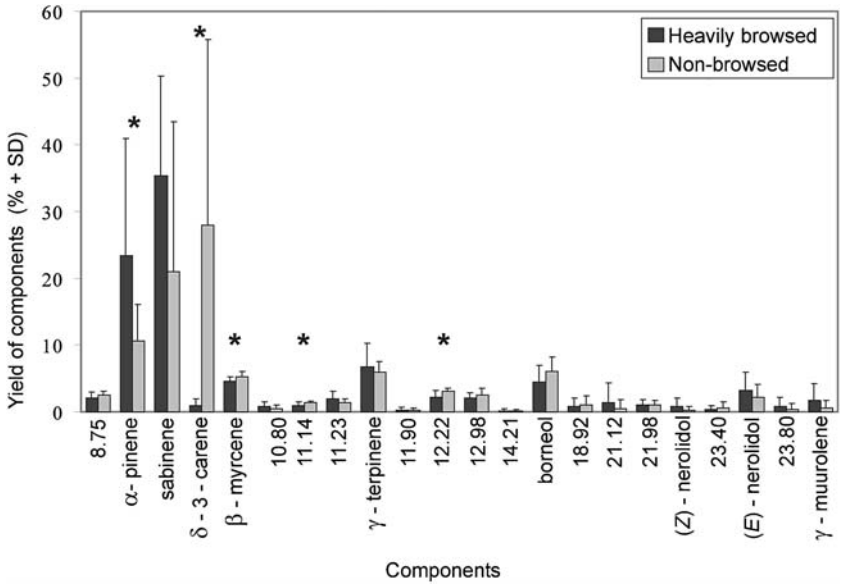

Fig. 4 Quantification of essential oil components of heavily browsed ($N=10$) and non-browsed juniper ($N=10$) shrubs from the Orgovány area as determined by GC. The identification of the oil components was based on a comparison of GC standards (Carl Roth; Karlsruhe, Germany). Component percentages were computed from GC (FID) peak areas by using the area normalization method. *Bars* represent the mean values+SD. Statistical difference of essential oil components of heavily browsed and nonbrowsed samples was verified by Student's *t* tests with Welch correction. *$P<0.05$, significant differences

This indicates that junipers at Bugac are older than the other two stands. Junipers at Bócsa and Orgovány seem to form stable populations with a linear decrease by age.

Discussion

It has been assumed that plant damage investigated at different temporal and spatial scales reflects selective herbivory for juniper forests in Hungary. The level of juniper damage was tested for a possible correlation with PSM concentration and the seasonal changes in browsing pressure, as plant damage can influence PSM concentrations in response to herbivore density (Tsujino and Yumoto 2004). Assuming that both the appearance and chemical composition of shrubs reflects grazing pressure, we found support for several predictions of plant–animal interaction. A diminishing impact toward the middle of the forests was expected in areas of sheep browsing damage. In contrast, an opposite spatial distribution of grazing damage should be found in rabbit-dense areas where damage is concentrated deep in the forest, as rabbits feed around their warrens to avoid open areas (Moreno et al. 1996). The level of damage was highest near a sheep pen (the activity center for the pastoral grazing system). There was no evidence of rabbit browsing in this location (Fig. 5).

Variation in the shape of juniper shrubs corresponded to their chemical composition (Fig. 4) with truncated shrubs containing the lowest oil yield. This indicates that PSMs might have led to avoidance of the undamaged shrubs (Provenza et al. 2003). Concentrations of volatile oils varied among junipers and seasons (Fig. 3). Even though our simplified method of essential oil extraction could underestimate the total oil yield (Venskutonis 1997;

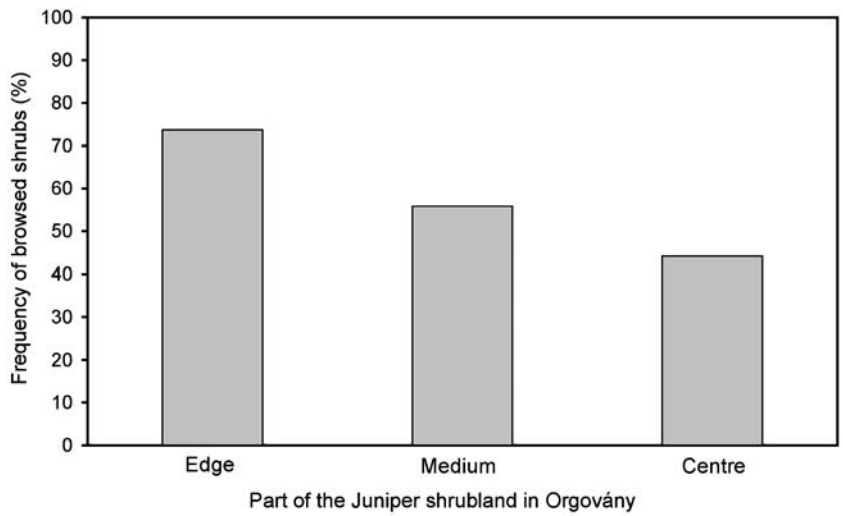

Fig. 5 Spatial distribution of damaged junipers along an axis from the edge to center of the juniper shrubland at Orgovány. *Bars* represent the frequency of juniper shrubs with any signs of browsing determined in 1 ha plots. In each plot, 100 individuals were classified. Mantel–Haenszel χ^2 test for linear trends showed damage frequency to decrease from edge to center, indicating that distance from the sheep fold was more important than presence of rabbits in determining browsing damage

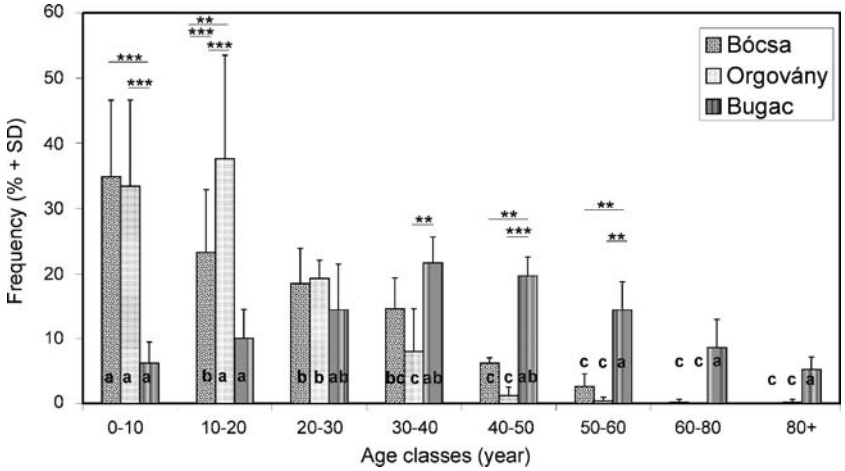

Fig. 6 Age structure of *J. communis* populations in the three study areas (Bócsa, Orgovány, and Bugac) of Kiskunság National Park, represented as the percentage of individuals belonging to the corresponding age class. In each area, the age distribution was determined from five different plots where stem diameter of the individuals at the border of the juniper forest (N=100) and the middle of the juniper forest (N=100) was measured. Age was determined by using the regression equation y=0.386x+4.97 based on counting age rings for 13 reference stems collected in the same locality. Mean values were tested for significant differences (P<0.05) by two-way repeated-measures ANOVA followed by Duncan's post hoc tests for all pairwise comparisons. Different letters indicate significant differences among age classes, while *P<0.05; **P<0.01; ***P<0.001 are significant differences among areas

Milojevic et al. 2008), differences among shrubs that show high or low damage levels are still meaningful. Certain chemicals might have contributed to reduced consumption, as browsed junipers contained essential oil with elevated levels of terpenoids (Fig. 4) similar to conifers (Vourc'h et al. 2001, 2002). In our case, the most frequent constituents, α-pinene and sabinene, were even more concentrated in the damaged plants than in those that were avoided. It should be noted that plants with low levels of α-pinene were rich (i.e., had high levels) of δ-3-carene, β-myrcene, and borneol, and whose concentration was high in damaged shrubs. This suggests that the complex composition of the oil, and not the concentration of a single component, may explain the variation in herbivore damage. Sheep avoid food when exposed to α-pinene or a mixture of monoterpenes (Narjisse et al. 1996), and lambs exposed to camphor and α-pinene reduced their food intake (Estell et al. 2005). The strong selective pressure exerted by black-tailed deer (*Odocoileus hemionus sitkensis*) contributed to the persistence of individual seedlings with high concentrations of chemical defenses (Vourc'h et al. 2001). In semiarid environments similar to our study site, aromatic plants often contain high monoterpenoid levels that can interfere with digestion (Carpenter et al. 1979). A tenfold increase in δ-3-carene was found in nonbrowsed junipers of Orgovány. In other studies, monoterpenes were either positively (e.g., cymene) or negatively (e.g., α-pinene, sabinene, β-pinene, myrcene, terpineol) correlated with goats' willingness to ingest juniper (Riddle et al. 1996). Black-tailed deer were repelled by α-pinene, myrcene, and sabinene (Vourc'h et al. 2002). The antiherbivore defense of red cedar (*Thuja plicata*) was directly proportional to the level of herbivory, as the essential oil yield and quality were adapted to the feeding pressure (Vourc'h et al. 2001).

PSM production may be seasonally coupled with herbivore browsing. Seasonal changes in essential oils have been documented for many coniferous species (Nerg et al. 1994; Riddle et al. 1996). Owens et al. (1998) reported a strong seasonal influence on volatile oil concentration in the leaves of ashe juniper (*Juniperus ashei*) and redberry juniper (*Juniperus pinchottii*). Essential oil concentrations were highest during the winter and spring months (January through May) and lowest in summer and fall. Similarly, the essential oil in thyme (*Thymus vulgaris*) was highest in midsummer (Badi et al. 2004) when browsing by goats is most intensive (Riddle et al. 1996). Production of volatile terpenoids (e.g., monoterpenes, sesquiterpenes, and homoterpenes) can be induced by herbivore feeding (Arimura et al. 2005), a mechanism that is well-studied in conifers (Trapp and Croteau 2001; Martin et al. 2003; McKay et al. 2003; Miller et al. 2005). In the present study, juniper oil had the lowest concentration during the summer period. The high concentration of essential oils in other seasons was remarkable. Due to management practices, sheep were present in the spring and autumn periods. Based on fecal analyses, rabbits most likely browsed junipers in winter (Matrai et al. 1998). As browsing by the main browsers were no longer present in the study area in the present sampling period, the induced plant protection system known for conifers was unlikely to be responsible for the seasonal changes observed. It is more likely that preexisting individual differences in PSMs production resulted in various levels of browsing damage, and that was still obvious at the time of sampling due to slow growth of junipers.

Differences in age distribution of junipers were expected among the areas due to local herbivores that affect the survival of shrubs. The difference in age distributions of compared stands might stem from herbivore species composition and foraging behavior of local herbivores. The foraging habits of European rabbits resulted in a high mortality of young shrubs at Bugac (Matrai et al. 1998), and age distribution of juniper stands reflects that impact (see Fig. 6). In spite of the recent browsing by sheep, the age distribution of the juniper population in Orgovány has remained the same as in Bócsa where neither sheep nor rabbits consumed juniper. Sheep forage mainly in the grassy patches and only start eating juniper when grass becomes scarce. The occasional browsing of tall junipers from the side did not affect the survival of junipers, but did modify their morphology. Rabbits, on the contrary, forage in well-hidden places close to shrubs and not only browse the bottom twigs of older plants, but can destroy young junipers as well, as our former field experiment proved (Matrai et al. 1998). For renewal of the juniper stands, it is essential that seedlings survive, especially as *J. communis* becomes fertile only after several years, and aging results in reduced fertility (Ward 2007). The long-term accumulation of such impacts could have resulted in the distorted age distribution of junipers at Bugac.

Herbivores may circumvent the negative effects of plants by physiological (Dearing et al. 2000) or behavioral means (Provenza 1995). The effect of plant metabolites can be reduced by several strategies; for example, certain herbivores can avoid tannin-containing plants by learned avoidance (Provenza 1995), and selective browsing minimizes the intake of harmful plants (Launchbaugh et al. 2001). Therefore, within the same plant population, individuals with a higher concentration of PSMs may enjoy better protection (Bryant et al. 1985). Juniper browsing by mule deer (*Odocoileus hemionus*) was inversely correlated to the essential oil yield (Schwartz et al. 1980a). This may be attributed to the oxygenated monoterpenes and sesquiterpenes in the oil that inhibit the microbial activity in the deer's rumen after ingestion of a certain number of branches (Schwartz et al. 1980b). As Welch et al. (1983) showed, PSM concentration was not the only determinant of food intake. More detailed studies are necessary.

Signs of a varying amount of juniper consumption were found by site and season. Such differences might stem either from plant toxicity, feeding responses of animals, or an interaction of both (Iason and Villalba 2006). Thyme was one of the main components of the summer diet of the Bugac rabbits (Matrai et al. 1998) and naive animals in captivity consumed small amounts if their mother was forced to eat thyme despite its toxicity (Altbacker et al. 1995). Rabbits could adjust their diet both by individual and social learning (Bilko et al. 1994). With a mixed diet, the accumulation of certain PSMs can be avoided and consumption increased (Marsh et al. 2006), and thereby the energy and toxin intake optimized (Bryant et al. 1985, 1992; Provenza et al. 2003) as can be observed in several generalist vertebrates. Nevertheless, some specialists are physiologically better adapted to ecological constraints and have a more efficient detoxification system than generalists (Dearing et al. 2000). Experimental manipulation of juniper chemical composition in food choice tests are currently underway in order to reveal how oil content and carene concentration reduce juniper consumption. Further studies that use different herbivore species and manipulate experience levels when alternative food sources are present would be valuable (Dearing et al. 2000; Villalba and Provenza 2005). Substantial changes in the grazing regime are considered as the major force of succession in semidesert shrub lands (Evans et al. 2006). Understanding the mechanisms that contribute to the interaction of plant defenses and browsing behavior of local herbivores is increasingly important.

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References

ALTBACKER, V., HUDSON, R., and BILKO, A. 1995. Rabbit-mothers diet influences pups later food choice. *Ethology* 99:107–116.

ARIMURA, G., KOST, C., and BOLAND, W. 2005. Herbivore-induced, indirect plant defences. *Biochim. Biophys. Acta* 1734:91–111.

BADI, H. N., YAZDANI, D., ALI, S. M., and NAZARI, F. 2004. Effects of spacing and harvesting time on herbage yield and quality/quantity of oil in thyme, *Thymus vulgaris* L. *Ind. Crops Prod* 19:231–236.

BILKO, A., ALTBACKER, V., and HUDSON, R. 1994. Transmission of food preference in the rabbit-the means of information-transfer. *Physiol. Behav* 56:907–912.

BRYANT, J. P., REICHARDT, P., and CLAUSEN, T. 1985. Plant carbon/nutrient balance-implications for chemical defense. *Abstr. Pap. Am. Chem. S* 190:91.

BRYANT, J. P., REICHARDT, P. B., and CLAUSEN, T. P. 1992. Chemically mediated interactions between woody-plants and browsing mammals. *J. Range Manag* 45:18–24.

CARPENTER, L. H., WALLMO, O. C., and GILL, R. B. 1979. Forage diversity and dietary selection by wintering mule deer. *J. Range Manag* 32:226–229.

DEARING, M. D., MANGIONE, A. M., and KARASOV, W. H. 2000. Diet breadth of mammalian herbivores: nutrient versus detoxification constraints. *Oecologia* 123:397–405.

DORMAN, H. J. D., and DEANS, S. G. 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol* 88:308–316.

ESTELL, R. E., FREDRICKSON, E. L., ANDERSON, D. M., HAVSTAD, K. M., and REMMENGA, M. D. 2005. Effect of previous exposure

of sheep to monoterpene odors on intake of alfalfa pellets treated with camphor or alpha-pinene. *Small Rumin. Res* 58:33–38.

EVANS, D. M., REDPATH, S. M., ELSTON, D. A., EVANS, S. A., MITCHELL, R. J., and DENNIS, P. 2006. To graze or not to graze? Sheep, voles, forestry and nature conservation in the British uplands. *J. Appl. Ecol* 43:499–505.

FREELAND, W. J., and JANZEN, D. H. 1974. Strategies in herbivory in mammals: the role of plant secondary compounds. *Am. Nat* 110:269–289.

GOMEZ, D., AZORIN, J., BASTIDA, J., VILADOMAT, F., and CODINA, C. 2003. Seasonal and spatial variations of alkaloids in *Merendera montana* in relation to chemical defense and phenology. *J. Chem. Ecol* 29:1117–1126.

HAUKIOJA, E., and NEUVONEN, S. 1985. Induced long-term resistance of birch foliage against defoliators—defensive or incidental. *Ecology* 66:1303–1308.

IASON, G. R., and VILLALBA, J. J. 2006. Behavioral strategies of mammal herbivores against plant secondary metabolites: the avoidance–tolerance continuum. *J. Chem. Ecol* 32:1115–1132.

KARAMAN, S., DIGRAK, M., RAVID, U., and ILCIM, A. 2001. Antibacterial and antifungal activity of the essential oils of *Thymus revolutus* Celak from Turkey. *J. Ethnopharmacol* 76:183–186.

KATONA, K., and ALTBACKER, V. 2002. Diet estimation by faeces analysis: sampling optimisation for the European hare. *Folia Zool* 51:11–15.

KERTESZ, M., SZABO, J., and ALTBACKER, V. 1993. The Bugac rabbit project. Part I: description of the study site and vegetation map. *Abstr. Bot* 17:187–196.

LAUNCHBAUGH, K. L., PROVENZA, F. D., and PFISTER, J. A. 2001. Herbivore response to anti-quality factors in forages. *J. Range Manag* 54:431–440.

MARSH, K. J., WALLIS, I. R., MCLEAN, S., SORENSEN, J. S., and FOLEY, W. J. 2006. Conflicting demands on detoxification pathways influence how common brushtail possums choose their diets. *Ecology* 87:2103–2112.

MARTIN, D., BOHLMANN, J., GERSHENZON, J., FRANCKE, W., and SEYBOLD, S. J. 2003. A novel sex-specific and inducible monoterpene synthase activity associated with a pine bark beetle, the pine engraver, *Ips pini*. *Naturwissenschaften* 90:173–179.

MATRAI, K., ALTBACKER, V., and HAHN, I. 1998. Seasonal diet of rabbits and their browsing effect on juniper in Bugac Juniper Forest (Hungary). *Acta Theriol* 43:107–112.

MCKAY, S. A. B., HUNTER, W. L., GODARD, K. A., WANG, S. X., MARTIN, D. M., BOHLMANN, J., and PLANT, A. L. 2003. Insect attack and wounding induce traumatic resin duct development and gene expression of (−)-pinene synthase in Sitka spruce. *Plant Physiol* 133:368–378.

MILLER, B., MADILAO, L. L., RALPH, S., and BOHLMANN, J. 2005. Insect-induced conifer defense. White pine weevil and methyl jasmonate induce traumatic resinosis, de novo formed volatile emissions, and accumulation of terpenoid synthase and putative octadecanoid pathway transcripts in Sitka spruce. *Plant Physiol* 137:369–382.

MILOJEVIC, S. Z., STOJANOVIC, T. D., PALIC, R., LAZIC, M. L., and VEJKOVIC, V. B. 2008. Kinetics of distillation of essential oil from comminuted ripe juniper (*Juniperus communis* L.) berries. *Biochem. Eng. J* 39:547–553.

MORENO, S., VILLAFUERTE, R., and DELIBES, M. 1996. Cover is safe during the day but dangerous at night: the use of vegetation by European wild rabbits. *Can. J. Zool* 74:1656–1660.

MYSTERUD, A. 2006. The concept of overgrazing and its role in management of large herbivores. *Wildlife Biol* 12:129–141.

NAGY, N. E., FRANCESCHI, V. R., SOLHEIM, H., KREKLING, T., and CHRISTIANSEN, E. 2000. Wound-induced traumatic resin duct development in stems of Norway spruce (Pinaceae): anatomy and cytochemical traits. *Am. J. Bot* 87:302–313.

NARJISSE, H., MALECHEK, J. C., and OLSEN, J. D. 1996. Influence of odor and taste of monoterpenoids on food selection by anosmic and intact sheep and goats. *Small Rumin. Res* 23:109–115.

NERG, A., KAINULAINEN, P., VUORINEN, M., HANSO, M., HOLOPAINEN, J. K., and KURKELA, T. 1994. Seasonal and geographical variation of terpenes, resin acids and total phenolics in nursery grown seedlings of Scots Pine (*Pinus sylvestris* L). *New Phytol* 128:703–713.

NOVAK, I., ZAMBORI-NEMETH, E., HORVATH, H., SEREGELY, Z., and KAFFKA, K. 2001. Evaluation of essential oils by gas-chromatography and a new method: "electronic nose". *J. Hortic. Sci* 7:85–89.

NOVAK, I., ZAMBORI-NEMETH, E., HORVATH, H., SEREGELY, Z., and KAFFKA, K. 2003. Study of essential oil components in different *Origanum* species by GC and sensory analysis. *Acta Aliment* 32:141–150.

OWENS, M. K., LIN, C. D., TAYLOR, C. A., and WHISENANT, S. G. 1998. Seasonal patterns of plant flammability and monoterpenoid content in *Juniperus ashei*. *J. Chem. Ecol* 24:2115–2129.

PROVENZA, F. D. 1995. Postingestive feedback as an elementary determinant of food preference and intake in ruminants. *J. Range Manag* 48:2–17.

PROVENZA, F. D., VILLALBA, J. J., DZIBA, L. E., ATWOOD, S. B., and BANNER, R. E. 2003. Linking herbivore experience, varied diets, and plant biochemical diversity. *Small Rumin. Res* 49:257–274.

RIDDLE, R. R., TAYLOR, C. A., KOTHMANN, M. M., and HUSTON, J. E. 1996. Volatile oil contents of ashe and redberry juniper and its relationship to preference by Angora and Spanish goats. *J. Range Manag* 49:35–41.

SCHWARTZ, C. C., NAGY, J. G., and REGELIN, W. L. 1980a. Juniper oil yield, terpenoid concentration, and anti-microbial effects on deer. *J. Wildl. Manage* 44:107–113.

SCHWARTZ, C. C., REGELIN, W. L., and NAGY, J. G. 1980b. Deer preference for juniper forage and volatile oil treated foods. *J. Wildl. Manage* 44:114–120.

TRAPP, S., and CROTEAU, R. 2001. Defensive resin biosynthesis in conifers. *Annu. Rev. Plant Physiol* 52:689–724.

TSUJINO, R., and YUMOTO, T. 2004. Effects of sika deer on tree seedlings in a warm temperate forest on Yakushima Island, Japan. *Ecol. Res* 19:291–300.

VEGH, A., editor. 1986. Pharmacopoea Hungarica. VII. ed. Medicina, Budapest. pp. 395–398.

VENSKUTONIS, P. R. 1997. Effect of drying on the volatile constituents of thyme (*Thymus vulgaris* L) and sage (*Salvia officinalis* L). *Food Chem* 59:219–227.

VILLAFUERTE, R., and MORENO, S. 1997. Predation risk, cover type, and group size in European rabbits in Donana (SW Spain). *Acta Theriol* 42:225–230.

VILLALBA, J. J., and PROVENZA, F. D. 2005. Foraging in chemically diverse environments: energy, protein, and alternative foods influence ingestion of plant secondary metabolites by lambs. *J. Chem. Ecol* 31:123–138.

VOURC'H, G., MARTIN, J. L., DUNCAN, P., ESCARRE, J., and CLAUSEN, T. P. 2001. Defensive adaptations of *Thuja plicata* to ungulate browsing: a comparative study between mainland and island populations. *Oecologia* 126:84–93.

VOURC'H, G., DE GARINE-WICHATITSKY, M., LABBE, A., ROSOLOWSKI, D., MARTIN, J. L., and FRITZ, H. 2002. Monoterpene effect on feeding choice by deer. *J. Chem. Ecol* 28:2411–2427.

WARD, L. K. 2007. Lifetime sexual dimorphism in *Juniperus communis* var. *communis*. *Plant Species Biol* 22:11–21.

WELCH, B. L., MCARTHUR, E. D., and DAVIS, J. N. 1983. Mule deer preference and monoterpenoids (essential oils). *J. Range Manag* 36:485–487.

WEZEL, A., and BENDER, S. 2004. Degradation of agro-pastoral village land in semi-arid southeastern Cuba. *J. Arid Environ* 59:299–311.

functions have been categorized as direct and indirect resistance mechanisms, respectively.

Nitrogen (N) application is an important practice in crop production. It can exert a variety of bottom-up effects and may significantly alter tritrophic interactions through qualitative and quantitative alteration of plant direct and indirect defensive compounds (McNeil and Southwood 1978; Stiling and Moon 2005). Soil nitrogen availability affects the expression of constitutive and induced plant defenses in a wide range of plant species (Stout et al. 1998; Cipollini and Bergelson 2001; Coviella et al. 2002). The level of expression of secondary metabolites may correlate positively (Cipollini and Bergelson 2001; Lou and Baldwin 2004), negatively (Stout et al. 1998; Hemming and Lindroth 1999), or not at all (Dudt and Shure 1994) with nitrogen availability among systems examined, but surprisingly few crop species have been examined in detail.

Nitrogen levels affect the release of plant volatile organic compounds (VOCs), which serve as cues for natural enemies to locate potential hosts/prey (Dicke et al. 1990; Choh et al. 2004). For example, in maize (*Zea mays* var Delprim), the maximal-induced volatile emission was detected after both mechanical wounding and addition of an elicitor from oral secretions of the noctuid *Spodoptera exigua* (Schmelz et al. 2003a) when N fertilization was the lowest. Similarly, celery with additional N had a lower quantity of constitutively emitted volatiles (Van Wassenhove et al. 1990). In contrast, Gouinguené and Turlings (2002) found that unfertilized maize plants (*Z. mays* var Delprim) emitted less volatiles following application of crude *Spodoptera littoralis* oral secretions when compared with those that had received a complete nutrient solution. Unfortunately, the role of N alone was unclear in this study because all the nutrient amounts were varied. In tobacco (*Nicotiana attenuata*), volatile emission levels were not affected by N, although low N availability attenuated the jasmonate and salicylate levels and reduced two N-containing antiherbivore defensive compounds, namely nicotine and trypsin proteinase inhibitors (Lou and Baldwin 2004). Based on this documented variability, the responses of any given plant species or crop cannot yet be readily predicted.

Changes in insect-induced phytohormone production or sensitivity mediated by nutrient availability may provide a mechanism for regulating the magnitude of plant defense responses. With jasmonates established as key regulators of plant responses to insect herbivores (Browse and Howe 2008), jasmonic acid (JA) serves as a useful marker in probing interactions between nutrients and induced defenses. In maize, direct positive relationships have been established between *S. exigua*-induced JA accumulation in the leaves and subsequent induced volatile emission (Schmelz et al. 2003b). In this system, low N availability resulted in higher levels of sustained JA accumulation and subsequent induced volatile emission, following treatment with insect-derived elicitors, than identically treated plants grown under medium N availability (Schmelz et al. 2003a). Despite some advances, few studies have considered the interactions between N fertilization, herbivore-induced JA, and defense in agronomically significant crops (Schmelz et al. 2003a; Lou and Baldwin 2004).

In this study, we investigated the defense response of cotton plants in relation to nitrogen fertilization. Specifically, we tested three hypotheses: (1) increased N fertilization will result in a reduced production of carbon-rich herbivore-induced defense responses, specifically JA and terpenoid aldehydes, in cotton leaves; (2) production of volatile organic compounds will be affected by N fertilization; and (3) the parasitoid *Cotesia marginiventris* will inflict higher mortality on sentinel beet armyworm larvae feeding on plants emitting higher VOC levels.

Methods and Material

Plants Cotton (*Gossypium hirsutum* cv. FiberMax 989) plants were grown using methods and nutrient solutions described by Chen et al. (2008), except as otherwise noted. Plants were fertilized with 100 ml of 112 ppm N nutrient solution daily for approximately 2 weeks, at which time they were of the same height and with leaves of similar size at the same leaf position were randomly assigned to different N treatments. Cotton plants were subsequently fertilized with respective N nutrient treatment solutions (42, 112, 196, and 280 ppm N) for approximately 2 weeks until experimentation. Leaching (watering without nutrients) followed every fourth N nutrient solution application to limit soil salinity. All experimental plants had three–five mature true leaves.

Insects Beet armyworm (*S. exigua*) larvae (reared on modified Pinto bean diet; Chen et al. 2008) and their parasitoids (*C. marginiventris*) were from laboratory colonies maintained in the Biological Control Laboratory at the University of Georgia in Tifton, GA, USA.

Plant Hormone and Volatile Production in Relation to N Fertilization To examine the effects of N fertilization on production of phytohormones and volatiles, a 2 (leaf position—local and systemic)×3 (N fertilization—42, 112, and 196 ppm N)×2 (herbivore infestation—control and 20 *S. exigua* larvae/plant) factorial experiment was designed. For the herbivore damage treatments, 20 3-day-old *S. exigua* larvae were caged on the third true leaf on the main stem for 48 h (see Chen et al. 2006 for cage description). In control treatments, a cage with no larvae was placed on the third true leaf to account for possible physiological changes caused by cages. The cages were checked twice daily for

larval escape and availability of leaf tissue for caterpillars. If all the leaf tissue within the cages were eaten, then the cages were moved to an undamaged location on the same leaf. Immediately following continuous feeding for 48 h, the insect-damaged leaves of all treatments were briefly and gently cleaned with a fine brush to remove larvae and debris. Damaged leaves were photographed digitally for herbivory assessment immediately following removal of larvae and debris, and approximately 150–200 mg fresh leaf tissue from each sample leaf were collected, weighed, stored in FastPrep® tubes containing approximately 1 g Zirmil beads (1.1 mm; SEPR Ceramic Beads and Powders, Mountainside, NJ, USA), and frozen in liquid N as described in Schmelz et al. (2004). Leaf damage was quantified by using the digital images as described in Chen et al. (2006). Samples were collected from leaf 3 (local, damaged, mature leaf) and leaf 6 (systemic; expanding at the time of experiment) and stored at −80°C until analysis. Each treatment was replicated four times.

Terpenoid Aldehyde Production in Relation to N Fertilization To examine the effects of N fertilization on terpenoid aldehyde production, the same 2 (leaf position)×3 (N fertilization)×2 (herbivore infestation) factorial experiment as phytohormone and volatile assessment was designed. At the beginning of the experiment, 20 3-day-old *S. exigua* larvae were caged on the third true leaf of the treatment plants to induce production of the terpenoid aldehydes (see Chen et al. 2006 for cage description). A cage with no larvae was placed on the third true leaf of control plants (zero larvae) to account for possible physiological changes caused by cages. Similarly, cages were checked twice daily for escape of larvae and availability of leaf tissue. Cages and *S. exigua* larvae were removed after 48 h of continuous feeding. The insect-damaged leaves of all treatments were briefly and gently cleaned with a fine brush to remove larvae and debris. A photo of the damaged leaves was taken immediately following removal of larvae and debris and excised at the distal end of the petiole and stored at −80°C until lyophilization. The leaf damage was quantified as in the previous experiment. The sixth true leaves (young and expanding leaves) of all treatment plants were also collected to quantify the effects of N fertilization on systemic production of terpenoid aldehydes. Each treatment was replicated four times.

C. marginiventris Foraging Tests To examine the effects of N fertilization on short-range host location by parasitoids, three cotton plants of one N treatment were placed in one end of 2×2×2 m cages covered with fine mesh. The mesh has square holes with 1 mm openings. Three plants of the other N treatment were placed in the opposite side of the cage. Plants were so arranged that the same N treatments were touching each other but were separated from the other treatment by at least 50 cm. N pairings tested were 42 vs. 196 and 112 vs. 280 ppm N. Forty (42 vs. 196 ppm N trial) or 30 (112 vs. 280 ppm N trial) neonate *S. exigua* larvae were placed on the top leaves of each cotton plant (a total of 120 and 90 larvae/treatment for 42 vs. 196 and 112 vs. 280 ppm N trials, respectively) and allowed to feed for 24 h before five *C. marginiventris* females were released into the center of the cage. *C. marginiventris* females were prepared as follows: 1-day-old male and female wasps were placed together in a plastic cage and 2-day-old *S. exigua* larvae were provided for 24 h for oviposition experience of females; the 2-day-old male and female wasps were then transferred to a new cage without *S. exigua* larvae but supplied with a cotton ball soaked in 5% honey solution for 24 h before experimentation. Remaining *S. exigua* larvae were recovered 24 h after parasitoid release and placed in groups of five in 5-ml plastic cups filled with 3 ml of modified Pinto bean diet. Parasitoid offspring emergence was checked daily. Recovery rate was calculated as the total number of *S. exigua* larvae recovered divided by 120 (42 vs. 196 ppm N trial) or 90 (112 vs. 280 ppm N trial). Parasitism rate was calculated as the number of *C. marginiventris* offspring that emerged from hosts, regardless of cocoon construction, divided by the total number of *S. exigua* larvae recovered. Total mortality was calculated as the total number of dead *S. exigua* larvae divided by the total number of larvae recovered.

Chemical Analyses The high performance liquid chromatography (HPLC) procedure outlined by Stipanovic et al (1988) was used to analyze terpenoid concentrations. Samples (100 mg of freeze-dried ground plant material) were shaken for 30 min in a capped 125-ml Erlenmeyer flask with 15 ml of glass beads, 10 ml of 3:1 hexane:ethyl acetate (HEA), and 100 μl of 10% HCL. The solution was filtered over a glass-fritted filter funnel into a 50-ml pear-shaped flask, and the beads and residue were rinsed three times with 3 ml of HEA. The solvent was left to evaporate in a 90°C water bath, and the residue in the flask was redissolved with four 150-μl HEA washes. Each wash was transferred to a Maxi-clean silica cartridge (Alltech, Breda, The Netherlands). The silica cartridges were dried with compressed air, and terpenoid compounds were eluted with 5 ml of isopropyl alcohol, acetonitrile, water, and ethyl acetate (35:21:39:5). The eluent was filtered through a 45-μm nylon filter and transferred to a crimp top vial. Twenty microliters of each sample were analyzed by an HPLC system (DIONEX Corp., Sunnyvale, CA, USA), using a single wavelength UV absorbance detector (λ= 272 nm) and a 250-mm long, 4.6-mm (*id*) Alltima C-18 column (Alltech, Breda, The Netherlands). The column was eluted with ethanol/methanol/isopropyl/alcohol/acetonitrile/water/ethylacetate/dimethylformamide/phosphoric acid

(16.7:4.6:12.1:20.2:37.4:3.8:5.1:0.1) at a flow rate of 1.25 ml per min and kept at 55°C during analysis (Stipanovic et al. 1988). Standard of gossypol (G) was purchased from Sigma Aldrich (UK), and standards of hemigossypolone (HGQ), heliocides 1 and 4 (H_1 + H_4), and heliocides 2 (H_2) and 3 (H_3) were kindly provided by Dr. R.D. Stipanovic. These standards were used to assess retention times of the individual terpenoids. Terpenoids were calculated as microgram per gram dried plant material.

Vapor phase extraction was used to estimate acidic phytohormones and herbivore-induced VOCs simultaneously, with analyses following those of Schmelz et al. (2004). Briefly, the plant metabolites were extracted with 300 µl of H_2O/1-propanol/HCl (1:2:0.005) and 1 ml dichloromethane ($MeCl_2$). The $MeCl_2$/1-propanol layer into which plant metabolites were extracted was then transferred to a glass vial, and 2 µl of 2.0 M trimethylsilyldiazomethane solution were added to form methyl esters of carboxylic acid containing analytes. Residual trimethylsilyldiazomethane was neutralized with excess acetic acid. Plant volatiles were trapped on filters containing 30 mg Super Q (Alltech Associates, Inc., Deerfield, IL, USA) at 200°C and eluted with $MeCl_2$. Samples were later analyzed with chemical ionization-gas chromatography/mass spectrometry (CI-GC/MS) profiling method. The settings of the CI-GC/MS profiling method are described elsewhere (Engelberth et al. 2003; Schmelz et al. 2004). Estimates of total JA and salicylic acid (SA) represent combined pools of endogenous methyl esters and free acids. Plant volatiles analyzed were (*Z*)-3-hexenal, (*E*)-2-hexenal, (*Z*)-3-hexenyl acetate, indole, α-pinene, β-pinene, myrcene, (*E*)-β-ocimene, β-caryophyllene, (*E*)-β-farnesene, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT), (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene (TMTT), α-bergamotene, α-humulene, γ-bisabolene, bisabolol, and limonene. Unless otherwise noted, quantification was based on the slopes of external standard calibration curves derived from the peak areas of $[M + H]^+$ parent molecular ions generated for each compound. Authentic standards for α-bergamotene and bisabolol were not available; thus, tentative identifications based on electron ionization spectra were made by using a National Institute of Standards and Technology database. To estimate quantities, the slopes generated for β-caryophyllene and γ-bisabolene were applied to the tentative α-bergamotene and bisabolol peak areas, respectively. All reagents and solvents used in the experiments were acquired from Sigma-Aldrich (St Louis, MO, USA) or previously established research standards.

Statistical Analyses The experimental design for phytohormone and volatile production assessment was a 2 (leaf position—young and mature)×3 (nitrogen levels—42, 112, and 196 ppm N)×2 (herbivore infestation—0 and 20 *S. exigua* larvae) factorial, so the amounts of chemical compounds were analyzed by a three-way analysis of variance (ANOVA). The data were square root (x+3/8) transformed for heteroscedasticity before analysis. The experimental design and analysis for terpenoid aldehyde production was the same as for phytohormone and volatile production assessment, except that untransformed data were analyzed. Leaf damage inflicted by *S. exigua* was analyzed by one-way ANOVA with data untransformed. Recovery rate, parasitism, and total mortality of *S. exigua* larvae were arcsine (square-root) transformed before being subjected to one-way ANOVA. All ANOVAs were conducted with PROC GLM in SAS (SAS Institute 1999), and means were separated by Bonferroni's t tests if the null hypothesis was rejected.

Results

Increased N Fertilization Decreased Herbivore-Induced JA Accumulation and Volatile Pools Nitrogen fertilization did not significantly influence feeding damage inflicted by inducing *S. exigua* larvae (P=0.38). The average leaf areas consumed on damaged leaves receiving 42, 112, and 196 ppm N were 13.93±1.30, 15.62±0.87, and 15.64±0.50 cm^2, respectively.

Leaf position marginally affected JA concentration (P=0.057). As expected, all herbivore-damaged leaves (local leaves) had higher JA content than undamaged leaves in the same position (P<0.001); however, the magnitude of herbivore-induced JA accumulation sharply and significantly decreased with increasing N levels (Fig. 1 (1)). After 48 h of herbivory, herbivore-damaged local leaves from 42 ppm N plants displayed 11-fold increases in JA while those from 196 ppm N plants were increased by only 1.5-fold. JA levels in the systemic leaves did not exhibit the sharp positive response to herbivory observed in the local leaves, but like the local leaves, the mean JA concentrations of the systemic leaves were highest in plants receiving the least N (Fig. 1 (2)).

Unlike JA, SA was either not significantly different by leaf position or was significantly reduced in herbivore-damaged local leaves compared to controls (Fig. 1 (3)). Herbivory resulted in a significant decrease in SA levels in the 112 ppm N group, while the lowest average concentrations of constitutive SA in local leaves were observed in the highest fertilization treatment (196 ppm N) (Fig. 1 (3)). Increasing plant N levels tended to decrease SA concentrations in systemic leaves of damaged and undamaged plants, although statistically significant differences were observed only in the damaged plants (P<0.05 or 0.001; Fig. 1 (4)).

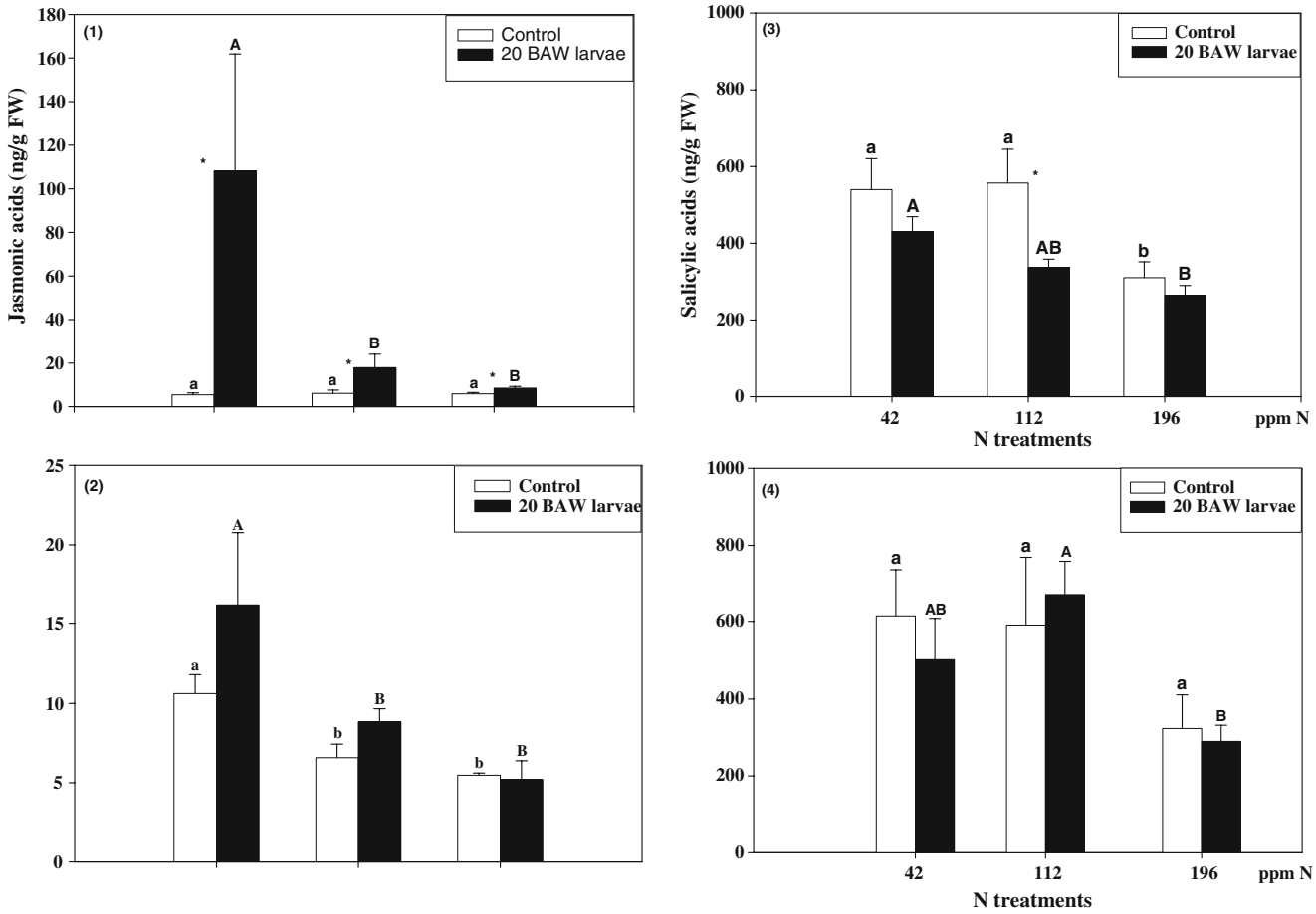

Fig. 1 Plant hormone production (mean ± SE ng g^{-1} fresh weight plant tissue) in response to nitrogen fertilization. *1* total jasmonic acid—local leaf (true leaf 3), *2* total JA—systemic leaf (true leaf 6), *3* total salicylic acid (SA)—local leaf, *4* total SA—systemic leaf. *Different lowercase letters above mean bars* denote significant difference between N treatments of control plants at α=0.05. *Different uppercase letters above mean bars* denote significant difference between N treatments of damaged plants at α=0.05; * Denotes significant difference between control and damaged plants of the same N treatment at α=0.05; damage induced by 20 3-day-old *S. exigua* (*BAW*) larvae

Effects of N on local production of volatiles are summarized in Table 1. Increasing N fertilization constrained leaf tissue concentrations of herbivore-induced volatiles. In plants grown under 42 ppm N, *S. exigua* herbivory significantly increased the local leaf tissue concentrations of seven volatile chemicals including (*Z*)-3-hexenal, (*E*)-2-hexenal, (*E*)-β-farnesene, DMNT, α-bergamotene, γ-bisabolene, and β-bisabolol (Table 1). As N fertilization increased to 112 ppm, only (*Z*)-3-hexenal and (*E*)-2-hexenal remained significantly different and increased in herbivore-attacked leaf tissues. At 196 ppm N, plants displayed an opposite pattern, with herbivory resulting in significantly lower levels of six predominant volatiles: β-caryophyllene, (*E*)-β-farnesene, α-bergamotene, α-humulene, γ-bisabolene, and β-bisabolol. DMNT, a widely occurring herbivore-induced plant volatile, exemplifies the above trend by exhibiting a significant 3.2-fold concentration increase in herbivore-damaged plants at the lowest N level, yet a steady decline in average fold induction under increasing N fertilization. Plant N addition increased constitutive levels of the lipoxygenase products (*Z*)-3-hexenal and (*E*)-2-hexenal (P<0.01 and P<0.001, respectively) in leaf extracts of undamaged control tissues (Table 1). Constitutive levels of (*Z*)-3-hexenyl acetate displayed the opposite trend, significantly decreasing in plants receiving 196 ppm N compared to those receiving 42 ppm N (P< 0.05). Aside from these lipoxygenase pathway products, N fertilization did not significantly influence the constitutive levels of volatiles in undamaged control leaves but instead demonstrated strong effects on the inducibility of volatiles in herbivore-attacked leaves.

Effects of N on systemic volatile production are summarized in Table 2. Increasing N fertilization decreased systemic production of six volatiles in undamaged mature leaves, while decreasing systemic production of nine volatiles in upper leaves of damaged plants (Table 2). Under 42 ppm N, herbivory in local leaves significantly increased systemic production of the volatiles (*Z*)-3-

Table 1 Plant volatile production (mean ± SE μg g^{-1} fresh mass) in response to various N levels—local leaf

Volatile compounds	42 ppm N		112 ppm N		196 ppm N	
	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]
(*Z*)-3-Hexenal	2.8±0.3b	17.0±2[d]	7.4±2.5b	19.5±2.7[e]	17.4±3.3a	19.6±4.3
(*E*)-2-Hexenal	2.6±0.2c	12.4±2.6[e]	10.6±2.3b	19.8±2.6[e]	22.0±3.6a	16.8±2.8
(*Z*)-3-Hexenyl acetate	0.3±0.06ab	0.4±0.1A	0.3±0.1a	0.1±0.02B	$0.1\pm6.2\times10^{-3}$b	0.1±0.03B
Indole	$1.2\times10^{-3}\pm 3.4\times10^{-4}$	0.01±0.01	$1.1\times10^{-3}\pm 7.7\times10^{-4}$	$1.6\times10^{-3}\pm 7.3\times10^{-4}$	$4.2\times10^{-4}\pm 1.3\times10^{-4}$	$1.4\times10^{-3}\pm 1.2\times10^{-3}$
α-Pinene	29.6±17.2	7.5±7.3	8.8±8.7	11.7±6.7	17.1±10.3	17.2±8.3
β-Pinene	10.7±5.3	6.8±2.4	4.0±2.2	4.5±1.9	6.2±2.7	6.2±2.4
Myrcene	15.7±8.4	19.2±6.8	8.4±2.7	7.7±2.7	13.9±5.8	13.2±3.1
(*E*)-β-Ocimene	3.3±1.7	5.0±1.4A	2.3±0.4	1.7±0.2B	3.5±0.7	2.2±0.5B
β-Caryophyllene	177.2±24.2	222.3±33.6A	127.4±14.9	109.0±11.1B	123.0±9.9	90.9±5.4B[e]
(*E*)-β-Farnesene	11.2±1.0	19.6±2.7A[e]	10.3±1.5	9.4±1.1B	11.9±0.8	8.8±0.2B[e]
DMNT	$4.7\times10^{-4}\pm 9.9\times10^{-3}$	0.2±0.02[f]	0.05±0.01	0.1±0.02	$0.04\pm8.3\times10^{-3}$	0.05±0.01
TMTT	$0.02\pm3.9\times10^{-3}$	$0.02\pm5.2\times10^{-3}$A	$0.01\pm3.0\times10^{-3}$	$9.6\times10^{-3}\pm 1.9\times10^{-3}$B	$0.01\pm1.3\times10^{-3}$	$9.6\times10^{-3}\pm 9.1\times10^{-4}$B
α-Bergamotene[a]	8.4±0.8	14.2±1.9A[e]	7.7±1.2	7.0±0.8B	8.8±0.6	6.7±0.2B[e]
α-Humulene	59.1±8.0	78.2±12.0A	43.9±5.0	37.7±4.1B	43.2±3.2	31.2±1.7B[e]
γ-Bisabolene	80.5±6.5	145.2±18.3A[e]	77.4±10.2	72.1±8.9B	88.4±6.8	65.2±2.3B[e]
Bisabolol[a]	153.4±14.2	267.5±40.4A[e]	142.6±21.2	130.4±15.0B	161.4±12.4	119.2±3.7B[e]
Limonene	13.0±6.4	7.4±2.6	5.0±2.5	5.8±2.3	7.7±3.2	7.3±2.6
Total	635.6±75.7	893.6±107.3A	521.3±35.0	501.3±36.1B	580.7±40.1	461.8±24.5B

DMNT (*E*)-4,8-dimethyl-1,3,7-nonatriene, *TMTT* (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene

[a] Tentative identification due to lack of authentic standard, identification based on comparison of mass spectra with spectra from the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health database

[b] Means followed by different lowercase letters denote significant difference among N treatments of control plants at $\alpha=0.05$

[c] Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at $\alpha=0.05$

[d] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.001$

[e] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.05$

[f] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.01$

hexenal, (*Z*)-3-hexenyl acetate, myrcene, and bisabolol (all $P<0.05$). Under 112 ppm N, systemic production of the volatiles (*Z*)-3-hexenal, (E)-2-hexenal, and DMNT was increased by herbivory (all $P<0.05$). However, at 196 ppm systemic production of none of the volatiles examined was affected by herbivory.

Constitutive and Herbivore-Induced Terpenoid Aldehyde Production Decreased Under Increased N Fertilization The effects of N fertilization and herbivory on production of nonvolatile terpenoid aldehydes of the local mature leaf (leaf 3) and the systemic young leaf (leaf 6) are summarized in Table 3. N fertilization did not significantly affect feeding damage caused by beet armyworm larvae ($P=0.50$).

The main terpenoids in the leaves were HGQ and heliocides (H_1, H_2, H_3, and H_4). Leaf position significantly affected production of HGQ, $H_1 + H_4$, H_2, H_3, and total terpenoids, and as expected, young leaves had greater terpenoids than mature leaves (HGQ: $P<0.001$; $H_1 + H_4$: $P<0.05$; H_2: $P<0.001$; H_3: $P<0.001$; total: $P<0.001$). Increased N fertilization decreased production of HGQ, H_2, H_3, and total terpenoid aldehydes (HGQ: $P<0.001$; H_2: $P<0.001$; H_3: $P<0.001$; total: $P<0.001$). *S. exigua* infestation increased HGQ and marginally elevated total terpenoid levels when all N treatments were pooled (HGQ: $P<0.05$; total: $P=0.06$). The interactions between leaf position and N were significant for HGQ, H_3, and total terpenoids (HGQ: $P<0.001$; H_3: $P<0.01$; and total: $P<0.05$, respectively). The interactions between leaf position and *S. exigua* infestation were significant for HGQ and total terpenoids (HGQ: $P<0.01$; total: $P<0.05$). The interactions between N and herbivore infestation were significant for HGQ ($P<0.01$). No other two-way and three-way interactions were observed ($P>0.05$).

N addition to control plants reduced constitutive expression of HGQ, H2, H3, and total terpenoid aldehydes in local leaves (all $P<0.01$) but did not affect terpenoid aldehydes of systemic leaves to the same degree. In contrast, increased N fertilization significantly decreased production of HGQ, H_2, H_3, and total terpenoid aldehydes

Table 2 Plant volatile production (mean ± SE μg g^{-1} fresh mass) in response to various N levels—systemic leaf

Volatile compounds	42 ppm N		112 ppm N		196 ppm N	
	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]	Control[b]	20 larvae[c]
(*Z*)-3-Hexenal	1.8±0.6b	4.6±0.6B[d]	5.2±1.1ab	13.7±2.5A[d]	11.5±3.2a	16.3±3.5A
(*E*)-2-Hexenal	4.2±1.6c	8.4±3.3B	15.1±2.3b	38.6±6.7A[d]	38.7±7.9a	35.5±2.5A
(*Z*)-3-Hexenyl acetate	0.2±0.05	1.0±0.3A[d]	0.1±0.01	0.3±0.09B	0.1±0.02	0.1±0.03B
Indole	$1.7\times10^{-4}\pm 1.1\times10^{-4}$	$1.5\times10^{-3}\pm 7.6\times10^{-4}$	$1.1\times10^{-4}\pm 4.8\times10^{-5}$	$1.9\times10^{-4}\pm 9.1\times10^{-5}$	$7.0\times10^{-4}\pm 1.3\times10^{-4}$	$3.4\times10^{-4}\pm 1.8\times10^{-4}$
α-Pinene	61.6±35.9	23.9±23.5	19.6±19.4	52.4±30.2	35.1±20.9	45.5±18.2
β-Pinene	19.5±11.0	7.5±6.1	5.8±5.4	14.4±8.1	10.9±6.5	12.7±4.8
Myrcene	297.7±65.5a	549.5±75.7A[d]	162.8±20.7b	173.8±18.3B	186.9±23.8ab	177.8±32.7B
(*E*)-β-Ocimene	148.5±69.8	404.4±113.8A	60.2±22.0	64.4±8.0B	64.3±22.2	46.5±14.4B
β-Caryophyllene	447.8±105.6a	661.4±115.0A	231.9±31.1b	228.6±6.5B	239.4±8.8	194.7±24.4B
(*E*)-β-Farnesene	32.5±5.1a	64.3±12.6A	20.0±1.0b	20.4±1.2B	22.4±2.6ab	18.1±0.6B
DMNT	$0.02\pm1.1\times10^{-3}$a	0.1±0.07A	$0.01\pm5.0\times10^{-3}$b	$0.03\pm5.1\times10^{-3}$AB[d]	$0.01\pm9.8\times10^{-4}$ab	$0.01\pm9.0\times10^{-4}$B
TMTT	$0.06\pm9.5\times10^{-3}$a	0.1±0.03A	$0.02\pm5.4\times10^{-3}$b	$0.03\pm4.1\times10^{-3}$B	$0.03\pm1.4\times10^{-3}$b	$0.02\pm5.4\times10^{-3}$B
α-Bergamotene[a]	30.6±15.7	11.8±5.0A	12.9±0.5	13.0±0.7B	14.0±1.6	11.4±0.4B
α-Humulene	158.5±39.2a	246.8±39.1A	80.8±10.5b	79.5±2.3B	83.8±4.0b	66.8±7.6B
γ-Bisabolene	226.3±40.2a	400.0±77.6A	145.3±6.2b	147.0±9.7B	152.5±19.1ab	126.0±5.4B
Bisabolol[a]	386.2±56.1a	770.3±153.0[d]	238.5±12.1b	246.9±16.7	272.9±32.0ab	170.2±57.1
Limonene	31.5±14.1	26.1±3.8A	11.6±4.9	18.2±7.2B	16.4±6.2	16.8±4.4B
Total	1,925.0±421.4	3,268.8±559.6A	1,113.1±96.0	1,198.4±72.0B	1,234.4±105.6	1,016.4±138.0B

DMNT (*E*)-4,8-dimethyl-1,3,7-nonatriene, *TMTT* (*E*,*E*)-4,8,12-trimethyl-1,3,7,11-trideca-tetraene

[a] Tentative identification due to lack of authentic standard, identification based on comparison of mass spectra with spectra from the National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health database

[b] Means followed by different lowercase letters denote significant difference among N treatments of control plants at $\alpha=0.05$

[c] Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at $\alpha=0.05$

[d] Significant difference between control and damaged plants within the same N treatment at $\alpha=0.05$

in systemic leaves of damaged plants (HGQ: $P<0.001$; H_2: $P<0.01$; H_3: $P<0.001$; total: $P<0.0001$). Under low N fertilization (42 ppm) HGQ, the predominant leaf terpenoid aldehyde displayed a 2.1-fold increase in the systemic leaves of herbivore-damaged plants (Table 3). With the exception of HGQ under 112 ppm N where herbivory diminished the concentration, no significant herbivore-induced terpenoid aldehyde production was detected at N fertilization levels above 42 ppm.

C. marginiventris Short-Range Foraging Not Affected by Plant N Fertilization N treatment did not significantly affect the recovery rate, parasitism rate, or total mortality of sentinel *S. exigua* larvae in the caged preference studies (Table 4).

Discussion

In this commercial variety of cotton, increased N fertilization dramatically impaired the herbivore-induced accumulation of plant defense markers, including JA, volatile pools, and also terpenoid aldehydes in systemic leaves. Consistent with the established role of JA as a positive regulator of herbivore-induced defenses (Reinbothe et al. 1994; Schmelz et al. 2003a; Browse and Howe 2008), low N (42 ppm) plants that exhibited the highest induced JA levels also exhibited robust increases in leaf tissue concentrations of volatiles in local and systemic leaves and terpenoid aldehydes in systemic leaves. At N fertilization levels above 42 ppm, the induction of most biochemical defense markers examined greatly declined or disappeared altogether. However, despite the large differences in herbivore-induced leaf-tissue volatile concentrations among the N fertilization regimes, parasitism of *S. exigua* larvae by adult female *C. marginiventris* did not differ among N fertilization regimes.

In this study, we considered JA, terpenoid aldehydes, and concentrations of leaf volatiles as markers for herbivore-induced defenses. Significant induced biochemical responses were demonstrable only at low N fertilization levels. In the local herbivore-damaged leaves

Table 3 Cotton leaf terpenoid aldehyde (mean ± SE μg g^{-1} dry mass) production in relation to N fertilization

	42 ppm N		112 ppm N		196 ppm N	
	Control	20 larvae[c]	Control	20 larvae[c]	Control	20 larvae[c]
Local leaf[a]						
Damage (cm^2)	0	14.5±2.1	0	15.9±0.5	0	14.9±2.1
HGQ	1,334.3±229.2a	1,206.0±293.2	1,110.8±84.8a[d]	736.0±110.3	574.3±13.8b	623.8±31.0
G	27.8±27.8	0	0	19.0±19.0	0	0
H1 + H4	467.5±29.2	428.75±101.5	578.0±141.4	780.5±368.2	291.0±36.9	297.0±17.3
H3	322.3±30.5a	249.3±29.3	298.5±13.6a	314.0±44.9	205.5±14.2b	208.8±21.5
H2	1,025.5±80.4a	740.3±95.8	808.0±50.4b	828.5±138.8	551.5±20.8c	569.8±71.3
Total	3,177.3±260.5a	2,624.3±495.0	2,795.3±226.5a	2,678.0±660.5	1,622.3±49.3b	1,699.3±62.5
Systemic leaf[b]						
HGQ	2,309.8±516.6	4,806.3±265.7A[d]	1,787.3±225.8	1,577.3±127.8B	1,406.5±237.1	1,529.3±515.8B
G	41.8±41.7	149.5±88.4	0	30.0±30.0	30.5±30.5	0
H1 + H4	484.0±76.8	1,041.0±246.5	699.8±160.2	864.5±323.2	403.5±51.4	845.8±563.5
H3	434.8±48.6	624.3±90.7A	391.3±41.5	379.3±12.1B	290.3±22.8	290.8±20.3B
H2	1,007.0±120.8	1,364.8±274.1A	1,013.5±122.6	1,013.5±47.2AB	781.3±65.9	754.8±79.6B
Total	4,277.3±584.3	7,985.8±647.3A[d]	3,891.8±479.1	3,864.5±496.3B	2,912.0±304.3	3,420.5±1,148B

Means followed by different lowercase letters denote significant difference among N treatments of control plants at α=0.05. Means followed by different uppercase letters denote significant difference among N treatments of damaged plants at α=0.05

HGQ hemigossypolone, *G* gossypol, H_{14} heliocides 1–4

[a] True leaf 3

[b] True leaf 6

[c] Number of 3-day-old *S. exigua* larvae used to induce plant resistance in mature leaf (true leaf 3)

[d] Significant difference between control and herbivore-damaged leaves at α=0.05.

of 42 ppm N plants, concentrations of volatiles in induced tissue undoubtedly relate to actual volatile emission at the larval feeding sites. In corn (*Z. mays*) leaves, herbivore-induced volatiles are readily detected in leaf tissue extracts (Schmelz et al. 2003c). In contrast, under higher fertilization levels (196 ppm N), we detected significant decreases in the volatile concentrations of herbivore-attacked leaves. This result makes projections of volatile emission more complex. Actual emission of constitutive volatiles at the feeding sites still would likely be substantial. However, if low levels of herbivore-induced JA fail to stimulate new volatile synthesis, low volatile concentrations in damaged leaf tissue may be the result of increased emission and eventual depletion of leaf volatile pools. In support of this hypothesis, Loughrin et al. (1994) found decreased emission of many constitutive and herbivore-inducible volatiles after the third day of continuous *S. exigua* feeding on cotton plants. One anomaly is the presence of significant amounts of (*Z*)-3-hexenal and (*E*)-2-hexenal in undamaged control tissue. Production of these C6 volatiles from C18 fatty acids proceeds through the sequential activity of C13 lipoxygenase and hydroperoxide lyase enzymes (Matsui 2006). Artificially increased levels of C6 volatiles in control tissue may have occurred during leaf harvesting or initial sample extraction. Despite this potentially elevated background, clear differences in (*Z*)-3-hexenal and (*Z*)-2-hexenal at both 42 and 112 ppm are indicative of herbivore-induced enzyme activity.

Table 4 Influence of host plant N fertilization on survival (mean ± SE %) of *S. exigua* and parasitism (mean ± SE %) by *C. marginiventris* in outdoor-cage choice tests

Fate of *S. exigua* larvae	42 vs. 196 ppm N		112 vs. 280 ppm N	
	42 ppm	196 ppm	112 ppm	280 ppm
Recovery rate[a]	62.00±5.20	70.50±6.56	72.50±3.94	77.83±1.98
Parasitism rate[b]	17.39±6.27	13.21±4.68	26.71±1.69	23.35±2.95
Total mortality[c]	23.30±7.62	21.46±7.47	38.75±1.77	40.16±5.63

[a] 42 vs. 196 ppm N: P=0.32; 112 vs. 280 ppm N: P=0.28

[b] 42 vs. 196 ppm N: P=0.72; 112 vs. 280 ppm N: P=0.34

[c] 42 vs. 196 ppm N: P=0.88; 112 vs. 280 ppm N: P=0.83

S. exigua herbivory significantly increased JA production in local leaves. While increased N fertilization had no significant effects on the constitutive JA levels in local leaves, it was inversely related to the induced JA in herbivore-damaged local leaves. As with many insect-inducible defenses, applications of JA on cotton similarly promoted terpenoid aldehyde accumulation (Opitz et al. 2008). What has not been previously explored is the relationship between insect-induced endogenous JA levels and the systemic induction of terpenoid aldehydes. While precise N availability was not specifically examined in

previous cotton studies, a strong reduction in herbivore-induced JA and systemic terpenoid aldehyde accumulation at higher N fertilization levels was unexpected. However, this N-mediated reduction in herbivore-induced JA, terpenoid aldehydes, and volatiles in cotton is surprisingly consistent with short-term responses described in corn following application of the *S. exigua* elicitor, volicitin (Schmelz et al. 2003a). In hydroponically grown corn seedlings, volicitin-induced levels of JA, and sesquiterpene volatiles dramatically increase as N availability decreases. In contrast, low N availability in tobacco (*Nicotiana attenuata*) suppresses the levels of JA and SA induced by insect oral secretions and likewise results in lower induced accumulation of nonvolatile defenses (Lou and Baldwin 2004). SA is a plant hormone widely considered to be induced in response to pathogen attack or pathogen-like damage caused by phloem-feeding insects, such as whiteflies and aphids (Walling 2000). N addition to cotton plants significantly decreased SA content of both control and damaged local leaves. However, in contrast to JA, insect herbivory generally reduced SA levels. This result is consistent with antagonistic interactions of JA and SA observed in tomato (Pena-Cortés et al. 1993) and tobacco plants (Niki et al. 1998) with respect to wound- and pathogen-induced defenses.

Herbivory by *S. exigua* larvae had no significant effects on the induction of terpenoid aldehydes under high (112 and 196 ppm) N conditions in this study but significantly increased induced terpenoid aldehydes of young leaves under low (42 ppm) N conditions. The lack of induction of terpenoid aldehydes by insect feeding in mature cotton leaves has been shown in another cotton variety (cv. Deltapine 90; McAuslane et al. 1997); however, the lack of systemic terpenoid aldehyde production under high N conditions contrasts with previous research (Alborn et al. 1996; McAuslane et al. 1997; McAuslane and Alborn 1998; Bezemer et al. 2003). We do not yet know if this is a specific response to N fertilization in *G. hirsutum* cv. FiberMax 989 or a more generalized pattern common to cotton cultivars. Many plants, including corn, tobacco, and cotton, exhibit significant cultivar or genetic variation in the production of both direct and indirect herbivore-induced defenses (Loughrin et al. 1995; Gouinguené and Turlings 2002; Wu et al. 2008).

Foliar terpenoid aldehydes in cotton function as dietary toxins for numerous lepidopteran insects and are typically dominated by HGQ, while heliocides (H_1, H_2, H_3, H_4) also occur at significant levels (Elliger et al. 1978; Stipanovic et al. 1988; Bezemer et al. 2003). N fertilization significantly reduced the constitutive (local mature leaves) and induced (systemic young leaves) accumulation of HGQ and most heliocides in this study. Plants with higher N may be more capable of compensating for biomass loss and therefore need not invest as much in defense as is the case for lower N plants. As shown by Chen et al. (2008), both *S. exigua* larvae and adult females preferred cotton leaves receiving high N fertilization for feeding and oviposition, respectively. Higher N content or weaker constitutive and inducible defenses of plants grown with high N fertilization or a combination of the two may contribute to this preference.

Young expanding leaves of plants with no previous herbivore damage had higher terpenoid aldehyde contents than mature leaves, which is consistent with predictions of the optimal defense (OD) theory (McKey 1974). The significance of defending the young leaves is underscored by our observation that terpenoid aldehyde titers in young leaves of control plants were not significantly affected by changing N levels, whereas increasing N led to significantly reduced terpenoid aldehyde content of mature leaves. OD theory predicts that plant parts having higher fitness value should be better defended. The greater fitness value of young expanding leaves over older leaves has been experimentally demonstrated in some plants (McKey 1979; Krischik and Denno 1983), as has the elevated accumulation of chemical defenses in these leaves (Ohnmeiss and Baldwin 2000; Bezemer et al. 2004). The general applicability of our results must be tempered by the extensive domestication of the cotton variety used in the present study. Nevertheless, the patterns observed tend to conform to adaptive theory and suggest that defensive responses may be intensified under nutrient deficiency.

For herbivore-induced indirect defenses, large differences in leaf-tissue volatile concentrations were found among the N fertilization regimes; however, parasitism of *S. exigua* larvae by adult female *C. marginiventris* was not different in caged choice tests with neonate larvae feeding on host plants grown under 42 and 196 ppm N. *C. marginiventris* females are known to exploit insect-induced volatiles for long-range attraction to hosts (Turlings et al. 1991a; Loughrin et al. 1995; Hoballah et al. 2002; Gouinguené et al. 2005), and both qualitative and quantitative differences in VOC blend are suggested to affect this attraction (Pickett 1999; Hoballah et al. 2002). At first glance, the lack of dose-dependent responses of the parasitoid to overall VOC levels is surprising, since attractiveness has been shown to increase at higher VOC release rates in some systems (Turlings et al. 1991b; Weissbecker et al. 1999; Hoballah et al. 2002). Unlike the commonly studied corn model, cotton foliage contains large preexisting pools of volatiles which are released immediately upon feeding damage (Elzen et al. 1985; Loughrin et al. 1994). In a short-range attraction assay, rankings for the total number of flights completed by female *C. marginiventris* suggested wasps preferred cotton plants systemically releasing herbivore-induced volatiles, followed by artificially damaged cotton plants, and lastly undamaged

control plants (Röse et al. 1998). While these results indicate that *C. marginiventris* has a strong attraction to herbivore-induced cotton volatiles, it also demonstrates an innate attraction to the volatiles from wounded cotton plants.

The lack of N effects on observed parasitism rates may be a result of the experimental arena. As parasitoids were exposed to plants in a closed field cage, they were unable to leave the plant patch. Under open-field conditions, they might have been more likely to ignore or abandon patches where plants release lower levels of VOCs. Parasitoid success is hierarchically divided into host habitat location, host location, host acceptance, and host suitability (Nordlund et al. 1981). In cages, the cues involved in host habitat location might be obscured, mixed, or even concentrated, thereby possibly rendering the results of limited ecological significance. Once parasitoids locate the plant–herbivore complex, additional cues including those associated with host frass can also be significant (Eller et al. 1988). Thus, the degree of difference in host searching cues between the treatments may have been insufficient to significantly modify parasitoid behaviors over the 24-h assay period. In the field, qualitative and quantitative differences of individual VOCs in the herbivore-induced blend are believed to significantly affect host searching behavior of parasitoids (De Moraes et al. 1998). The apparent disparity of VOC levels and parasitoid host finding also might be attributable to limitations of chemical analytical methods. VOCs of leaf extracts analyzed in the study may not necessarily reflect the parasitoid-orienting VOCs released into the air. Nevertheless, it is possible that the relatively large preexisting pools of plant volatiles (known to be emitted during larval herbivory) across all N fertilization treatments overshadow weak vs. strong herbivore-induced biochemical defenses and impair the ability of *C. marginiventris* to discriminate, at short range, host *S. exigua* larvae on these physiologically different host plants.

In summary, N fertilization rates and herbivory exerted variable effects on cotton plant chemistry, with low N input (42 ppm N) and herbivore damage inducing significant increases in JA, volatiles, and in systemic accumulation of terpenoid aldehydes relative to higher N rates. However, increased N fertilization of cotton plants suppressed *S. exigua*-induced plant hormones and led to reduced production of various terpenoid aldehydes in undamaged mature leaves and systemic young leaves above mature leaves that had been fed on by *S. exigua* larvae. However, although increased N fertilization significantly diminished herbivore-induced leaf volatile concentrations, parasitism of *S. exigua* larvae by *C. marginiventris* in field cages did not differ among N treatments. This suggests that parasitoids were unable to differentiate in the field cages among the varying VOC titers induced by different N treatments.

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References

ALBORN, H. T., RÖSE, U. S. R., and MCAUSLANE, H. J. 1996. Systemic induction of feeding deterrents in cotton plants by feeding of *Spodoptera* spp. larvae. *J. Chem. Ecol.* 22:919–932.

BERENBAUM, M. R. 1995. The chemistry of defense: theory and practice. *Proc. Natl. Acad. Sci. U.S.A.* 92:2–8.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., and WÄCKERS, F. L. 2003. Interactions between above- and belowground insect herbivores as mediated by the plant defense system. *Oikos* 101:555–562.

BEZEMER, T. M., WAGENAAR, R., VAN DAM, N. M., VAN DER PUTTEN, W. H., and WÄCKERS, F. L. 2004. Above- and below-ground terpenoid aldehyde induction in cotton, *Gossypium herbaceum*, following root and leaf injury. *J. Chem. Ecol.* 30:53–67.

BROWSE, J., and HOWE, G. A. 2008. New weapons and a rapid response against insect attack. *Plant Physiol.* 146:832–838.

CHEN, Y., RUBERSON, J. R., and OLSON, D. 2008. Nitrogen fertilization rate affects feeding, larval performance, and oviposition preference of the beet armyworm, *Spodoptera exigua*, on cotton. *Entomol. Exp. Appl.* 126:245–255.

CHEN, Y., RUBERSON, J. R., LEWIS, W. J., and BEDNARZ, C. 2006. Herbivore feeding and induction of systemic resistance in cotton plants, pp. 1510–1520, *in* Proceedings, Beltwide Cotton Conferences, National Cotton Council, Memphis, Tennessee.

CHOH, Y., SHIMODA, T., OZAWA, R., DICKE, M., and TAKABAYASHI, J. 2004. Exposure of lima bean leaves to volatiles from herbivore-induced conspecific plants results in emission of carnivore attractants: active or passive process? *J. Chem. Ecol.* 30:1305–1317.

CIPOLLINI, D. F., and BERGELSON, J. 2001. Plant density and nutrient availability constrain constitutive and wound-induced expression of trypsin inhibitors in *Brassica napus*. *J. Chem. Ecol.* 27:593–610.

COVIELLA, C. E., STIPANOVIC, R. D., and TRUMBLE, J. T. 2002. Plant allocation to defensive compounds: interactions between elevated CO_2 and nitrogen in transgenic cotton plants. *J. Exp. Bot.* 53:323–331.

DE MORAES, C. M., LEWIS, W. J., PARÉ, P. W., ALBORN, H. T., and TUMLINSON, J. H. 1998. Herbivore-infested plants selectively attract parasitoids. *Nature* 393:570–573.

DICKE, M., SABELIS, M. W., TAKABAYASHI, J., BRUIN, J., and POSTHUMUS, M. A. 1990. Plant strategies of manipulating predator–prey interactions through allelochemicals: prospects for application in pest control. *J. Chem. Ecol.* 16:3091–3118.

DUDT, J. F., and SHURE, D. J. 1994. The influence of light and nutrients on foliar phenolics and insect herbivory. *Ecology* 75:86–98.

DUFFY, S. S., and STOUT, M. J. 1996. Antinutritive and toxic components of plant defense against insects. *Arch. Insect Biochem. Physiol.* 32:3–37.

ELLER, J. J., TUMLINSON, J. H., and LEWIS, W. J. 1988. Beneficial arthropod behavior mediated by airborne semiochemicals. II. Olfactometric studies of host location by the parasitoid *Microplitis croceipes* (Cresson) (Hymenoptera: Braconidae). *J. Chem. Ecol.* 14:425–434.

ELLIGER, C. A., CHAN, B. G., and WAISS, A. C. Jr. 1978. Relative toxicity of minor cotton terpenoids compared to gossypol. *J. Econ. Entomol.* 71:161–164.

ELZEN, G. W., WILLIAMS, H. J., BELL, A. A., STIPANOVIC, R. D., and VINSON, S. B. 1985. Quantification of volatile terpenes of glanded and glandless *Gossypium hirsutum* cultivars and lines by gas chromatography. *J. Agric. Food. Chem.* 33:1079–1082.

ENGELBERTH, J., SCHMELZ, E. A., ALBORN, H. T., CARDOZA, Y. J., HUANG, J., and TUMLINSON, J. H. 2003. Simultaneous quantification of jasmonic acid and salicylic acid in plants by vapor phase extraction and gas chromatography-chemical ionization-mass spectrometry. *Analyt. Biochem.* 321:242–250.

FIRN, R. D., and JONES, C. G. 2006. Do we need a new hypothesis to explain plant VOC emissions? *Trends Plant Sci.* 11:112–113.

GOUINGUENÉ, S. P., and TURLINGS, T. C. J. 2002. The effects of abiotic factors on induced volatile emission in corn plants. *Plant Physiol.* 129:1296–1307.

GOUINGUENÉ, S., PICKETT, J. A., WADHAMS, L. J., BIRKETT, M. A., and TURLINGS, T. C. J. 2005. Antennal electrophysiological responses of three parasitic wasps to caterpillar-induced volatiles from maize (*Zea mays mays*), cotton (*Gossypium herbaceum*), and cowpea (*Vigna unguiculata*). *J. Chem. Ecol.* 31:1023–1038.

HEMMING, J. D. C., and LINDROTH, R. L. 1999. Effects of light and nutrient availability on aspen: growth, phytochemistry, and insect performance. *J. Chem. Ecol.* 25:1687–1714.

HOBALLAH, M. E. F., TAMÒ, C., and TURLINGS, T. C. J. 2002. Differential attractiveness of induced odors emitted by eight maize varieties for the parasitoid *Cotesia marginiventris*: is quality or quantity important? *J. Chem. Ecol.* 28:951–968.

KRISCHIK, V. A., and DENNO, R. F. 1983. Individual, population, and geographic patterns in plant defense, pp. 463–512, in R. F. Denno, and M. S. McClure (eds.). Variable Plants and Herbivores in Natural and Managed SystemsAcademic, New York, New York, USA.

LOU, Y., and BALDWIN, I. T. 2004. Nitrogen supply influences herbivore-induced direct and indirect defenses and transcriptional responses in *Nicotiana attenuata*. *Plant Physiol.* 135:496–506.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., TURLINGS, T. C. J., and TUMLINSON, J. H. 1994. Diurnal cycle of emission of induced volatile terpenoids by herbivore-injured cotton plant. *Proc. Natl. Acad. Sci. U.S.A.* 91:11836–11840.

LOUGHRIN, J. H., MANUKIAN, A., HEATH, R. R., and TUMLINSON, J. H. 1995. Volatiles emitted by different cotton varieties damaged by feeding beet armyworm larvae. *J. Chem. Ecol.* 21:1217–1227.

MATSUI, K. 2006. Green leaf volatiles: hydroperoxide lyase pathway of oxylipin metabolism. *Curr. Opin. Plant Biol.* 9:274–280.

MCAUSLANE, H. J., and ALBORN, H. T. 1998. Systemic induction of allelochemicals in glanded and glandless isogenic cotton by *Spodoptera exigua* feeding. *J. Chem. Ecol.* 24:399–416.

MCAUSLANE, H. J., ALBORN, H. T., and TOTH, J. P. 1997. Systemic induction of terpenoid aldehydes in cotton pigment glands by feeding of larval *Spodoptera exigua*. *J. Chem. Ecol.* 23:2861–2879.

MCKEY, D. 1974. Adaptive patterns in alkaloid physiology. *Am. Nat.* 108:305–320.

MCKEY, D. 1979. The distribution of secondary compounds within plants, pp. 55–133, in G. A. Rosenthal, and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites Academic, New York, New York, USA.

MCNEILL, S., and SOUTHWOOD, T. R. E. 1978. The role of nitrogen in the development of insect/plant relationships, pp. 77–98, in J. S. Harborne (ed.). Aspects of Plant and Animal CoevolutionAcademic, London.

NIKI, T., MITSUHARA, I., SEO, S., OHTSUBO, N., and OHASHI, Y. 1998. Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. *Plant Cell Physiol.* 39:500–507.

NORDLUND, D. A., JONES, R. L., and LEWIS, W. J. 1981. Semiochemicals, Their Role in Pest Control. Wiley, New York, USA.

OHNMEISS, T., and BALDWIN, I. T. 2000. Optimal defense theory predicts the ontogeny of an induced nicotine defense. *Ecology* 81:1765–1783.

OPITZ, S., KUNERT, G., and GERSHENZON, J. 2008. Increased terpenoid accumulation in cotton (*Gossypium hirsutum*) foliage is a general wound response. *J. Chem. Ecol.* 34:508–522.

PENA-CORTÉS, H., ALBRECHT, T., PRAT, S., WEILER, E. W., and WILLMITZER, L. 1993. Aspirin prevents wound-induced gene expression in tomato leaves by blocking jasmonic acid biosynthesis. *Planta* 191:123–128.

PEÑUELAS, J., and LLUSIÀ, J. 2004. Plant VOC emissions: making use of the unavoidable. *Trends Ecol. Evol.* 19:402–404.

PICHERSKY, E., SHARKEY, T. D., and GERSHENZON, J. 2006. Plant volatiles: a lack of function or a lack of knowledge? *Trends Plant Sci.* 11:421.

PICKETT, J. 1999. Insect-Plant Interactions and Induced Plant Defence. Wiley, New York, USA.

REINBOTHE, S., MOLLENHAUER, B., and REINBOTHE, C. 1994. JIPs and RIPs: the regulation of plant gene expression by jasmonates in response to environmental cues and pathogens. *Plant Cell* 6:1197–1209.

RÖSE, U. S. R., LEWIS, W. J., and TUMLINSON, J. H. 1998. Specificity of systemically released cotton volatiles as attractants for specialist and generalist parasitic wasps. *J. Chem. Ecol.* 24:303–319.

SAS INSTITUTE 1999. SAS/STAT User's guide, version. 8th edn.SAS Institute, Inc., Cary, NC.

SCHMELZ, E. A., ALBORN, H. A., ENGELBERTH, J., and TUMLINSON, J. H. 2003a. Nitrogen deficiency increases volicitin-induced volatile emission, jasmonic acid accumulation, and ethylene sensitivity in maize. *Plant Physiol.* 133:295–306.

SCHMELZ, E. A., ALBORN, H. T., BANCHIO, E., and TUMLINSON, J. H. 2003b. Quantitative relationship between induced jasmonic acid levels and volatile emission in *Zea mays* during *Spodoptera exigua* herbivory. *Planta* 216:665–673.

SCHMELZ, E. A., ENGELBERTH, J., ALBORN, H. T., O'DONNELL, P., SAMMONS, M., TOSHIMA, H., and TUMLINSON, J. H. 2003c. Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. *Proc. Natl. Acad. Sci. U.S.A.* 100:10552–10557.

SCHMELZ, E. A., ENGELBERTH, J., TUMLINSON, J. H., BLOCK, A., and ALBORN, H. T. 2004. The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. *Plant J.* 39:790–808.

STILING, P., and MOON, D. C. 2005. Quality or quantity: the direct and indirect effects of host plants on herbivores and their natural enemies. *Oecologia* 142:413–420.

STIPANOVIC, R. D., ALTMAN, D. W., BEGIN, D. L., GREENBLATT, G. A., and BENEDICT, J. H. 1988. Terpenoid aldehydes in upland cottons: analysis by aniline and HPLC methods. *J. Agric. Food Chem.* 36:509–515.

STOUT, M. J., BROVONT, R. A., and DUFFEY, S. S. 1998. Effects of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, *Lycopersicon esculentum*. *J. Chem Ecol.* 24:945–963.

TURLINGS, T. C. J., TUMLINSON, J. H., HEATH, R. R., PROVEAUX, A. T., and DOOLITTLE, R. E. 1991a. Isolation and identification of allelochemicals that attract the larval parasitoid, *Cotesia marginiventris* (Cresson), to the microhabitat of one of its hosts. *J. Chem. Ecol.* 17:2235–2251.

TURLINGS, T. C. J., TUMLINSON, J. H., ELLER, F. J., and LEWIS, W. J. 1991b. Larval-damaged plants: sources of volatile synomones that guide the parasitoid *Cotesia marginiventris* to the microhabitat of its hosts. *Entomol. Exp. Appl.* 58:75–82.

VAN WASSENHOVE, F. A., DIRINCK, P. J., SCHAMP, N. M., and VULSTEKE, G. A. 1990. Effects of nitrogen fertilizers on celery volatiles. *J. Agric. Food Chem.* 38:220–226.

WÄCKERS, F. L., and BONIFAY, C. 2004. How to be sweet? Extrafloral nectar allocation by *Gossypium hirsutum* fits optimal defense theory predictions. *Ecology* 85:1512–1518.

WALLING, L. L. 2000. The myriad plant responses to herbivores. *J. Plant Growth Regul.* 19:195–216.

WEISSBECKER, B., VAN LOON, J. J. A., and DICKE, M. 1999. Electroantennogram responses of a predator, *Perillus bioculatus*, and its prey, *Lepinotarsa decemlineata*, to plant volatiles. *J. Chem. Ecol.* 25:2313–2325.

WU, J., HETTENHAUSEN, C., SCHUMAN, M. C., and BALDWIN, I. T. 2008. A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. *Plant Physiol.* 146:927–939.

2004; Pawlik et al. 2007). In addition, competition can be avoided or limited by upward growth or by inward burrowing into the substratum (Jackson 1979; Woodin and Jackson 1979).

Allelopathic agents may allow sessile species to compete effectively for space. These agents can inhibit adjacent organisms through direct contact or by releasing mucus or exudations into the water (Becerro et al. 1997). Allelopathy often can be inferred from the presence of chemical substances (Uriz et al. 1996; Becerro et al. 1997; Hay et al. 1998). As a first step, the chemical properties of the suspected allelopathic organism need to be segregated from other physical and biological characteristics (shape, color, texture). Crude organic extracts are fractionated to give the corresponding fractions and pure compounds. Chemical substances are then delivered to the target organism at natural concentrations through an assumedly inert carrier medium. The potential existence of allelopathy is inferred if the chemical substances are active and if there is a plausible mechanism for their natural delivery, e.g., direct contact, through mucus, by exudation (Sullivan et al. 1983; Targett and Schmahl 1984; Porter and Targett 1988; de Nys et al. 1991; Miyamoto et al. 1994; Thacker et al. 1998; Nishiyama and Bakus 1999; Engel and Pawlik 2000; Nishiyama et al. 2004; Chaves-Fonnegra et al. 2005; Pawlik et al. 2007). The exact mechanism by which allelopathy occurs is not clearly elucidated in most cases.

Water-soluble (polar) compounds can diffuse in water and act relatively far from their source (Hadfield and Scheuer 1985; Woodin 1993; Slattery et al. 1997). However, such compounds need to be continuously produced to maintain minimally active concentrations (Woodin 1993). Water-insoluble (non-polar) compounds or compounds with mixed polarity can act when competitors are in direct contact or at close range (Schmitt et al. 1995; Turon et al. 1996). Such compounds may be deployed through mucus and have longer residence times (Sullivan et al. 1983; Miyamoto et al. 1994). Bioactive compounds are often stored in specialized cells and then released by exocytosis (Thompson et al. 1983; Walker et al. 1985; Uriz et al. 1996; Turon et al. 2000). In some cases, there must be a tactile stimulus for release to occur (de Nys et al. 1991).

Some sponges induce bleaching and death of underlying or adjacent coral polyps, thus attaching onto and spreading over the dead areas (Jackson and Buss 1975; Sullivan et al. 1983; Target and Schmahl 1984; Sullivan and Faulkner 1985; Porter and Targett 1988). However, few studies have shown that marine sponges produce allelopathic compounds that may be used in the competition for space. Siphonodictidine, isolated from the coral excavating sponge *Aka* (= *Siphonodictyon*) sp., was the first example of a potentially allelopathic substance produced by a marine sponge (Sullivan et al. 1983). This compound decreased respiration rates and was lethal at high concentrations to the coral *Acropora formosa*. Siphonodictidine, along with siphonodictyals from *Aka coralliphaga*, was presumed to be present in the abundant mucus that these sponges secrete that kills surrounding coral (Sullivan et al. 1983; Sullivan and Faulkner 1985). However, bioassays used pure compound solutions in sea water, and they did not directly address allelopathy. Subsequent studies are more ecologically relevant. For example, the regeneration of holes experimentally made in the sponge *Scopalina lophyropoda* was inhibited when rubbed with the co-occurring sponge *Crambe crambe*, which is known to possess an array of bioactive metabolites (Turon et al. 1996). The use of allelopathy by *C. crambe* revealed a significant trend for other sponges to be distributed away from this sponge (Turon et al. 1996). 7-Deacetoxyolepupuane from the sponge *Dysidea* sp. caused necrosis by contact with the sponge *Cacospongia* sp. when incorporated into agar strips (Thacker et al. 1998). Engel and Pawlik (2000) recorded lateral growth inhibition in fast growing sponges when they grew on gels containing crude extracts of other sponges. Pawlik et al. (2007) also demonstrated a decrease in photosynthetic activity of coral zooxanthellae when sponge extracts included in gels are brought into contact with live coral tissue in the field.

In coral reefs, sponges that simultaneously excavate and encrust the calcareous substratum are strong competitors for space (Pang 1973; Rützler 2002; López-Victoria et al. 2006). These sponges are able to displace and kill live coral tissue, apparently by undermining the polyp's skeletal support through excavating tissue filaments and fronts (Schönberg and Wilkinson 2001; Rützler 2002; López-Victoria et al. 2003, 2006; Chaves-Fonnegra and Zea 2007). Among these sponges, Caribbean *Cliona tenuis* is one of the fastest in displacing and killing coral tissue (López-Victoria et al. 2003, 2006). It currently occupies up to 10% of the hard reef substratum in some wave-exposed reef zones (Williams et al. 1999; Rützler 2002; López-Victoria and Zea 2004, 2005). Furthermore, the crude extracts and some fractions of *C. tenuis* included in gels and brought into contact with live coral are able to kill tissue (Chaves-Fonnegra et al. 2005) and decrease the photosynthetic yield of associated zooxanthellae (Pawlik et al. 2007, e.g., *Cliona langae*).

The aim of this study was to examine whether an allelopathic process—through external contact or at a distance—is involved in coral death by *C. tenuis*. The following investigations were carried out: (1) We tested whether experimentally induced proximity or contact of sponge tissue with coral tissue is deleterious to coral; (2) we searched for the substance(s) responsible through bioassay-guided fractionation; (3) we tested the toxic

effects of extracts, fractions, and pure substances against live corals. The chemical structure and the total synthesis of compound **1** (Fig. 1, herein named clionapyrrolidine A), which were isolated from a fraction that showed a potent allelopathic effect of *C. tenuis* against corals, have been published (Castellanos et al. 2006a). For comparison, we included in this study the recently synthesized *N*-acetylhomoagmatine (compound **2**, Fig. 1; Castellanos et al. 2006b). This compound is a suspected allelopathic agent originally isolated from the polar fraction of the excavating sponge *Cliona celata* from the Northeastern Atlantic (Lenis et al. 1996).

Methods and Materials

Source of Studied Material and Locations The sponge *C. tenuis* was collected at a depth of 3–6 m in the northern windward fringing fore-reef of the Islas del Rosario, Pajarales sector, SW of the city of Cartagena, Colombian Caribbean (for description of the sites, see López-Victoria and Zea 2004). Field and laboratory bases were the Oceanarium of Islas del Rosario and INVEMAR at Santa Marta, in NE Colombia. Extraction and chemical studies were carried out at the Universidad Nacional de Colombia in Bogotá and Universidade da Coruña, Spain.

Allelopathy by Contact or by Proximity of Live Sponge Tissue to Coral Tissue Fragments of *C. tenuis* of approximately 10×10 cm were cut from sponge-colonized dead branches of the coral *Acropora palmata*. The fragments were allowed to heal inside a basket for 2 days. To determine the effect of direct contact of sponge tissue on coral tissue, fragments were placed on each of ten hemispherical colonies of the coral *Siderastrea siderea*. Each fragment was strapped in place with a piece of plastic netting tied to surrounding nails with cable ties. Fragments of the same dead coral branches wrapped in cotton fabric (to prevent grinding of the live coral) previously soaked in sea water for 2 days were employed as controls. Live fragments and controls were lifted after 24 h, and coral and sponge tissue were compared for the presence/absence of tissue death in the contact zones. Significance of the treatment effects was established by Fisher's exact test (Siegel and Castellan 1988; see details below) from the number of cases in which the coral–sponge contact elicited coral or sponge death but the controls did not.

To determine whether there may be an allelopathic effect at distance by exudation, a healed sponge fragment was placed at a distance of up to 2 cm from the side of each of six colonies of *S. siderea*. Fragments were mounted and tightly tied onto a bed of plastic netting held by two adjacent 3-in. steel nails. Beds were placed horizontally to prevent fragments from shading adjacent coral tissue. A control fragment (as above, but cleaned in bleach and not covered with fabric) was also positioned in each colony in the same way. After 6 months, fragments were removed, and the presence/absence of coral or sponge tissue death was recorded.

Bioassay-Guided Fractionation of C. tenuis Organic Extract The sponge and its excavated substratum (which extends 2 cm below the surface) were collected by using a hammer and chisel, broken further into small fragments, frozen, and sent to Bogotá by air. Fragments were extracted exhaustively first with MeOH and then with CH_2Cl_2 (2:1 sample/solvent volume ratio). The displaced volume of each sample was measured when it was immersed in MeOH. Extracts were filtered, concentrated under reduced pressure, and combined to give the total extract. Five subsamples of fragments were used to determine the volumetric equivalent of the extract (weight of extract per volume of sponge tissue) without substratum. The displaced volume of each subsample was measured first. Subsamples were then extracted as described above, and the dried extract was weighed. Extracted sponge tissue + substratum were digested in 15% HCl to dissolve carbonates, and the displaced volume of the sponge tissue was measured (26.0±5.6% of the total volume). The volumetric equivalent referred to this value, whose mean was 0.082±0.007 g ml^{-1} of sponge. This mean value was used subsequently as the natural concentration of the crude extract.

The total extract (93 g) was partitioned between CH_2Cl_2 and H_2O (Fig. 2). The CH_2Cl_2 partition was evaporated under reduced pressure (non-polar fraction, 29%) and partitioned between 10% aqueous MeOH (400 ml) and hexane (2×400 ml). The hexane partition was evaporated to obtain the hexane fraction (12%). Water was added to the MeOH partition until the mixture became 50% aqueous MeOH. This material was partitioned with CH_2Cl_2 (3×400 ml) to give the methanol (7%) and dichloromethane (10%) fractions after removal of the solvent under reduced pressure. The initial aqueous partition was evaporated and further partitioned between 1:1 water and *n*-BuOH saturated

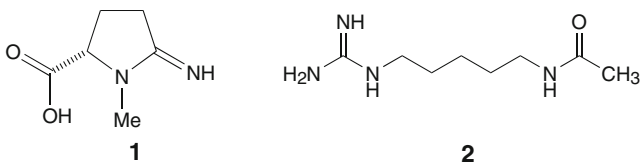

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Fig. 1 Structure of (**1**) Clionapyrrolidine A [(−)-(5*S*)-2-imino-1-methylpyrrolidine-5-carboxylic acid], isolated from the most active water fraction of the sponge *C. tenuis* from the Caribbean, and of (**2**) *N*-acetylhomoagmatine, obtained from a polar fraction of the sponge *C. celata* from the Northeastern Atlantic

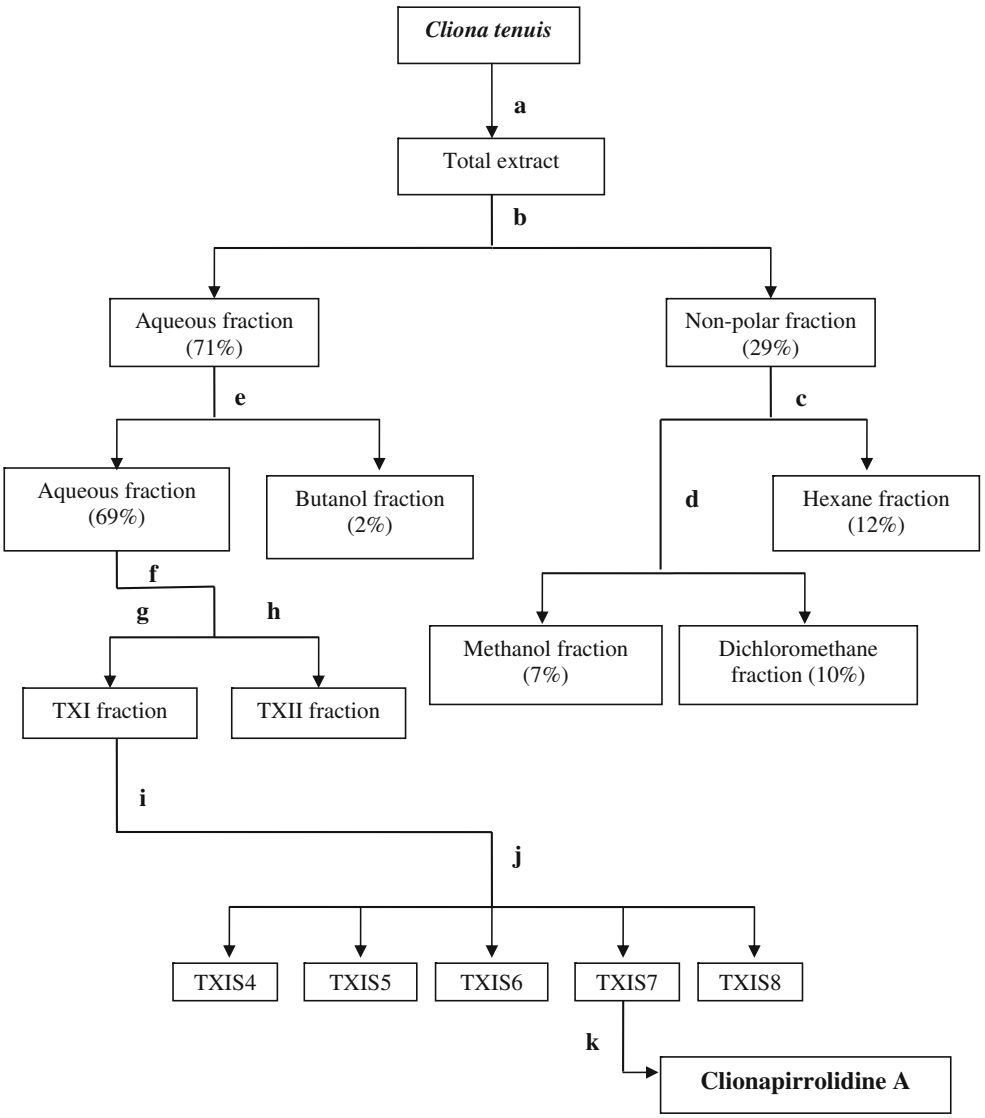

Fig. 2 *C. tenuis* bioassay-guided fractionation protocol. *a* MeOH and CH_2Cl_2 extraction (2:1 sample/solvent volume ratio). *b* Partition between CH_2Cl_2 and H_2O. *c* Partition between 10% aqueous MeOH and hexane. *d* Addition of water to obtain a 50% aqueous MeOH mixture and then partition with CH_2Cl_2. *e* Partition between water and *n*-BuOH saturated with water. *f* Column chromatography on XAD-4 resin. *g* Washing with distilled water. *h* Washing with methanol and with acetone. *i* Addition of methanol to achieve sequential precipitation of inorganic components, which were removed. *j* Column chromatography on Sephadex LH-20 (10% MeOH/H_2O). *k* RP-HPLC purification on a Discovery® HS F5 column [using acetonitrile/water (8:2) and 0.05% formic acid]

with water to afford the aqueous (69%) and butanol (2%) fractions after evaporation.

The total extract and fractions were assayed for activity by contact against corals (see below). This enabled us to identify an active fraction (aqueous), further fractionated and assayed. The active fraction was loaded onto XAD-4 resin, washed with distilled water (fraction TXI), then with methanol, and finally acetone (fraction TXII). Fraction (TXI) was further fractionated by slow addition of methanol in order to achieve sequential precipitation of inorganic components, which were removed. Filtration and evaporation of the methanol filtrate to dryness yielded a residue (14 g) that was chromatographed on Sephadex LH-20 (10% MeOH/H_2O) to afford 12 fractions (TXIS1-S12). The most active fraction (TXI-S7, 80 mg) was purified by RP-HPLC on a Discovery® HS F5 column, using acetonitrile/water (8:2) and 0.05% formic acid, to yield 3.5 mg of clionapyrrolidine A (Fig. 1). Natural concentration of this compound was calculated to be 0.1056 mg ml^{-1}. This compound was characterized by NMR and MS-ESI techniques, and its structure was confirmed by total synthesis of a 100 mg sample (Castellanos et al. 2006a). Spectroscopic data for clionapyrrolidine A are as follows: (+)-HRMS-ESI *m/z*: 143.0815 $[M + H]^+$ (calculated 143.0815). ^1H NMR (500 MHz, D_2O) δ (parts per million): 2.95 (H-2, m, 2H), 2.51 (H-3, m, 1H), 2.12 (H-3′, m, 1H), 4.37 (H-4, dd, J=9.5, 4.2, 1H), 3.04 (H_3-6, s, 3H). ^{13}C NMR (125 MHz, D_2O) δ (parts per million): 169.55 (C-1, C), 30.12 (C-2, CH_2), 24.29 (C-3, CH_2), 70.36 (C-4, CH), 31.74 (C-6, CH_3), 177.43 (C-7, C).

Allelopathy by Contact Against Live Coral Using Gels - Following the method of Pawlik et al. (2007), gels made of Phytagel™ were used as carrier media in which extracts,

fractions, and pure compounds were included. These agar-based gels are made of complex sugars that do not have active functional groups, and they slowly release the extract contents when exposed to sea water (Henrikson and Pawlik 1995). Treatment gels were prepared by adding 0.37 g of Phytagel powder to 35 ml of deionized water at room temperature. Once the powder was completely wet, it was heated with constant stirring until it boiled—at which point it became translucent. Subsequently, 1 ml of a solution (or dispersion) of the extract, fraction, or compound in solvent (CH_2Cl_2 for non-polar fractions, H_2O for the aqueous fractions, MeOH for all others) was added to the gel. The gel, still hot to avoid bubble formation, was poured into opaque plastic caps (28 mm diam, 5 mm high, approx. volume 3 ml) of 35-mm commercial photographic film canisters. These caps had been mounted previously on strips of duct tape assembled to resemble a wristwatch for subsequent attachment onto the coral. Gels were allowed to cool and solidify at room temperature and were kept refrigerated in sealed plastic bags for use within 24 h. When solidified gels had an elevated convex surface; they were leveled to a flat surface with a razor blade to prevent excessive smothering when placed on the coral.

Assays were carried out at Santa Marta Bay (8–10 m in depth) and the Islas del Rosario (5–6 m in depth). Gels in their mounts were placed on live corals and tied with cable ties (through holes made in the tape) to nails driven into the coral head (where possible in the dead periphery). Mounts were stretched with enough force to avoid free movement but loose enough to minimize gel pressure on the coral. As pressure and shading by gels always produced coral tissue bleaching beneath them, several alternative approaches were tried for gel placement to reduce pressure and smothering. In the first, three small supporting "legs" were added to the caps that contained gels in order to keep them at a distance 1–2 mm above the surface. In this way, the coral tissue would touch the gel only at night when polyps extended to feed. In a second approach, gels were placed loosely on coral tissue in such a way that the only pressure exerted involved the weight of the caps and the gels. This was done by holding the supporting caps (without duct tape mountings) with two nails placed directly on each side of the caps by means of copper wire rings. Finally, we also prepared strips of gel (6×2 cm, 2–3 mm thick) in the manner described by Thacker et al. (1998), placing them between two sewn pieces of soft plastic mosquito netting (as described by Gochfeld et al. 2006). The netting was then loosely tied onto the coral tissue surface for a few days. The lack of pressure and smothering in these experiments did not elicit coral death with active extracts and fractions, so we carried out the bioassay-guided fractionation as described above. It was assumed that in competition for space, direct contact between sponges and neighbors also entails some degree of pressure, abrasion, and smothering.

Gels with total extract and the first round of fractions were prepared at concentrations between two and three times the natural concentration in order to enhance any effect. Two different control gels were prepared. The first control was prepared as described previously, without substances from the sponge but with the addition of the maximum amount of solvent used to dissolve substances in each assay. In the second control gel, marine salt (evaporated from local sea water) was added at the volumetric equivalent concentration. This sample was used as a control for assays with the complete aqueous fraction (which had not yet been obtained in a salt-free form).

In each experiment, one set of each of the various treatment and control gels was placed in each of four to ten coral colonies. Gels were left in place for 1–4 days, after which they were lifted, and the presence/absence of dead coral tissue was recorded. As coral tissue under control gels always bleached, it often was difficult to distinguish bleaching from coral tissue death. Further observations were carried out at 3 to 93 days thereafter in order to confirm coral death or recovery under treatment gels or recovery from bleaching under control gels. Fisher's exact test (Siegel and Castellan 1988) was used to determine whether differences were statistically significant. This process involved counting the number of cases in which treatment gels elicited coral tissue death out of the number of control gels that recovered. The null hypothesis was that compounds contained in *C. tenuis* did not produce a deleterious effect on coral tissue beyond the physical effect of shading and transient smothering produced by the gel itself. Different coral species were used at various stages, with the major fractions tested initially on *Montastraea cavernosa*, the most abundant coral around Santa Marta. However, thereafter, the coral *S. siderea* was used because it has smaller polyps and a smoother and more level surface, thus allowing the placement of gels with lower pressure. Pure synthetic clionapyrrolidine A (**1**) was tested against the latter coral at a concentration of 0.19 mg ml^{-1}, which is 1.8 times its natural concentration.

Synthetic *N*-acetylhomoagmatine (**2**) from *C. celata* also was tested in this assay in order to compare its effect to that of clionapyrrolidine A. Compound **2** also was tested because of its structural similarity—a guanidine residue bound to an aliphatic chain—to the compound siphonodictidine, which is known to be lethal to corals (Sullivan et al. 1983). As an approximation to the natural concentration, a level of 0.042 mg ml^{-1} of **2** was used as the reference, a value calculated from its known weight per gram of crude extract (Lenis et al. 1996). In this calculation, we assumed *C. celata* to have the same proportion of

sponge tissue to un-eroded substratum volume and weight of total extract to tissue volume as *Cliona delitrix*. The latter is a Caribbean sponge with a similar excavation pattern to that of *C. celata* and has been previously studied by us (unpublished data).

Toxicity Against Live Coral The non-polar CH_2Cl_2 partition, the aqueous-butanol, and aqueous fractions of *C. tenuis* were evaluated in this assay along with clionapyrrolidine A and *N*-acetylhomoagmatine. Fragments of the branching coral *Madracis auretenra* (=*Madracis mirabilis*; see Locke et al. 2007) were collected in the Santa Marta area and brought to continuous flow aquaria at INVEMAR. Specimens were broken into similarly sized branches (up to 5 cm tall, often being bifurcated at the tip) and kept in a vertical position on plastic mesh. Specimens were allowed to acclimate for 3 days and fed with live plankton or nauplii of the brine shrimp, *Artemia salina*. In the assay, three branches were placed separately in glass bottles with 70 ml of sea water and constant aeration and then fed with nauplii (added in 5 ml of sea water). In this way, the polyps would extend out of their calices to feed and thus confirm the good health of the specimens. Within 2 h, solutions of the fractions and test substances in 5 ml of the corresponding solvent were added to each bottle to give a total volume of 80 ml, in amounts according to the required final concentration. Solvents were CH_2Cl_2/MeOH for the CH_2Cl_2 partition, MeOH for the butanol fraction, distilled water for the aqueous fraction and clionapyrrolidine A, and EtOH for *N*-acetylhomoagmatine. Final concentrations were the natural ones for fractions, 0.1372 mg ml^{-1} for clionapyrrolidine A (1.3 times the natural concentration) and 0.1 mg ml^{-1} for *N*-acetylhomoagmatine. The latter concentration was that used in respiration inhibition assays with siphonodictidine from the excavating sponge *Aka* sp. (see Sullivan et al. 1983). Three replicate bottles were used for each treatment and control. The maximum amount of solvent used in the controls was added to the water. Assays were carried out separately for fractions and for pure compounds. After 2 h, coral branches were moved back to the aquaria with clean circulating water and again fed with nauplii. The behavior of the polyps was observed between 1 and 24 h later. The retracted percentage was quantified over an area of approximately 1 cm^2 in the middle of the branch, usually below the apical bifurcation. Branches were monitored for up to 1 week to confirm whether retracted polyps remained so and thus were dead. The percentage polyp retraction (taken as percentage lethality) at 24 h was used as the analysis variable. Differences in percent lethality between treatments and controls were compared separately for each assay by using the Kruskal–Wallis test (Sokal and Rohlf 1981).

Results

Allelopathy by Contact or Proximity of Live Sponge Tissue None of the ten *C. tenuis* fragments produced tissue death on live tissue of *S. siderea* when both were in contact for 24 h (Fisher's exact test, $P=1$). Coral tissue underneath both sponge and control fragments with cloth showed only a few scratches. In contrast, the area of sponge fragments that had been in close proximity or in contact with the coral had dermal tissue digested in all ten cases (as evidenced by the lack of a brown color and uneven surface). Sponge tissue away from coral contact was normal. Thus, the external defenses of the coral prevented any possible sponge allelopathy by quickly killing sponge tissue that was in contact or in close proximity. In contrast, the six *C. tenuis* and six control fragments placed within 2 cm of *S. siderea* tissue for 6 months did not have any discernible effect on coral tissue (Fisher's exact test, $P=1$), nor was sponge tissue affected. Hence, allelopathy did not occur over this distance by release of substances into the medium.

Allelopathy by Contact with Extract, Fractions, and Pure Compounds Included in Gels Despite initial bleaching and in a few cases some small patches of necrosis, coral tissue under all control gels recovered within days to weeks after the experiments. In cases where fractions were active, after gel retrieval, coral tissue was bleached and necrotic under the entire area of the gels. Coral tissue was thereafter completely lost under gels, and the dead area was colonized by turf algae and filled with sediment. Gels with the total extract of *C. tenuis* had a significant deleterious effect against *M. cavernosa* after 4 days of contact (Fig. 3, tissue killed under gels in all four cases and in five out of six cases, respectively, in two separate experiments, Fisher's exact test, $P<0.05$). Of the five major fractions, only the aqueous one had a significant effect (Fig. 3, tissue killed under all four gels, $P<0.05$; in the other fractions, tissue was killed under up to two out of four gels, $p>0.05$). Further experiments with the most polar fractions (butanol, aqueous) on the coral *S. siderea* confirmed that the aqueous fraction had the highest level of activity (nine out of ten cases after 1 day of contact; nine out of nine cases after 4 days of contact (both $P<0.05$) vs. two out of ten cases after 1 day of contact for the butanol fraction, $P>0.05$; Fig. 3).

Further fractionation of the aqueous fraction in XAD-4 resin produced two fractions, one eluted with water (TXI) and another with organic solvent (TXII). Although both fractions showed a significant effect on the coral *S. siderea* after 1 day of contact (Fisher's exact test, $P<0.05$, $N=10$), TXI was the most active (nine of ten vs. five of ten; Fig. 3). Only five (S4–S8) out of 12 fractions obtained from TXI after chromatography on a Sephadex LH-20 column had

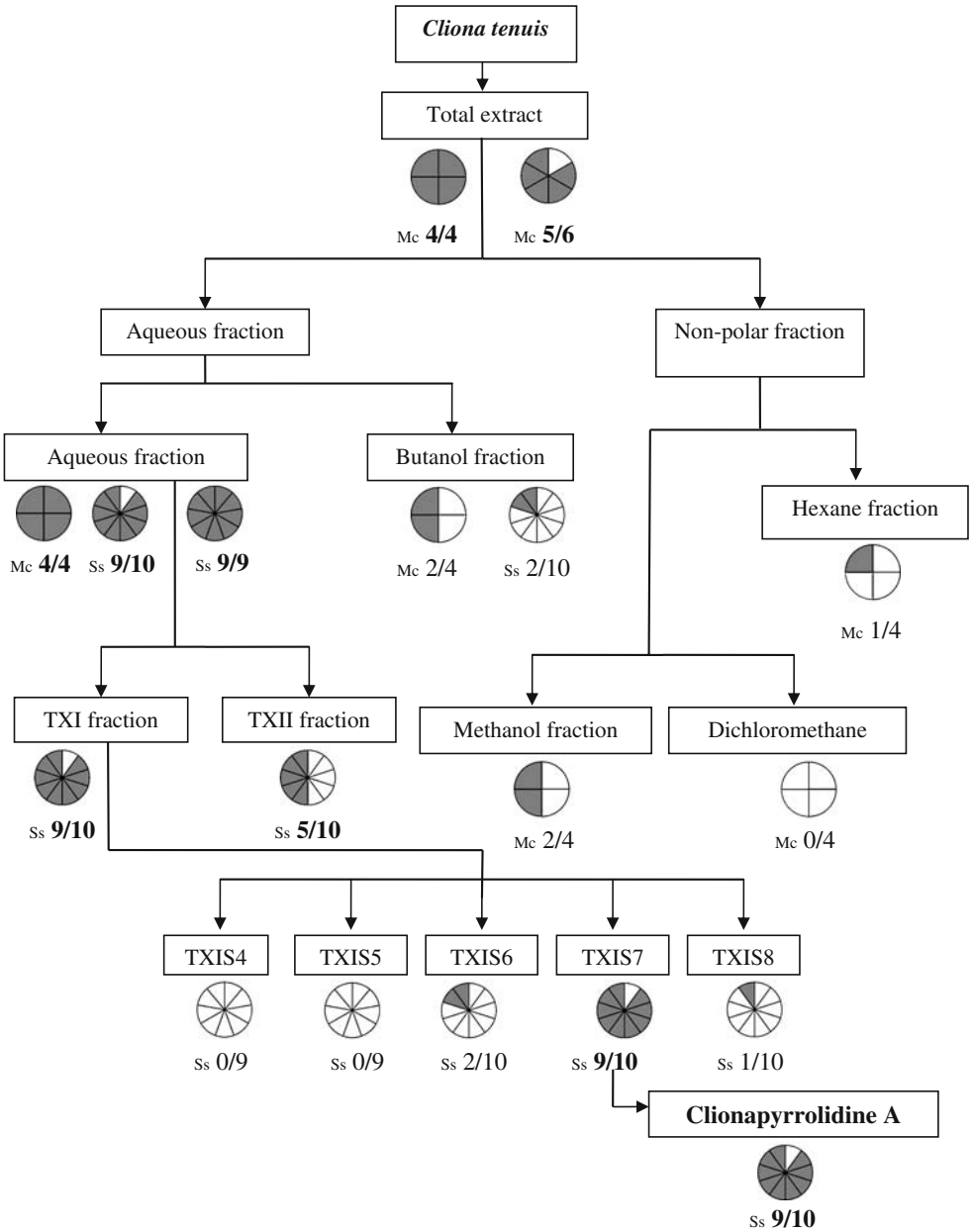

Fig. 3 Bioassay-guided fractionation of *C. tenuis* extract using the allelopathy by gel contact assay. Pie charts and numbers below show the number of cases in which there was coral tissue mortality over the total number of replicate coral colonies (controls recovered in all cases). For the values shown in *bold*, there is a statistically significant effect of tissue mortality (Fisher's exact test, $P<0.05$). *Mc M. cavernosa*, *Ss S. siderea*. Concentrations used in *M. cavernosa* and *S. siderea* were, respectively, three and two times the natural volumetric concentrations. Gels were placed on coral tissue for 4 days in *M. cavernosa* and for 1 day in *S. siderea* (excepting the repeated assay with the aqueous fraction and final assay with pure clionapyrrolidine A, in which contact lasted 4 days). Effects were verified from 2 days to 2.5 months after gel retrieval

sufficient material (50 mg) for testing. From those fractions, the allelopathic activity after 1 day of contact on *S. siderea* was significant only in fraction S7 (nine out of ten cases, $P<0.05$, Fig. 3). HPLC purification on a Discovery® HS F5 column of the active fraction S7 led to the isolation of clionapyrrolidine A (**1**), the major organic component of the fraction. This fraction also contained inorganic salts and unidentified sugars, which were not tested for activity.

Bioassays with synthetic clionapyrrolidine A in contact with *S. siderea* for 4 days, with monitoring over the next 23 days, resulted in a significant effect (nine out of ten gels killed coral tissue after 4 days of contact, Fisher's exact test, $P<0.05$, Fig. 3). These results show clearly that clionapyrrolidine A is responsible for the activity displayed by the crude extract. In contrast, *N*-acetylhomoagmatine (**2**) from *C. celata*, when placed against *M. cavernosa* for 4 days and monitored for up to 1 month later, did not have a deleterious effect on the coral tissue (zero of ten cases, $P>0.05$). This experiment ruled out the suspected allelopathy of this compound.

Toxicity Against Live Coral In the toxicity assays with the non-polar CH_2Cl_2 partition, as well as those with the butanol and aqueous fractions of *C. tenuis* at natural volumetric concentrations, all polyps of the coral *M. auretenra* remained retracted throughout the 2-h exposure

period. Controls remained extended and fed on *Artemia* nauplii during the first 2 h and throughout the experiment. After 24 h and thereafter, however, there was a great deal of polyp recovery on treated corals (polyp lethality at 24 h; aqueous fraction 37.0±23%; butanol fraction 18.9±12%; non-polar partition 7.0±7.0%). A significant difference was not observed among these treatments, and the controls had zero polyp lethality (Kruskal–Wallis test, KW=1.9, $P>0.05$, $N=3$). Pure clionapyrrolodine A at 1.3 times the natural concentration (0.1372 mg ml^{-1}) and *N*-acetylhomoagmatine (0.1 mg ml^{-1}), tested in a separate assay, also caused 100% of polyps of all three replicates to retract during the 2 h of exposure. However, all polyps had relaxed in clean water after a further 2 to 24 h and were out feeding, thus showing zero lethality. Hence, even though there may exist a partial lethal effect at the fraction level (not significant because of the low replication of the assay), pure clionapyrrolidine A and *N*-acetylhomoagmatine are non-lethal in a 2-h exposure period.

Discussion

The use of bioassay-guided fractionation enabled us to show that *C. tenuis* produces a substance (clionapyrrolidine A) that, at concentrations similar to the natural level within the sponge tissue, kills coral tissue upon forced contact, i.e., in combination with pressure and smothering. This is the first report of a pure chemical substance produced by a sponge that kills coral tissue upon direct contact. One of the major tasks of chemical ecology in general and of sponge ecology in particular is to identify ecological roles associated with the diverse production of secondary metabolites (Becerro 2008). However, the mechanisms by which *C. tenuis* actually uses clionapyrrolidine A to kill coral, while displacing it in competition for space, is not known. As *C. tenuis* does not directly overgrow or smother corals, other mechanisms must be sought. The discussion below concerns the possible ways in which allelopathy may or may not occur in *C. tenuis*–coral interactions.

In portions of *C. tenuis*–coral borders that have not been bitten by corallivorous fish (López-Victoria et al. 2003, 2006), there are unhealthy signs in the coral polyps that confront advancing *C. tenuis*. These include paling, partial bleaching, and half polyps. The latter are the result of partial death or partial detachment from the skeleton (Chaves-Fonnegra et al. 2005). However, these signs do not seem to be the result of the release by *C. tenuis* of clionapyrrolidine A or any other allelopathic substances to the external medium. Our experiments showed that coral tissue was not affected on placing sponge fragments less than 2 cm from the coral for 6 months. Siphonodictidine from the excavating sponge *Aka* (=*Siphonodictyon*) sp. inhibits coral respiration when dissolved in the medium at low concentration (0.0001 mg ml^{-1}) and kills corals through cell lyses at higher concentration (0.1 mg ml^{-1}; Sullivan et al. 1983; Sullivan and Faulkner 1985). In contrast, clionapyrrolidine A is only slightly toxic (non-lethal, eliciting temporary polyp contraction during 2-h exposure) to coral polyps at close to its natural concentration (1.3 times, 0.14 mg ml^{-1}). Even if allelopathic compounds are released, substances such as water-soluble clionapyrrolidine A are probably washed away too quickly by currents for any allelopathic effect to occur (see also Pawlik et al. 2007). Indeed, only through HPLC purification on a Discovery® HS F5 column was it possible to separate clionapyrrolidine A from the aqueous partition (see also Castellanos et al. 2006a). This may explain why a deleterious effect was not observed with the *C. tenuis* extract and active fractions when gels were used in caps slightly elevated from the coral surface or when loosely tied gel strips were tested. In a natural situation, a carrier medium such as mucus would be required to deliver a water-soluble compound to the neighboring coral tissue. Mucus production in *C. tenuis* was not observed. In this context, siphonodictidine previously had been assumed, although not yet proven, to kill coral upon contact when delivered in the abundant mucus produced by the sponge (Sullivan et al. 1983; Sullivan and Faulkner 1985). Unhealthy signs observed in coral tissue that confront *C. tenuis* may thus be the result of processes occurring below the coral polyps, where the sponge is excavating and undermining the polypar skeletal support. Mechanisms that involve the use of clionapyrrolidine A in these processes should be explored.

Allelopathy by *C. tenuis*–coral external tissue contact cannot occur because the external defense mechanisms of the coral kill the contacting sponge tissue, as shown by our experiments. We previously observed that healed cores of *C. tenuis* (López-Victoria and Zea 2004) and *Cliona varians* (unpublished results) implanted in holes surrounded by live coral tissue were killed by the coral defensive appendages within 24 h. It appears that in the normal situation, the undermining by excavating tissue guarantees the weakening of the border polyps or enough separation of the external tissues, thus limiting coral defense. However, there is one natural situation in which forced *C. tenuis*–coral contact occurs. During heavy surge, loose dead coral branches colonized by *C. tenuis* are thrown onto massive live corals. The sponge subsequently is able to invade the massive coral, and then it advances and displaces live tissue by undermining (López-Victoria and Zea 2004). Presumably, the initial crush damages both coral and sponge tissue. Allelopathic compounds may be liberated when producer cells lyse. This release, in combination with smothering by

the fallen branch, could elicit coral tissue weakening and death. This process occurs in a manner similar to allelopathy by contact experiments with gels carried out in this study. The sponge tissue leaning against the coral could then reach down to the now polyp-free coral skeleton and start a new colonization (López-Victoria and Zea 2004). Predator deterrent compounds also are thought to be released when predators bite and crush sponge tissue (Pawlik 1997). Similarly, abrasion and smothering of coral tissue by a non-excavating erect sponge growing close by could elicit release of allelochemicals by the sponge and aid in its subsequent attachment to the dead or dying portion of the coral. Rubbing fragments of a sponge known to have strong bioactive compounds against holes made in another non/toxic sponge inhibited regeneration and regrowth of the hole (Turon et al. 1996). A red alga is known to release chloromertensene, a toxic chlorinated terpene, when abrading neighboring octocorals and sponges, inducing local tissue necrosis (de Nys et al. 1991).

In summary, *C. tenuis* contains clionapyrrolidine A, and this substance, when included in gels, kills coral tissue upon forced contact. This is the first report of a pure chemical substance produced by a sponge that kills coral tissue upon direct contact. Natural forced *C. tenuis*–coral contact occurs during storms, and this process results in local coral death and new colonization by the sponge. However, under normal circumstances, in which *C. tenuis* encrusts and excavates a coral colony and approaches live coral tissue sideward and from underneath, coral death neither occurs through natural external tissue contact nor at distance through mucus or exudation of allelochemicals. Clionapyrrolidine A, however, could be used to kill coral tissue when *C. tenuis* excavating tissue reaches coral polyps from below.

In contrast to clionapyrrolidine A, *N*-acetylhomoagmatine from *C. celata*, when incorporated into gels, did not produce coral death upon forced contact. At concentrations similar to the natural level of clionapyrrolidine A, a solution of *N*-acetylhomoagmatine was only temporality toxic to live coral. These results suggest that the guanidine moiety, which is also present in siphonodictidine, is not a sufficiently strong structural motif to explain the strong deleterious effect of this compound on corals.

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References

Becerro, M. A. 2008. Quantitative trends in sponge ecology research. *Mar. Ecol.* 29:167–177.

Becerro, M. A., Turon, X., and Uriz, M. J. 1995. Natural variation of toxicity in encrusting sponge *Crambe crambe* (Schmidt) in relation to size and environment. *J. Chem. Ecol.* 21:1931–1946.

Becerro, M. A., Turon, X., and Uriz, M. J. 1997. Chemically-mediated interactions in benthic organisms: the chemical ecology of *Crambe crambe* (Porifera, Poecilosclerida). *Hydrobiologia* 356:77–89.

Castellanos, L., Duque, C., Zea, S., Espada, A., Rodríguez, J., and Jiménez, C. 2006a. Isolation and synthesis of (−)-(5*S*)-2-imino-1-methylpyrrolidine-5-carboxylic acid from *Cliona tenuis*. Revised structure of pyrostatin B. *Org. Lett.* 8:4967–4970.

Castellanos, L., Duque, C., Rodríguez, J., and Jiménez, C. 2006b. Synthesis of acetylhomoagmatine. *Mar. Drugs* 4:286–289.

Chaves-fonnegra, A. and Zea, S. 2007. Observations on reef coral undermining by the Caribbean excavating sponge *Cliona delitrix* (Demospongiae, Hadromerida), pp. 247–254 *in* M. R. Custódio, E. Hajdu, G. Lôbo-Hajdu, and G. Muricy (eds.). Sponges—biodiversity, innovation, sustainability. Proceedings of the 7th International Sponge Symposium, Río de Janeiro, Brazil.

Chaves-fonnegra, A., López-victoria, M., Parra-velandia, F., and Zea, S. 2005. Ecología química de las esponjas excavadoras *Cliona aprica*, *C. caribbaea*, *C. delitrix* y *C. tenuis*. *Bol. Inst. Invest. Mar. Cost.* 34:43–67.

de Nys, R., Coll, J. C., and Price, I. R. 1991. Chemically mediated interactions between the red alga *Plocamium hamatum* (Rhodophyta) and the octocoral *Sinularia cruciata* (Alcyonacea). *Mar. Biol.* 108:315–320.

Engel, S., and Pawlik, J. R. 2000. Allelopathic activities of sponge extracts. *Mar. Ecol. Prog. Ser.* 207:273–281.

Gochfeld, D., Harrison, L., Olson, J. Lesser, M. P., Ankisetty, S., and Slattery, M. 2006. Allelopathic effects of the Caribbean sponge *Svenzea zeai* on the coral *Montastraea annularis*. Abstracts, 7th Int. Sponge Symp., Búzios, Río de Janeiro:116.

Hadfield, M. G., and Scheuer, D. 1985. Evidence for a soluble metamorphic inducer in *Phestilla sibogae*: ecological, chemical and biological data. *Bull. Mar. Sci.* 37:556–566.

Hay, M. E., Stachowicz, J. J., Cruz-Rivera, E., Bullard, S., Deal, M. S., and Lindquist, N. 1998. Bioassays with marine and freshwater macroorganisms, pp. 32–122, in K. F. Haynes, and J. G. Millar (eds.). Methods in chemical ecology: bioassay methodsKluwer Academic, London.

Henrikson, A. A., and Pawlik, J. R. 1995. A new antifouling assay method: results from field experiments using extracts of four marine organisms. *J. Exp. Mar. Biol. Ecol.* 194:157–165.

Jackson, J. B. C. 1979. Morphological strategies of sessile animals, pp. 499–555, in G. Larwood, and B. R. Rosen (eds.). Biology and systematics of colonial organismsAcademic, London.

Jackson, J. B. C., and Buss, L. 1975. Allelopathy and spatial competition among coral reef invertebrates. *Proc. Nat. Acad. Sci. U S A* 72:5160–5163.

Lang, J. C. 1973. Interspecific aggression by scleractinian corals. II. Why the race is not only to the swift. *Bull. Mar. Sci.* 23:260–279.

Lang, J. C., and Chornesky, E. H. 1990. Competition between scleractinian reef corals — a review of mechanisms and effects, pp. 209–252, in Z. Dubinsky (ed.). Ecosystems of the world 25: coral reefsElsevier, Amsterdam.

Lenis, L. A., Núñez, L., Jiménez, C., and Riguera, R. 1996. Isonitenin and acetylhomoagmatine, new metabolites from the sponges *Spongia officinalis* and *Cliona celata* collected at the Galician coast (N.W. Spain). *Nat. Prod. Lett.* 8:15–23.

LOCKE, J. M., WEIL, E., and COATES, K. A. 2007. A newly documented species of *Madracis* (Scleractinia: Pocilloporidae) from the Caribbean. *Proc. Biol. Soc. Wash.* 120:14–226.

LÓPEZ-VICTORIA, M., and ZEA, S. 2004. Storm-mediated coral colonization by an excavating Caribbean sponge. *Clim. Res.* 26:251–256.

LÓPEZ-VICTORIA, M., and ZEA, S. 2005. Current trends of space occupation by encrusting excavating sponges on Colombian coral reefs. *Mar. Ecol.* 26:33–41.

LÓPEZ-VICTORIA, M., ZEA, S., and WEIL, E. 2003. New aspects on the biology of the excavating sponge complex *Cliona caribbaea–C. langae–C. aprica*. *Boll. Mus. Ist. Biol. Univ. Genova* 68:425–432.

LÓPEZ-VICTORIA, M., ZEA, S., and WEIL, E. 2006. Competition for space between encrusting excavating Caribbean sponges and other coral reef organisms. *Mar. Ecol. Prog. Ser.* 312:113–121.

MIYAMOTO, T., YAMADA, K., IKEDA, N., KOMORI, T., and HIGUCHI, R. 1994. Bioactive terpenoids from Octocorallia, I. Bioactive diterpenoids: litophynols A and B from the mucus of the soft coral *Litophyton* sp. *J. Nat. Prod.* 57:1212–1219.

NISHIYAMA, G. K., and BAKUS, G. J. 1999. Release of allelochemicals by three tropical sponges (Demospongiae) and their toxic effects on coral substrate competitors. *Mem. Qld. Mus.* 44:411–417.

NISHIYAMA, G. K., BAKUS, G. J., and KAY, C. A. 2004. The effects of sponges and sponge metabolites on the settlement, growth and association of substratum competitors. *Boll. Mus. Ist. Biol. Univ. Genova* 68:499–508.

PANG, R. K. 1973. The systematics of some Jamaican excavating sponges (Porifera). *Postilla* 161:1–75.

PAUL, V. J., and PUGLISI, M. 2004. Chemical mediation of interactions among marine organisms. *Nat. Prod. Rep.* 21:189–209.

PAWLIK, J. R. 1993. Marine invertebrates chemical defenses. *Chem. Rev.* 93:1911–1922.

PAWLIK, J. R. 1997. Fish predation on Caribbean reef sponges: An emerging perspective of chemical defenses. Proc. 8th Int. Coral. Reef. Sym. 2:1255–1258.

PAWLIK, J. R., STEINDLER, L., HENKEL, T. P., BEER, S., and ILAN, M. 2007. Chemical warfare on coral reefs: Sponge metabolites differentially affect coral symbiosis *in situ*. *Limnol. Oceanogr.* 52:907–911.

PORTER, J. W., and TARGETT, N. M. 1988. Allelochemical interactions between sponges and corals. *Biol. Bull.* 175:230–239.

RÜTZLER, K. 2002. Impact of crustose clionid sponges on Caribbean reef corals. *Acta Geol. Hisp.* 37:61–72.

SCHMITT, T. M., HAY, M. E., and LINDQUIST, N. 1995. Constraints on chemically mediated coevolution: multiple functions for seaweed secondary metabolites. *Ecology* 76:107–123.

SCHÖNBERG, C. H. L., and WILKINSON, C. R. 2001. Induced colonization of corals by a clionid bioeroding sponge. *Coral Reefs* 20:69–76.

SIEGEL, S., C, and Jr, N. J. 1988. Non-parametric statistics for the behavioral sciences. 2nd edn.McGraw Hill, New York.

SLATTERY, M., HAMANN, M. T., MCCLINTOCK, J. B., PERRY, T. L., PUGLISI, M. P., and YOSHIDA, W. Y. 1997. Ecological roles for water-borne metabolites from Antarctic soft corals. *Mar. Ecol. Prog. Ser.* 161:133–144.

SOKAL, R. R., and ROHLF, F. J. 1981. Biometry: the principles and practice of statistics in biological research. 2nd edn.Freeman, San Francisco.

SULLIVAN, B. W., and FAULKNER, D. J. 1985. Chemical studies of the burrowing sponge *Siphonodictyon coralliphagum*, pp. 45–50, in K. Rützler (ed.). New perspectives in sponge biologySmithsonian Institution Press, Washington DC.

SULLIVAN, B., FAULKNER, D. J., and WEBB, L. 1983. Siphonodictidine, a metabolite of the burrowing sponge *Siphonodictyon* sp. that inhibits coral growth. *Science* 221:1175–1176.

TARGETT, N. M., and SCHMAHL, G. 1984. Chemical ecology and distribution of sponges in the Salt River Canyon, St. Croix, U.S. V.I. USA. NOAA Technical memorandum OAR NURP-1. Rockville, Maryland.

THACKER, R. W., BECCERRO, M. A., LUMBANG, W. A., and PAUL, V. J. 1998. Allelopathic interactions between sponges on a tropical reef. *Ecology* 79:1740–1750.

THOMPSON, J. E., BARROW, K. D., and FAULKNER, D. J. 1983. Localization of two brominated metabolites, aerothionin and homoaerothionin, in spherulous cells of the marine sponge *Aplysina fistularis* (=*Verongia thiona*). *Acta Zool.* 64:199–210.

TURON, X., BECERRO, M. A., URIZ, M. J., and LLOPIS, J. 1996. Small-scale association measures in epibenthic communities as clues for allelochemical interactions. *Oecologia* 108:351–360.

TURON, X., BECERRO, M. A., and URIZ, M. J. 2000. Distribution of brominated compounds within the sponge *Aplysina aerophoba*: coupling of X-ray microanalysis with cryofixation techniques. *Cell Tissue Res.* 301:311–322.

URIZ, M. J., BECERRO, M. A., TUR, J. M., and TURON, X. 1996. Location of toxicity within the Mediterranean sponge *Crambe crambe* (Demospongiae: Poecilosclerida). *Mar. Biol.* 124:583–590.

WALKER, R. P., THOMPSON, J. E., and FAULKNER, D. J. 1985. Exudation of biologically-active metabolites in the sponge *Aplysina fistularis*. II. Chemical evidence. *Mar. Biol.* 88:27–32.

WILLIAMS, E. H., BARTELS, P. J., and BUNKLEY-WILLIAMS, L. 1999. Predicted disappearance of coral-reef ramparts: a direct result of major ecological disturbances. *Global Change Biol.* 5:839–845.

WOODIN, S. A. 1993. Allelochemical inhibition of recruitment in a sedimentary assemblage. *J. Chem. Ecol.* 19:517–530.

WOODIN, S. A., and JACKSON, J. B. C. 1979. Interphyletic competition among marine benthos. *Amer. Zool.* 19:1029–1043.

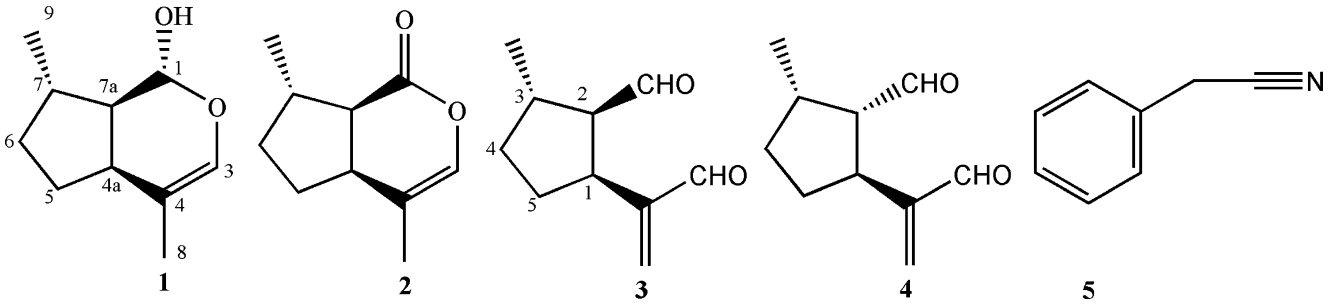

Fig. 1 **1** (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol, **2** (4a*S*,7*S*,7a*R*)-nepetalactone, **3** (1*S*,2*R*,3*S*)-dolichodial, **4** (1*S*,2*S*,3*S*)-dolichodial, **5** phenylacetonitrile

behavioral response is greater with the ratio identified from conspecific oviparae (Dawson et al. 1990; Hardie et al. 1990; Lilley and Hardie 1996; Boo et al. 2000). However, some experiments have suggested that the two iridoids do not always convey species integrity. For example, the ratio of (4a*SR*,7*SR*,7a*RS*)-nepetalactone/(1*RS*,4a*SR*,7*SR*,7a*RS*)-nepetalactol released from different species of *Cryptomyzus* oviparae were similar, but *Cryptomyzus galeopsidis* (European blackcurrant aphid) males could distinguish between the sex pheromone released by conspecific oviparae and the sex pheromone from other species of *Cryptomyzus* (Guldemond et al. 1992). Male *C. galeopsidis* also could discriminate between a synthetic pheromone blend 30:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1)/(4a*S*,7*S*,7a*R*)-nepetalactone (2) and volatiles from conspecific oviparae (Guldemond et al. 1993). A stronger, positive behavioral response to the conspecific oviparae occurred, suggesting that the sex pheromone of some aphids is likely to comprise more than two components.

The holocyclic heteroecious aphid *Dysaphis plantaginea* (rosy apple aphid) is the second most important pest of apples in Europe and North America after the codling moth (*Cydia pomonella*) (Graf 1999; Wyss et al. 1999; Blommers et al. 2004). It colonizes apple (*Malus domestica*) as its primary host and plantain (*Plantago* spp.) as the secondary host (Blackman and Eastop 2000). In apple, *D. plantaginea* impairs shoot growth, reduces the formation of flowers over winter, gives rise to leaf curl, and causes malformation and reduction in size of the fruit (Forrest and Dixon 1975; Blommers et al. 2004). Apple yield can be reduced by as much as 45% (De Berardinis et al. 1994; Blommers et al. 2004). Stewart-Jones et al. (2007) determined that *D. plantaginea* oviparae release (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (1) and (4a*S*,7*S*,7a*R*)-nepetalactone (2) in a 4:1 ratio.

In this paper, we investigate whether chemicals in addition to the nepetalactone and nepetalactols are released from oviparae as part of the aphid sex pheromone. The identification of other chemicals that may play a role in species integrity will add a new dimension to our understanding of aphid sex pheromones, and additional chemicals could be exploited as part of integrated pest management systems. To investigate the role of other components, *D. plantaginea* is used as a model.

Methods and Materials

Insects *Dysaphis plantaginea* oviparae were collected from an apple (*Malus silvestris* c.v. Braburn) orchard (Leckford Fruit Farm, Leckford Estate, Hampshire, UK). Male *D. plantaginea* were obtained by rearing on apple (*M. silvestris* c.v. Braburn) in a controlled environment room (12L/12D regime; photophase 16±0.5°C; scotophase 12±0.5°C).

The aphid parasitoid *Aphidius ervi* was purchased as mummies from Koppert Biological Systems (Product: Ervipar). The mummies were placed into a Petri dish (9-cm diameter, Scientific Laboratory Supplies), and the adult parasitoids emerged into a ventilated polypropylene breeding cage (30×30×30 cm, Bugdorm 1, Watkins & Doncaster, Kent, UK). Honey solution (1:1 honey/water) on cotton wool was provided as a food source. Emergence cages were kept in a controlled environment room (20°C, 25–40% RH, 16L/8D regime). All parasitoids used in laboratory experiments were naïve-mated females, 1–3 d old.

Isolation of Volatiles The base of an excised apple (*M. silvestris* c.v. Braburn) branch, bearing leaves infested with *D. plantaginea* oviparae (various adult ages), was placed in a glass vessel (500 ml) containing water. The branch was then placed into a glass entrainment vessel (1.5 L). A metal plate containing a hole for the apple branch was clipped to the base of the glass vessel. As a hole was present in the entrainment set-up (an open system), air that had been purified by passage through an activated charcoal filter (BDH, 10–14 mesh, 50 g) was pushed into (700 ml min^{-1}) and pulled out of (600 ml min^{-1}) the vessel. Excised leaves heavily infested with *D. plantaginea* oviparae (various adult ages) were also placed in a glass vessel (500 ml). Air that had been purified by passage through an activated charcoal filter (BDH, 10–14 mesh, 50 g) was

pulled (600 ml min^{-1}) out of the vessels. Volatiles were also collected from uninfested apple leaves and an uninfested apple branch with leaves as controls.

Volatiles were trapped onto Porapak Q 50/80 (50 mg; Supelco, Bellefonte, PA, USA) held in glass tubing (5 mm outer diameter) by two plugs of silanized glass wool. The Porapak Q was conditioned by washing with redistilled diethyl ether (5 ml) and heating at 132°C for 2 hr under a stream of purified N_2. After the air entrainment, volatiles were eluted from the Porapak with redistilled diethyl ether (750 µl), and samples were stored in a freezer (−22°C). Because preliminary tests showed that the quantities of pheromone released were low, subsequent entrainment using Porapak Q was carried out over a 4-d period. The procedure was repeated for two additional 4-d periods.

Analysis of Volatiles Air entrainment samples were analyzed by gas chromatography (GC) on both polar (DB-wax, 30 m×0.32 mm inner diameter × 0.5 µm film thickness) and non-polar (HP-1, 50 m×0.32 mm inner diameter×0.5-µm film thickness) capillary columns with a HP5890 GC (Agilent Technologies, UK) fitted with a cool-on-column injector, a deactivated retention gap (1 m×0.53 mm inner diameter), and a flame ionization detector (FID). The GC oven temperature was maintained at 30°C for 1 min after sample injection and then raised by 5°C min^{-1} to 150°C, then 10°C min^{-1} to 240°C. The carrier gas was hydrogen. Peak enhancement by co-injection with a chemical standard was done to confirm tentative identification of the chemicals present. A multiple-point external standard method was used to quantify the amount of identified chemical components present in the air entrainment samples. Coupled gas chromatography-mass spectrometry (GC-MS) analysis was performed on a Thermofinnigan Instrument MAT95 XP double-focusing magnetic sector mass spectrometer coupled to a TRACE GC fitted with an HP-1 column and integrated data system (Fisons Instruments, Manchester, UK). The GC oven temperature was maintained at 30°C for 5 min and then programmed at 5°C min^{-1} to 250°C. Ionization was by electron impact at 70 eV, 250°C (source temperature).

Electrophysiology The air entrainment sample collected from *D. plantaginea* oviparae was tested by using coupled gas chromatography-electroantennography (GC-EAG) with male *D. plantaginea*. Two Ag–AgCl glass electrodes were filled with saline solution [composition as in Maddrell (1969), but without glucose]. The head was excised and placed into the indifferent electrode, and the tips of the antennae were severed and inserted into the recording electrode. The signals were passed through a high impedance amplifier (UN-06, Syntech®, The Netherlands) and analyzed by using a customized software package (EAG 2000 and GC-EAG 2000, Syntech®, The Netherlands). Preparations were held in a humidified, charcoal filtered air stream (1 l min^{-1}) coming from a glass tube outlet positioned 0.5 cm from the preparation.

Separation of the volatiles was achieved by GC on a non-polar (HP-1, 50 m×0.32 mm inner diameter×0.52-µm film thickness) capillary column using an HP5890 GC (Agilent Technologies, UK) fitted with a cold on-column injector and a FID. The oven temperature was maintained at 30°C for 2 min and then programmed at 5°C min^{-1} to 100° C and then at 10°C min^{-1} to 250°C. The carrier gas was hydrogen. The outputs from the EAG amplifier and the FID were monitored simultaneously and analyzed with the software package (EAG 2000, Syntech®, The Netherlands). Chromatograms were compared visually by overlaying traces on a light box and matching corresponding EAG peaks. Six replicates were done.

EAG recordings were also made from male and gynoparous *D. plantaginea* with three of the identified chemicals (10 µg). The delivery system employed a filter paper in a disposable Pasteur pipette cartridge. The stimulus (2 sec duration, 100 ml min^{-1}) was delivered into a purified air stream (900 ml min^{-1} during stimulus delivery, 1 l min^{-1} before and after stimulus delivery) flowing continuously over the preparation. Solutions of synthetic compounds were made in distilled hexane and applied to a filter paper strip. The solvent was allowed to evaporate for 30 sec before the strip was placed in the cartridge. The control stimulus was hexane. Fresh cartridges were prepared immediately prior to each experiment. Responses were compared for significant differences by using Student's *t* test. Six replicates were done.

Chemical Standards (1*R*,4a*S*,7*S*,7a*R*)-Nepetalactol (**1**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) were synthesized as stated in Dawson et al. (1996). Phenylacetonitrile (benzyl cyanide, **5**) was obtained from Sigma-Aldrich, UK (99% purity).

(1*S*,2*R*,3*S*)-Dolichodial (**3**) (Fig. 1) was extracted from cat thyme, *Teucrium marum* (Jekka's Herb Farm, Bristol, UK). The aerial parts of *T. marum* (102.58 g) were extracted with chloroform (2×800 ml) for 24 hr at ambient temperature. The solvent was removed under reduced pressure to yield a golden-brown gum (2.97 g). The extract was subjected to liquid chromatography over Florisil (100–200 mesh, Sigma-Aldrich) with hexane/diethyl ether (1:1) to yield a pale oil (912 mg). Bulb-to-bulb distillation using a Kugelrohr apparatus (90°C, 2 mmHg) yielded four fractions, one of which was shown by comparison of MS, ^{1}H and ^{13}C NMR data with literature values (Pagnoni et al. 1976) to contain (1*S*,2*R*,3*S*)-dolichodial (**3**) and (1*S*,2*S*,3*S*)-dolichodial (**4**) (Fig. 1) in a 9:1 ratio (Bellesia et al. 1983a).

Four-Way Olfactometer Aphid behavioral assays were done by using a Perspex four-way olfactometer [modified from Pettersson (1970), 120 mm diameter]. Air was removed from the center of the olfactometer by a vacuum pump, buffered by a 2-l jar and adjusted with a flow meter to 400 ml min^{-1}. Air was thus pulled through each of the four side arms at 100 ml min^{-1}, and again verified with airflow meters. Teflon tubing was used to attach a glass vessel (25 ml) and a flow meter to each of the four side arms. Polytetrafluoroethylene tape was used to ensure airtight seals between the olfactometer and the Teflon tubing. All five holes were covered with a layer of muslin to prevent access by the aphid during bioassays. To remove any visual stimuli, the olfactometer was placed in the center of a black-walled box with an observation opening at the front and lit from above with diffuse uniform lighting.

Two experiments were conducted. First, the response of male *D. plantaginea* to different amounts of (1*S*,2*R*,3*S*)-dolichodial (**3**) (10, 1, 0.1, and 0.01 µg) and hexane (control) was tested. Second, two ratios, 4:1:0 (40:10:0 µg) and a 4:1:0.05 (40:10:0.5 µg) of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**) and hexane (control) were tested. An aliquot of the test solution was applied with a micropipette (Drummond "microcaps," Drummond Scientific Co., USA) to a filter paper strip (solvent allowed to evaporate for 30 sec). The filter paper was placed into one of the glass vessels (25 ml). The three control vessels were similarly treated with the same volume of solvent on the filter paper. Sixteen replicates were done. All treatments were tested in a randomized block experimental design.

The four-way olfactometer arena was split into five areas (four areas by each arm and a central area). A single aphid was introduced into the central area. The time spent and number of entries into each area was recorded by using specialist software (OLFA, Udine, Italy) over a 16-min period. The apparatus was rotated 90° every 4 min to eliminate bias. The proportion of time spent in each of the four side areas was logit-transformed with a correction factor in order to avoid extreme values (Rawlings et al. 1998). The proportion of entries into each of the four areas out of the total number of entries was also calculated. The data were compared by analysis of variance (ANOVA) with randomized blocking (Montgomery 1997), as implemented in Genstat 8.0 (Payne et al. 2005). The analysis was used to look at two parameters: (1) the difference between the treated and control arms and (2) the difference between the three control arms.

Four-Choice Olfactometer A four-choice olfactometer (Vamvatsikos 2006) [modified from Douloumpaka and van Emden (2003)] was used to test the behavioral response of naïve female *A. ervi* to (1*S*,2*R*,3*S*)-dolichodial (**3**). The olfactometer was made from four cylindrical Perspex tubes (test-odor chambers, 9.5×2.5 cm internal diameter) each connected to a flow meter by Teflon tubing and a central arena by four smaller pieces of Perspex tubing (4×0.5 cm internal diameter). These small tubes protruded 2 cm into the cylindrical Perspex tubes but were flush with the internal surface of the central arena. The central arena comprised a Perspex tube (4.5 cm internal diameter×2.5 cm height) and a Perspex lid covered the pot firmly, forming an air-tight central arena. The lid was fitted with a small tube connecter (0.5 cm internal diameter) by Teflon tubing to a flow meter and a vacuum pump, buffered by a 2-l jar.

Two experiments were conducted. First, the response of *A. ervi* to different amounts of (1*S*,2*R*,3*S*)-dolichodial (**3**) (10, 1, 0.1, and 0.01 µg), a control (hexane), and a positive control [1:1 (10:10 µg) (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)] was tested. Second, two ratios 1:1:0 (10:10:0 µg) and a 1:1:0.05 (10:10:0.5 µg) of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**), a control (hexane), and a positive control [1:1 (10:10 µg) (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)] were tested. Glinwood (1998) showed that, in a four-way olfactometer, mated naïve female *A. ervi* spent significantly more time in the test arm containing 1:1 (10:10 µg) (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**) compared to the control arms (10 µl of hexane), hence the use of this ratio.

An aliquot of the test solution was applied with a micropipette (Drummond "microcaps," Drummond Scientific Co., USA) to a filter paper strip (solvent allowed to evaporate for 30 sec). The filter paper was placed into two of the cylindrical, Perspex test-odor chambers. The two control test-odor chambers were similarly treated with the same volume of solvent on the filter paper. Sixteen replicates were done. All treatments were tested in a randomized block experimental design.

Twelve mated naïve female *A. ervi* were drawn into the central arena of the olfactometer by using a pooter. The insects were left to acclimatize for 15 min before the experiment commenced. To remove any visual stimuli, the olfactometer was placed in the center of a black-walled box. Air was removed from the center of the olfactometer at a flow rate of 1.6 l min^{-1}. Air was thus pulled through each of the four side arms at 400 ml min^{-1}. After 30 min, parasitoids that had entered the odor chambers were counted. The whole system (central arena and side arms) was rotated 90° clockwise after each replicate to cancel out any directional bias in the apparatus.

Chi-square analysis was performed on the results from the four-choice olfactometer. The choice chi (which tests

the significance of variation between choices) and the heterogeneity chi (which tests the significance of variation between the replicates) were taken into consideration (Gomez 1984).

Results

Electrophysiology Coupled GC-EAG analysis with male *D. plantaginea* revealed four EAG-active compounds in the air entrainment sample collected from *D. plantaginea* oviparae (Fig. 2). Gas chromatography-mass spectrometry and peak enhancement by co-injection using non-polar (HP-1) and polar (DB-Wax) columns confirmed that the peaks were phenylacetonitrile (**5**), (1*SR*,2*RS*,3*SR*)-dolichodial (**3**), (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**), and (4a*S*,7*S*,7a*R*)-nepetalactone (**2**). (1*R*,4a*S*,7*S*,7a*R*)-Nepetalactol (**1**), (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), and (1*SR*,2*RS*,3*SR*)-dolichodial (**3**) were present in a 4:1:0.05 ratio. These compounds were not detected in the air entrainment samples of apple leaves without *D. plantaginea*.

Male and gynoparous *D. plantaginea* showed a significantly greater EAG response to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**), (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), and (1*S*,2*R*,3*S*)-dolichodial (**3**) compared with the control (hexane) (Fig. 3).

Behavioral Response of Male Dysaphis plantaginea Male *D. plantaginea* spent more time in (P=0.025) and made a higher proportion of entries (P=0.012) into the arm containing 1 µg (1*S*,2*R*,3*S*)-dolichodial (**3**) compared to the control arms (Fig. 4). Male *D. plantaginea* also spent more time (P=0.016) in the arm containing 0.1 µg (1*S*,2*R*,3*S*)-dolichodial (**3**) compared to the control arms. The time male *D. plantaginea* spent in and the proportion of entries into the arms containing 10 µg and 0.01 µg of (1*S*,2*R*,3*S*)-dolichodial (**3**) were not significantly different compared to the control arms. A significant difference was not seen between any of the control arms.

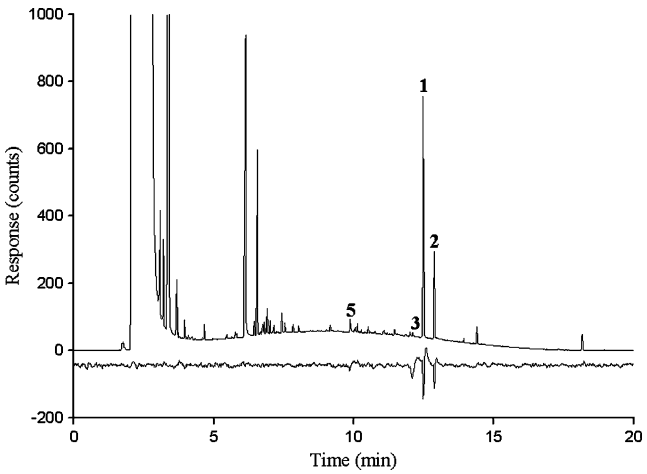

Fig. 2 Example of a coupled GC-EAG trace of male *Dysaphis plantaginea* responses to an air entrainment sample from conspecific oviparae. Top trace corresponds to the FID detector on the GC, and the bottom trace corresponds to the antennal response of the insect preparation. *Numbers* refer to chemicals *1* (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**), *2* (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), *3* (1*S*,2*R*,3*S*)-dolichodial (**3**) and *5* phenylacetonitrile (**5**)

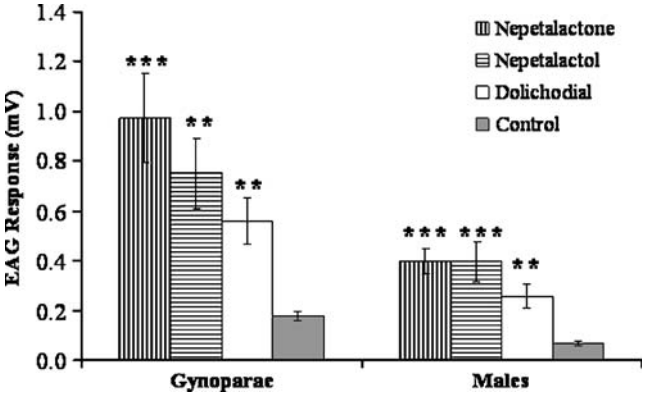

Fig. 3 Electrophysiological response (mean±SE) of male and gynoparous *Dysaphis plantaginea* to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**), (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), and (1*S*,2*R*,3*S*)-dolichodial (**3**) standards. *Asterisks* indicate significant differences from the control (solvent) determined using Student's *t* test (see text) (**P<0.01, ***P<0.001, N=6)

Male *D. plantaginea* spent a greater proportion of time in (P=0.014) and made a higher proportion of entries (P< 0.001) into the arm of the olfactometer where the three-component mixture [4:1:0.05 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**)] was present compared to the control arms (Fig. 5). The proportion of time spent in and the proportion of entries into the arms containing the two-component mixture [4:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)] compared to the control arms were not significantly different. A significant difference was not observed between any of the control arms.

Behavioral Response of Aphidius ervi More naïve-mated female *A. ervi* were counted in the arms containing the positive control (P<0.001), 10 µg (P<0.001), and 1 µg (P< 0.01) of (1*S*,2*R*,3*S*)-dolichodial (**3**) compared to the control arms (Fig. 6). The number of naïve-mated female *A. ervi* counted in the arms containing 0.1 and 0.01 µg (1*S*,2*R*,3*S*)-dolichodial (**3**) was not significantly different compared to the control arms.

The number of naïve-mated female *A. ervi* counted in arms containing 1:1:0.05 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**) was not significantly different compared to the arms containing 1:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**) (Fig. 7).

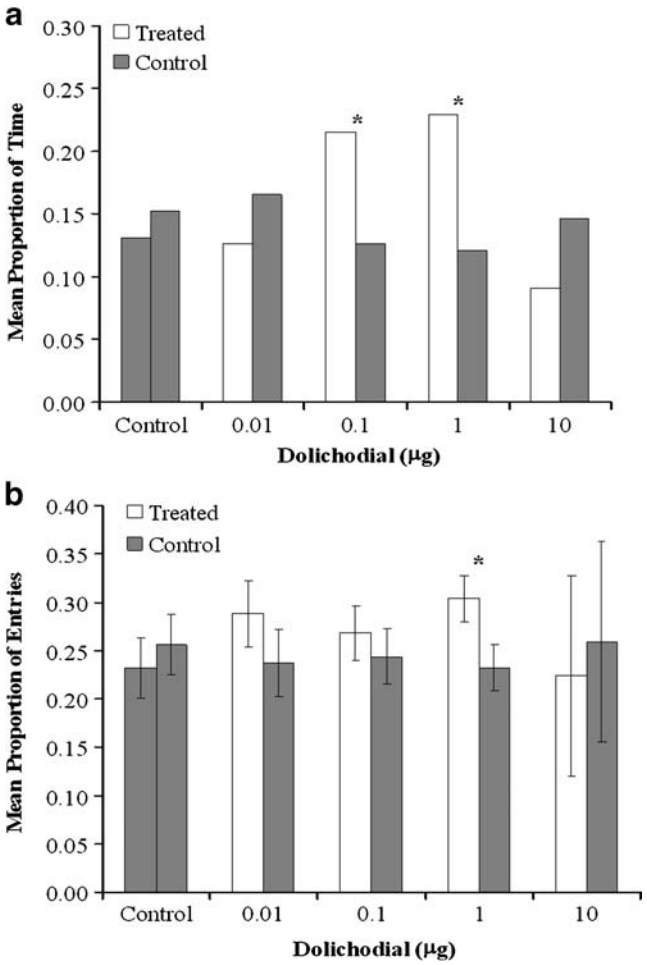

Fig. 4 The response, **a** back-transformed mean proportion of time spent and **b** mean proportion of entries±SE, by male *Dysaphis plantaginea* to different amounts of (1*S*,2*R*,3*S*)-dolichodial (**3**) in the four-way olfactometer. *Asterisks* indicate significant differences from the control (solvent) determined using ANOVA (see text) (**P*<0.05, ***P*<0.01, ****P*<0.001, *N*=16)

Discussion

Electrophysiological responses by male *D. plantaginea* to four chemicals present in volatiles collected from *D. plantaginea* oviparae were recorded. The EAG-active chemicals were identified as (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**), (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), (1*S*,2*R*,3*S*)-dolichodial (**3**), and phenylacetonitrile (**5**). These four compounds were not detected in the air entrainment sample collected from the host plant, suggesting that they are either released by oviparae or by the plant in response to oviparae feeding. As previously discussed, (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) may be components of *D. plantaginea* sex pheromone (Stewart-Jones et al. 2007).

Past research has identified phenylacetonitrile (**5**) as an insect and a plant volatile, involved in insect–plant (Leal et al. 1994; Bartlet et al. 1997) and insect–insect (Norris and Pener 1965; Obeng-Ofori et al. 1993; Torto et al. 1994; Loughrin et al. 1995) interactions. With regard to plant volatiles, phenylacetonitrile (**5**) has been identified from leaf tissue (Macleod et al. 1981; Loughrin et al. 1995) and flowers (Tatsuka et al. 1990; Knudsen et al. 1993; Leal et al. 1994) but, relevant to this study, occurs as a major volatile from apple fruit (Boeve et al. 1996). This may suggest that phenylacetonitrile (**5**) present in the air entrainment sample of *D. plantaginea* oviparae originated from the aphid/plant complex. Thus, phenylacetonitrile (**5**) is most likely not a component of the aphid sex pheromone, but male *D. plantaginea* may utilize it synergistically with the sex pheromone components to locate conspecific oviparae (Powell and Hardie 2001). As phenylacetonitrile (**5**) is thought not to be a component of the aphid sex pheromone, no behavioral studies were conducted.

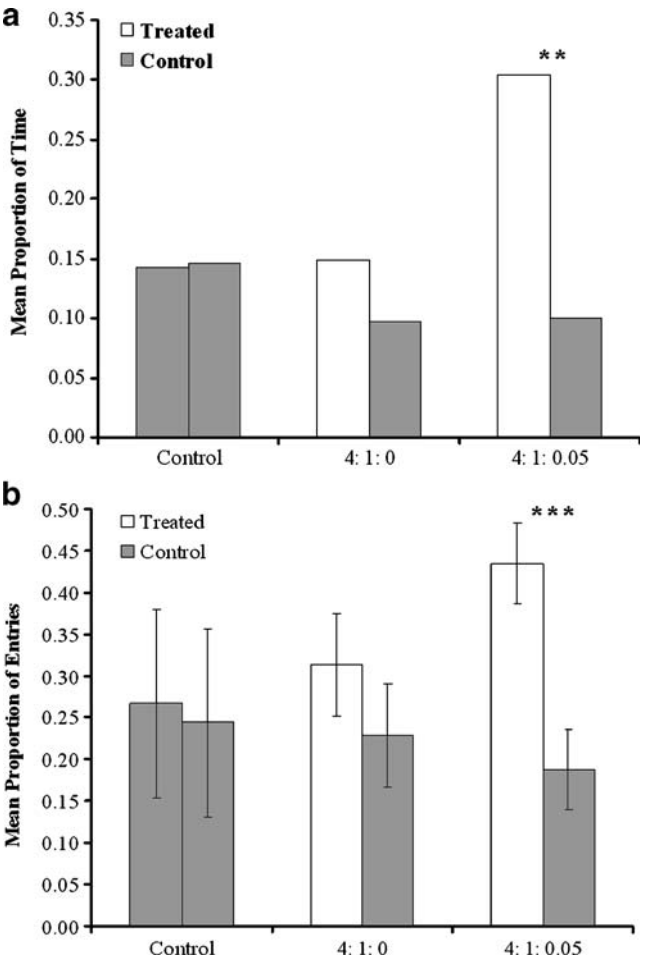

Fig. 5 The response (back-transformed mean proportion of time-spent) by male *Dysaphis plantaginea* to different ratios of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**) in a four-way olfactometer. *Asterisks* indicate statistically significant differences from the control (solvent) determined using ANOVA (see text) (****P*<0.001, *N*=16)

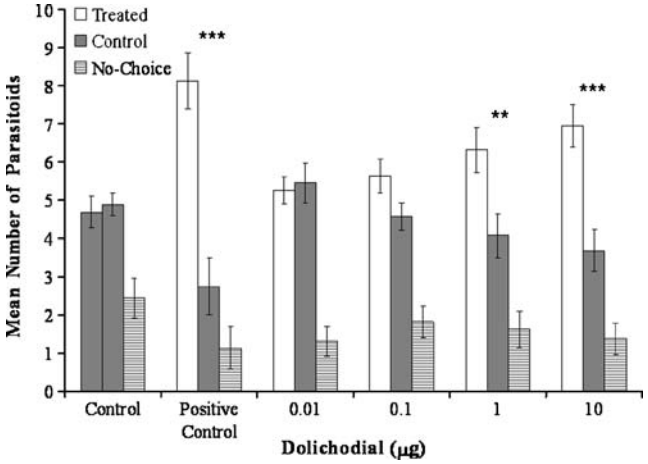

Fig. 6 The response (mean number±SE) of naïve-mated female *Aphidius ervi* to different amounts of (1*S*,2*R*,3*S*)-dolichodial (**3**) in a four-choice olfactometer. *Asterisks* indicate statistically significant differences from the control (solvent) determined using χ^2-test (see text) (**P<0.01, ***P<0.001, N=16)

The fourth compound, (1*S*,2*R*,3*S*)-dolichodial (**3**), is released by insects. *Dolichoderus* and *Iridomyrmex* species of ants release (1*S*,2*R*,3*S*)-dolichodial (**3**), and it may play a defense or trail role (Cavill and Hinterberger 1960; Cavill and Houghton 1974; Cavill et al. 1982). The diastereoisomer anisomorphal [(1*S*,2*S*,3*S*)-dolichodial (**4**) (Pagnoni et al. 1976)] is a major component of the defense secretion of the Southern walking stick insect, *Anisomorpha buprestoides* (Meinwald et al. 1962). In addition, (1*S*,2*R*,3*S*)-dolichodial (**3**) is structurally related to (4a*S*,7*S*,7a*R*)-nepetalactone (**2**), a known component of the aphid sex pheromone. Both are methylcyclopentanoid terpenes thought to originate biosynthetically from citronellol. Dawson et al. (1996) suggested that citronellol may be a precursor for the cyclopentanoids biosynthesized in aphids, as during studies on the composition of the sex pheromone of several species of aphid, including *D. plantaginea*, citronellol accompanied the cylcopentanoid sex pheromone components but was electrophysiologically and behaviorally inactive. Although the biosynthetic pathways from citronellol to (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) in *Nepeta cataria* (Lamiaceae=Labiatae) (Bellesia et al. 1984) and (1*S*,2*R*,3*S*)-dolichodial (**3**) in cat thyme, *T. marum* (Bellesia et al. 1983b), are thought to be different, when [10–^{3}H](1*S*,2*R*,3*S*)-dolichodial was fed to cut stalks of *N. cataria*, partial incorporation into (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) was observed (Bellesia et al. 1984). This suggests that the (1*SR*,2*RS*,3*SR*)-dolichodial (3) present in the air entrainment sample of *D. plantaginea* oviparae may originate from the oviparae and may be a third component of the sex pheromone.

In behavioral assays, (1*S*,2*R*,3*S*)-dolichodial (**3**) elicited a response by male *D. plantaginea*. This suggests that (1*S*,2*R*,3*S*)-dolichodial (**3**) may be an attractant or an arrestant. This is the first time that electrophysiological and behavioral responses by any aphid morph to (1*S*,2*R*,3*S*)-dolichodial (**3**) have been reported. Behavioral responses were not recorded when the two-component mixture [4:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)] was present in the bioassay. However, when (1*S*,2*R*,3*S*)-dolichodial (**3**) was present in a three component mixture [4:1:0.05 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**)] with a ratio equivalent to the ratio in the air entrainment sample, a behavioral response by male *D. plantaginea* was recorded.

These behavioral data add weight to the possibility that (1*S*,2*R*,3*S*)-dolichodial (**3**) is a component of the aphid sex pheromone. In addition, mass spectrometric analysis on air entrainment samples collected at Rothamsted Research from *Rhopalosiphum padi* (bird-cherry-oat aphid), *Aphis fabae* (black bean aphid), *Cryptomyzus maudamanti*, and *Cryptomyzus ribis* (redcurrent blister aphid) oviparae all contain a chemical with the same mass spectra as (1*S*,2*R*,3*S*)-dolichodial (**3**) (Pickett, Wadhams and Woodcock, unpublished data). As discussed, biological evidence suggests that the sex pheromone of the *Cryptomyzus* species is likely to comprise more than just the (4a*SR*,7*SR*,7a*RS*)-nepetalactone and (1*RS*,4a*SR*,7*SR*,7a*RS*)-nepetalactol. As (1*S*,2*R*,3*S*)-dolichodial (**3**) elicits a behavioral response in male *D. plantaginea*, this chemical may also be a component of the sex pheromone of *Cryptomyzus* species and play a role in species integrity.

(1*S*,2*R*,3*S*)-Dolichodial (**3**) not only elicits a behavioral response by male *D. plantaginea* but also an

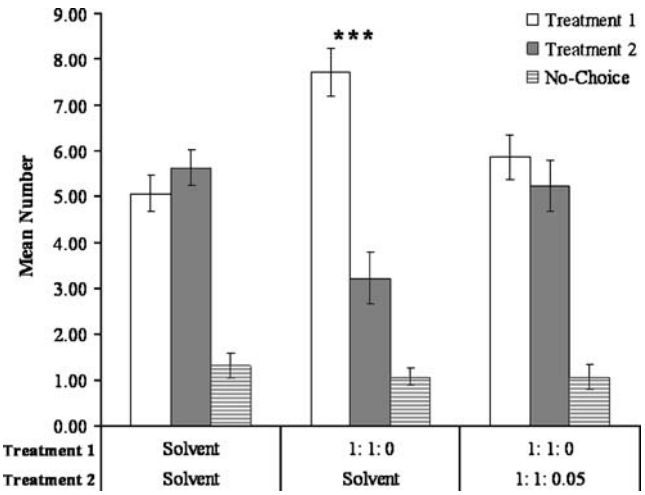

Fig. 7 The response (mean number±SE) of naïve-mated female *Aphidius ervi* to different ratios of (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**)/(1*S*,2*R*,3*S*)-dolichodial (**3**) in a four-choice olfactometer. *Asterisks* indicate statistically significant differences between treatments 1 and 2 determined using a χ^2-test (see text) (***P<0.001, N=16)

electrophysiological response by gynoparous *D. plantaginea*. Electrophysiological and behavioral responses by gynoparae to (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**) and (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) have been reported previously (Hardie et al. 1994; Lösel et al. 1996; Park et al. 2000; Zhu et al. 2006). It was suggested that the aphid sex pheromone may act as an aggregation pheromone for gynoparae in order to locate conspecific oviparae on suitable host plants (Lilley and Hardie 1996; Powell and Hardie 2001; Zhu et al. 2006). Therefore, if (1*S*,2*R*,3*S*)-dolichodial (**3**) is part of the aphid sex pheromone, an EAG response in gynoparae would be expected. Behavioral assays are required to determine whether this compound elicits a behavioral response by gynoparae.

Aphid pheromones provide an ideal method for parasitoids to locate hosts, as they are specific to aphids. If (1*S*,2*R*,3*S*)-dolichodial (**3**) is part of the sex pheromone of certain aphid species, it may be perceived by *A. ervi* as a pheromone component and, therefore, should elicit a behavioral response. In this paper, a behavioral response by naïve-mated female *A. ervi* to (1*S*,2*R*,3*S*)-dolichodial (**3**) was indeed recorded. However, when a choice was available between the two-component mixture and the three-component mixture, no significant differences were recorded in a four-choice olfactometer. A behavioral response of naïve-mated female *A. ervi* toward a 1:1 (1*R*,4a*S*,7*S*,7a*R*)-nepetalactol (**1**)/(4a*S*,7*S*,7a*R*)-nepetalactone (**2**) mixture and to (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) alone has already been reported (Glinwood 1998). This suggests that the presence of (4a*S*,7*S*,7a*R*)-nepetalactone (**2**) may be needed only to elicit a strong behavioral response by *A. ervi*.

Air entrainments with oviparae on artificial diets and radio-labeling studies could be conducted to assess whether (1*SR*,2*RS*,3*SR*)-dolichodial (**3**) is released from the oviparae or from the host/aphid complex. In addition, enantiomeric studies need to be conducted to determine whether (1*S*,2*R*,3*S*)-dolichodial (**3**) or its enantiomer (1*R*,2*S*,3*R*)-dolichodial is present in the air entrainment sample. The absolute stereochemical configuration is presumed to be (1*R*,2*S*,3*R*)-dolichodial (**3**), as this enantiomer is behaviorally active and is structurally related to (4a*S*,7*S*,7a*R*)-nepetalactone (**2**). If (1*SR*,2*RS*,3*SR*)-dolichodial (**3**) is a third component of the aphid sex pheromone, it will add a new dimension to the ratios of sex pheromone components and may play an important role in species integrity.

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References

BARTLET, E., BLIGHT, M. M., LANE, P., and WILLIAMS, I. H. 1997. The responses of the cabbage seed weevil *Ceutorhynchus assimilis* to volatile compounds from oilseed rape in a linear track olfactometer. *Entomol. Exp. Appl.* 85:257–262.

BELLESIA, F., PAGNONI, U. M., PINETTI, A., and TRAVE, R. 1983a. Teucrein, a new iridolactol from *Teucrium marum*, and its biosynthetic relationship with dolichodial. *J. Chem. Res. Synop.* 12:328–329.

BELLESIA, F., PAGNONI, U. M., PINETTI, A., and TRAVE, R. 1983b. The biosynthesis of dolichodial in *Teucrium marum*. *Phytochemistry* 22:2197–2201.

BELLESIA, F., GRANDI, R., PAGNONI, U. M., PINETTI, A., and TRAVE, R. 1984. Biosynthesis of nepetalactone in *Nepeta cataria*. *Phytochemistry* 23:83–87.

BIRKETT, M. A., and PICKETT, J. A. 2003. Aphid sex pheromones: from discovery to commercial production. *Phytochemistry* 62:651–656.

BLACKMAN, R. L., and EASTOP, V. F. 2000. Aphids on the Worlds Crops. 2nd edn. p. 414. Wiley, Chichester.

BLOMMERS, L. H. M., HELSEN, H. H. M., and VAAL, F. W. N. M. 2004. Life history data of the rosy apple aphid *Dysaphis plantaginea* (Pass.) (Homoptera: Aphididae) on plantain and as migrant to apple. *J. Pest Sci.* 77:155–163.

BOEVE, J. L., LENGWILER, U. B., TOLLSTEN, L., DORN, S., and TURLINGS, T. C. J. 1996. Volatiles emitted by apple fruitlets infested by larvae of the European apple sawfly. *Phytochemistry* 42:373–381.

BOO, K. S., CHOI, M. Y., CHUNG, I. B., EASTOP, V. F., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 2000. Sex pheromone of the peach aphid, *Tuberocephalus momonis* and optimal blends for trapping males and females in the field. *J. Chem. Ecol.* 26:601–609.

CAMPBELL, C. A. M., DAWSON, G. W., GRIFFITHS, D. C., PETTERSSON, J., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Sex attractant pheromone of damson-hop aphid *Phorodon humuli* (Homoptera: Aphididae). *J. Chem. Ecol.* 16:3455–3465.

CAVILL, G. W. K., and HINTERBERGER, H. 1960. The chemistry of ants. IV. Terpenoid constituents of some *Dolichoderus* and *Iridomyrmex* species. *Aust. J. Chem.* 13:514–519.

CAVILL, G. W. K., and HOUGHTON, E. 1974. Volatile constituents of argentine ant, *Iridomyrmex humilis*. *J. Insect Physiol.* 20:2049–2059.

CAVILL, G. W. K., ROBERTSON, P. L., BROPHY, J. J., CLARK, D. V., DUKE, R., ORTON, C. J., and PLANT, W. D. 1982. Defensive and other secretions of the Australian cocktail ant, *Iridomyrmex nitidiceps*. *Tetrahedron* 38:1931–1938.

DAWSON, G. W., GRIFFITHS, D. C., JANES, N. F., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1987. Identification of an aphid sex-pheromone. *Nature* 325:614–616.

DAWSON, G. W., GRIFFITHS, D. C., MERRITT, L. A., MUDD, A., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid semiochemicals: a review, and recent advances on the sex-pheromone. *J. Chem. Ecol.* 16:3019–3030.

DAWSON, G. W., PICKETT, J. A., and SMILEY, D. W. M. 1996. The aphid sex pheromone cyclopentanoids: synthesis in the elucidation of structure and biosynthetic pathways. *Bioorg. Med. Chem.* 4:351–361.

DE BERARDINIS, E., BARONIO, P., and BAUMGARTNER, J. 1994. The effect of aphid (*Dysaphis plantaginea* Pass., Homoptera: Aphididae) feeding on apple fruit-growth. *Ecol. Model.* 72:115–127.

DOULOUMPAKA, S., and VAN EMDEN, H. F. 2003. A maternal influence on the conditioning to plant cues of *Aphidius colemani*

Viereck, parasitizing the aphid *Myzus persicae* Sulzer. *Physiol. Entomol.* 28:108–113.

FORREST, J. M. S., and DIXON, A. F. G. 1975. The induction of leaf-roll galls by the apple aphids *Dysaphis devecta* and *D. plantaginea*. *Ann. Appl. Biol.* 81:281–288.

GLINWOOD, R. T. 1998. Response of aphid parasitoids to aphid sex pheromones: laboratory and field studies, Ph.D. Thesis. The University of Nottingham.

GOLDANSAZ, S. H., DEWHIRST, S. Y., BIRKETT, M. A., HOOPER, A. M., SMILEY, D. W. M., PICKETT, J. A., WADHAMS, L. J., and MCNEIL, J. N. 2004. Identification of two sex pheromone components of the potato aphid, *Macrosiphum euphorbiae* (Thomas). *J. Chem. Ecol.* 30:819–834.

GOMEZ, K. A. 1984. Statistical Procedures for Agricultural Research. p. 680. Wiley, New York.

GRAF, B. 1999. Optimising the control of rosy apple aphid *Dysaphis plantaginea* (Pass.) (Homoptera: Aphididae). *IOBC/WPRS Bull.* 22:71–76.

GULDEMOND, J. A., DIXON, A. F. G., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1992. The role of host plant odour and sex pheromones in mate recognition in the aphid Cryptomyzus. Proceedings of the 8th International Symposium of Insect-Plant Relationships, 119–121.

GULDEMOND, J. A., DIXON, A. F. G., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1993. Specificity of sex-pheromones, the role of host-plant odor in the olfactory attraction of males, and mate recognition in the aphid *Cryptomyzus*. *Physiol. Entomol.* 18:137–143.

HARDIE, J., HOLYOAK, M., NICHOLAS, J., NOTTINGHAM, S. F., PICKETT, J. A., WADHAMS, L. J., and WOODCOCK, C. M. 1990. Aphid sex pheromone components: age-dependent release by females and species-specific male response. *Chemoecology* 1:63–68.

HARDIE, J., VISSER, J. H., and PIRON, P. G. M. 1994. Perception of volatiles associated with sex and food by different adult forms of the black bean aphid, *Aphis fabae*. *Physiol. Entomol.* 19:278–284.

KNUDSEN, J. T., TOLLSTEN, L., and BERGSTROM, L. G. 1993. Floral scents—a checklist of volatile compounds isolated by headspace techniques. *Phytochemistry* 33:253–280.

LEAL, W. S., ONO, M., HASEGAWA, M., and SAWADA, M. 1994. Kairomone from dandelion, *Taraxacum officinale*, attractant for scarab beetle *Anomala octiescostata*. *J. Chem. Ecol.* 20:1697–1704.

LILLEY, R., and HARDIE, J. 1996. Cereal aphid responses to sex pheromones and host-plant odours in the laboratory. *Physiol. Entomol.* 21:304–308.

LÖSEL, P. M., LINDEMANN, M., SCHERKENBECK, J., MAIER, J., ENGELHARD, B., CAMPBELL, C. A. M., HARDIE, J., PICKETT, J. A., WADHAMS, L. J., ELBERT, A., and THIELKING, G. 1996. The potential of semiochemicals for control of *Phorodon humuli* (Homoptera: Aphididae). *J. Pest Sci.* 48:293–303.

LOUGHRIN, J. H., POTTER, D. A., and HAMILTON-KEMP, T. R. 1995. Volative compounds induced by herbivory act as aggregation kairomones for the Japanese beetle (*Popillia japonica* Newman). *J. Chem. Ecol.* 21:1457–1467.

MACLEOD, A. J., PIERIS, N. M., and GIL, V. 1981. Volatile components of sugar beet leaves. *Phytochemistry* 20:2292–2295.

MADDRELL, S. H. 1969. Secretion by malpighian tubules of *Rhodnius*: movements of ions and water. *J. Exp. Biol.* 51:71–97.

MARSH, D. 1972. Sex pheromone in the aphid *Megoura viciae*. *Nature* 238:31–32.

MARSH, D. 1975. Responses of male aphids to female sex pheromone in *Megoura viciae* Buckton. *J. Entomol. Ser. A. Physiol. Behav.* 50:43–64.

MEINWALD, J., CHADHA, M. S., HURST, J. J., and EISNER, T. 1962. Defense mechanisms of arthropods. 9. Anisomorphal, the secretion of a phasmid insect. *Tetrahedron* 1:29–33.

MONTGOMERY, D. C. 1997. Design and Analysis of Experiments. 4th edn. p. 704. Wiley, New York.

NORRIS, M. J., and PENER, M. P. 1965. A inhibitory effect of allatectomized males and females on the sexual maturation of young male adults of *Schistocerca gregaria* (Forskal) (Orthoptera: Acrididae). *Nature* 208:1122.

OBENG-OFORI, D., TORTO, B., and HASSANALI, A. 1993. Evidence for mediation of two releaser pheromones in the aggregation behavior of the gregarious desert locust, *Schistocerca gregaria* (Forskal) (Orthoptera, Acrididae). *J. Chem. Ecol.* 19:1665–1676.

PAGNONI, U. M., PINETTI, A., TRAVE, R., and GARANTI, L. 1976. Monoterpenes of *Teucrium marum*. *Aust. J. Chem.* 29:1375–1381.

PARK, K. C., ELIAS, D., DONATO, B., and HARDIE, J. 2000. Electroantennogram and behavioural responses of different forms of the bird cherry-oat aphid, *Rhopalosiphum padi*, to sex pheromone and a plant volatile. *J. Insect Physiol.* 46:597–604.

PAYNE, R. W., MURRAY, D. A., HARDING, S. A., BAIRD, D. B., and SOUTAR, D. M. 2005. GenStat® for Windows®—Introduction. 8th edn. p. 359. VSN International, Hemel Hempstead.

PETTERSSON, J. 1970. An aphid sex attractant I. Biological studies. *Entomol. Scan.* 1:63–73.

POWELL, G., and HARDIE, J. 2001. The chemical ecology of aphid host alternation: how do return migrants find the primary host plant? *Appl. Entomol. Zoolog.* 36:259–267.

RAWLINGS, J. O., PANTULA, S. G., and DICKEY, D. A. 1998. Applied Regression Analysis: A Research Tool. p. 657. Springer, New York.

STEWART-JONES, A., DEWHIRST, S. Y., DURRANT, L., FITZGERALD, J. D., HARDIE, J., HOOPER, A. M., PICKETT, J. A., and POPPY, G. M. 2007. Structure, ratios and patterns of release in the sex pheromone of an aphid, *Dysaphis plantaginea*. *J. Exp. Biol.* 210:4335–4344.

TATSUKA, K., SUEKANE, S., SAKAI, Y., and SUMITANI, H. 1990. Volatile constituents of kiwi fruit flowers: simultaneous distillation and extraction versus headspace sampling. *J. Agric. Food Chem.* 38:2176–2180.

TORTO, B., OBENG-OFORI, D., NJAGI, P. G. N., HASSANALI, A., and AMIANI, H. 1994. Aggregation pheromone system of adult gregarious desert locust *Schistocerca gregaria* (Forskal). *J. Chem. Ecol.* 20:1749–1762.

VAMVATSIKOS, P. G. 2006. Olfactory behaviours associated with host-aphid location in a generalist parasitoid wasp, PhD. University of London.

WYSS, E., VILLIGER, M., HEMPTINNE, J. L., and SCHARER, H. M. 1999. Effects of augumentative release of eggs and larvae of the ladybird beetle, Adalia bipuncata, on the abundance of the rosy apple aphid, *Dysaphis plantaginea*, in organic apple orchards. *Entomol. Exp. Appl.* 90:167–173.

ZHU, J. W., ZHANG, A. J., PARK, K. C., BAKER, T. C., LANG, B., JURENKA, R., OBRYCKI, J. J., GRAVES, W. R., PICKETT, J. A., SMILEY, D., CHAUHAN, K. R., and KLUN, J. A. 2006. Sex pheromone of the soybean aphid, *Aphis glycines* Matsumura, and its potential use in semiochemical-based control. *Environ. Entomol.* 35:249–257.

sized de novo via the mevalonate pathway (Seybold et al. 1995) in the anterior midguts of male *I. pini* (Hall et al. 2002), *Ips confusus* (Ginzel et al., unpublished data), and probably also in other *Ips* spp. Although early steps in the pathway are well understood in *I. pini*, final steps are still being characterized. The ipsdienol biosynthetic pathway branches from the main mevalonate pathway via the activity of a dual function enzyme, geranyldiphosphate synthase/myrcene synthase (GPPS/MS) (Gilg et al. 2005, Unpublished data). Achiral myrcene, whether produced endogenously (Martin et al. 2003) or ingested with the phloem, is then hydroxylated to ipsdienol by CYP9T2 (Sandstrom et al. 2006). Since CYP9T2 from a western *I. pini* population produces ipsdienol in vitro in a ~4:1 (*R*)-(−):(*S*)-(+) ratio, other enzymes must act to increase the proportion of (*R*)-(−)-ipsdienol before it is released. Indeed, Vanderwel (unpublished data) proposed that an oxidoreductase system oxidizes ipsdienol to ipsdienone and then stereo-selectively reduces it to (*R*)-(−)-ipsdienol, and a similar mechanism was proposed to determine the final enantiomeric composition of ipsdienol in *Ips paraconfusus* (Fish et al. 1984) (Fig. 1). Genetic analyses of eastern and western *I. pini* populations suggest that, while a single locus contributes significantly to the observed distribution of enantiomers (Domingue et al. 2006), other factors are involved, and the stereoselectivity of the cytochrome P450 may be important in some instances (Domingue and Teale 2008).

Although significant progress has been made in understanding pheromone biosynthesis and regulation in *I. pini*, studies that involve species with different pheromone ratios would be useful to investigate the mechanism(s) determining pheromonal ipsdienol composition, and to provide insight into the evolution of pheromone systems. To this end, we have begun studies of pheromone biosynthesis in the pinyon ips, *I. confusus*. *I. confusus* is reproductively isolated from *I. pini* by both its host preference and pheromone system. *I. confusus* infests and kills pinyon pines (*Pinus monophylla* and *Pinus edulis*) (Furniss and Carolin 1977), whereas *I. pini* in the southwestern USA largely infests weakened or fallen trees and branches of *Pinus ponderosa* (ponderosa pine), *Pinus contorta* (lodgepole pine), and *Pinus jeffreyi* (Jeffery pine) (Kegley et al. 2002). The aggregation pheromone blend emitted by *I. confusus* consists of about 99%-(S)-(−)-ipsenol and 95%-(*S*)-(+)-ipsdienol (Young et al. 1973), very different from the approximately 95%-(*R*)-(−)-ipsdienol produced by the western North American *I. pini* (Miller et al. 1997).

Here, we report the isolation and characterization of a CYP9T2 ortholog, IcCYP9T1, from *I. confusus* in order to assess its role in pheromone biosynthesis and in determining the enantiomeric ratio of the key pheromone component, ipsdienol.

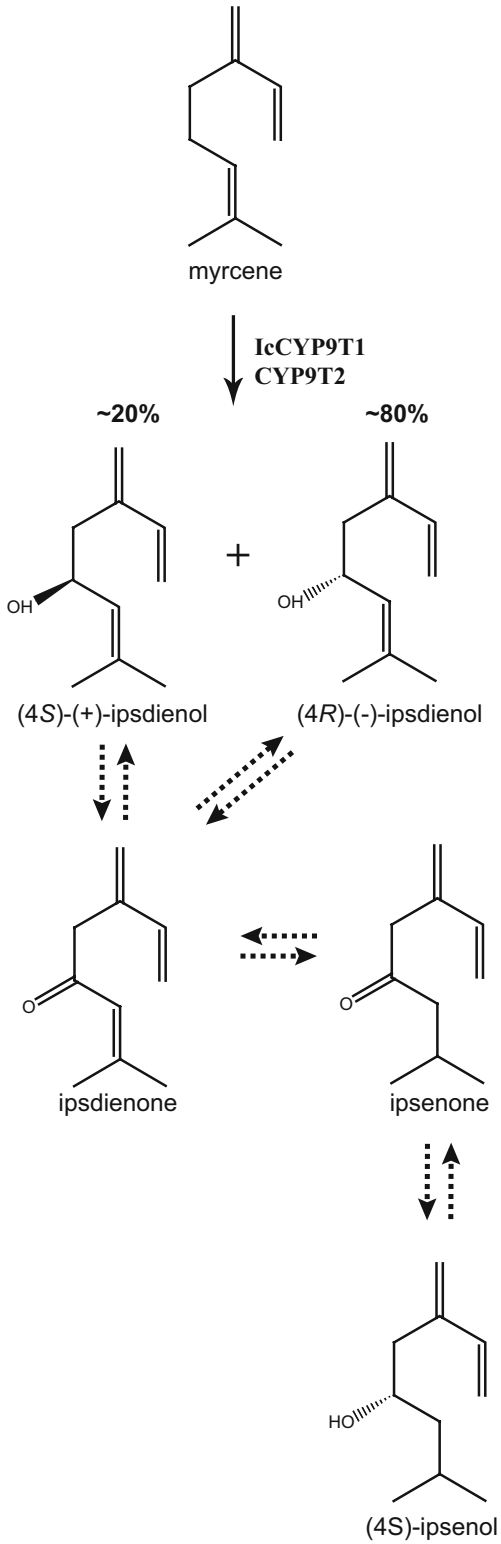

Fig. 1 Pheromone production in *Ips pini* and *Ips confusus*. Recombinant IcCYP9T1 and CYP9T2 are able to convert myrcene, which is ingested in the diet and produced de novo, to a ~5:1 mixture of (*R*)-(−):(*S*)-(+)-ipsdienol. Hypothesized pheromone-biosynthetic reactions, to achieve the crucial enantiomeric compositions of mostly (−)-ipsdienol for western North American *I. pini* populations and mostly (−)-ipsenol and (+)-ipsdienol for *I. confusus*, are shown as *dashed lines*

Methods and Materials

Insects Immature *I. confusus* were obtained from infested *P. edulis* bolts collected from the Bureau of Land Management (BLM), land east of Carson City, Nevada, USA. The insects were reared in a greenhouse to adults as per Browne (1972) and emerged adults (i.e., those that exited the brood tree) were collected daily. Adults were separated by sex according to Lanier and Cameron (1969) and were stored for up to 2 weeks at 4°C in moist paper towels.

Experimental All reagents and chemicals, cloning procedures, cell culture, recombinant protein production, microsome preparation, and enzyme assays were performed essentially as described previously (Sandstrom et al. 2006), with the following modifications:

cDNA isolation Two male *I. confusus* were inserted into separate small holes drilled into the phloem of fresh *P. edulis* bolts, secured with wire mesh, and allowed to feed for 23 h before their midguts were dissected and flash frozen in liquid nitrogen. The midguts were kept overnight at −80°C, and RNA was isolated by using the RNeasy Plant Mini Kit (Qiagen). To obtain a cDNA homologous to CYP9T2, first-strand cDNA synthesis and 3′ and 5′-Rapid amplification of cDNA ends (RACE) was achieved with the Clontech SMART™ RACE cDNA amplification kit (Clontech, Mountain View, CA, USA). *CYP9T2*-specific primers ("Ic9T2F1", GTGGCAAAAACTAACACCGCTGAAGAC; "Ic9T2R1", GTCTTCAGCGGTGTTAGTTTTTGCCAC, and "Ic9T2R2", CCGATTCATAGGTGACTTTGTTCCC), and the provided anchor primer (10× Universal Primer A Mix) were used as described in the manufacturer's instructions. Amplified products from the RACE PCR reactions were visualized on agarose gels, cloned into pST-Blue-1 AccepTor™ Vector (Novagen, San Diego, CA, USA), and transformed in *E. coli* NovaBlue Singles™ Competent Cells (Novagen). Recombinants were identified by colony PCR as described by the AccepTor™ Vector Kit protocol. Purified PCR products were sequenced by primer walking by using the ABI BigDye Terminator Cycle Sequencing Ready Reaction Kit v3.1 on an ABI3730 DNA Analyzer at the Nevada Genomics Center (UNR). All sequence analysis was done with Vector NTIv.9 software (Informax, N. Bethesda, MD, USA). The full-length cDNA was assembled from the overlapping RACE products and confirmed by PCR amplification of first strand cDNA with primers flanking the putative open reading frame (ORF). The complete deduced amino acid sequence was submitted to the P450 Nomenclature Committee (David Nelson, personal communication) and was given the name *Ips confusus CYP9T1* (IcCYP9T1).

Expression Analysis mRNA levels were determined by quantitative Real Time RT-PCR (qRT-PCR). cDNA templates for the fed male and female anterior midgut time-course studies were prepared previously (J. Bearfield, unpublished). Briefly, beetles were fed for 4, 8, 16, or 32 h, while unfed controls were held in plastic cups in the dark. There were six biological replicates that each included five beetles. For the tissue-distribution study, males and females were either allowed to feed on the phloem of *P. edulis* bolts or kept as unfed controls in plastic cups in the dark for 26 h and then dissected in water under a stereomicroscope. Tissues from the head, anterior midgut, posterior midgut, hindgut, fat body, and carcasses of eight insects were pooled and frozen in N_2(l). There were four male and female biological replicates.

Real time PCR primers with minimal potential for primer–dimer formation were identified by using Vector NTI (Version 7.1, Invitrogen) among primers suggested by Primer Express software (Applied Biosystems, Foster City, CA, USA). Relative gene expression was determined with the ΔΔCT method (Livak and Schmittgen 2001). The results were normalized with an internal control gene, *cytoplasmic actin*, which is unaffected by feeding in *I. confusus* (J. Bearfield, unpublished).

Cloning The *IcCYP9T1* ORF was amplified by PCR by using Ic9T1F1 (GCACCATGGTGGTCGGGTTGGTT) and IcCYP9T1R1 (tagged; GCCTCGAGGGTTCAAATG CAAGGT) primers, directionally cloned into the *NcoI* and *Xho*I sites of pENTR4 (Invitrogen) by standard methods (Sambrook et al. 1989), and transformed into DH5α cells. The reverse primer ensured that the construct would have a C-terminal extension containing a V5 epitope and the polyhistidine tag (encoded by the vector). An untagged version was not constructed because products and their relative abundance were statistically identical in assays of both the untagged and tagged versions of CYP9T2 (Sandstrom et al. 2006). The recombinant plasmid, pENTR4-IcCYP9T1V5H6, was confirmed by sequencing.

Recombinant IcCYP9T1 Protein Detection IcCYP9T1 production was determined by western blotting using 1:10,000 Anti-V5 primary antibody (Invitrogen), 1:20,000 Goat Anti-Mouse secondary antibody (Biorad, Hercules, CA, USA), and SuperSignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA).

Enzyme Assays Recombinant IcCYP9T1 assays were performed as described by Sandstrom et al. (2006).

Gas Chromatography–Mass Spectrometry A Thermo Finnigan Polaris Q ion trap coupled with a Trace gas chromatograph was used for the gas chromatography–

mass spectrometry (GC-MS) analyses. The GC was fitted with a 60 m×0.25 mm i.d. DB-5 capillary column (J&W Scientific, Palo Alto, CA, USA) and was programmed from 50 (1 min hold) to 200°C at 5°C min^{-1}, and then to 320°C at 10°C min^{-1}. Split injection (100:1 ratio) with a constant column flow of 1.5 ml min^{-1} of helium was used. The enantiomeric composition of ipsdienol was determined by chiral separation by using the same instruments with a CycloSil-B (30 m×0.25 mm i.d., 0.25 μm film thickness) column (J&W Scientific) at 100°C and with a helium flow rate of 1.3 ml min^{-1}.

Results

DNA Isolation and Expression The cDNA for the CYP9T2 ortholog from *I. confusus* was isolated by PCR techniques. The 1,699 bp cDNA contained a 1,596 nt ORF encoding a 532 amino acid (a.a.) protein flanked by 74 nt and 26 nt 5′ and 3′ UTRs, respectively, and is therefore likely full-length. The predicted translation product has a molecular mass of 61 kDa and a pI of 8.9 (Gasteiger et al., 2003). It was designated *I. confuses* CYP9T1 (IcCYP9T1) by the P450 Nomenclature Committee (David Nelson, personal communication). The primary structure has an N-terminal membrane anchor and typical P450 conserved residues, including the WxxxR, ExxR, and PxxFxPERF ('PERF') motifs, and the canonical heme-binding domain (PFxxGxRxCxG) surrounding the heme-cysteine ligand (Cys475; Fig. 2) (Feyereisen 2005). BLAST searches (Altschul et al. 1990) indicated IcCYP9T1 is most similar to coleopteran CYP9s (not shown). It has a 98% a.a. identity (7 a.a. different) with the functionally uncharacterized partial length CYP9T1 from *I. paraconfusus* (IpCYP9T1) (Huber et al. 2007) and is 94% identical (34 a.a. different) to the *I. pini* myrcene hydroxylase CYP9T2 (Sandstrom et al. 2006) (Fig. 2).

Message levels were determined by qRT-PCR by using *cytoplasmic actin* as a normalizing gene. *IcCYP9T1* mRNA levels in males increased substantially over time upon feeding, with a maximal induction of approximately 400-fold at 32 h (Fig. 3a). In contrast, *IcCYP9T1* mRNA levels in females were repressed at 4, 8, and 16 h and only minimally induced at 32 h by feeding (Fig. 3a). *IcCYP9T1* mRNA localized predominantly to the anterior midgut of fed males (Fig. 3b). Levels in females were substantially lower than those in males, with no clear induction in any tissue upon feeding (Fig. 3b).

Fig. 2 Alignment of the deduced a.a. sequences of IcCYP9T1, IpCYP9T1 (ABF06554) and CYP9T2 (ABG74909). Conserved residues are indicated by a *dash*. The missing portion of IpCYP9T1 is indicated by a *gap* (a.a. 1–20). The membrane anchor domain is *underlined*. Conserved WxxxR, ExxR, and PxxF motifs are *boxed*. The heme-binding domain (PFxxGxRxCxG) is *shaded*

Enzyme Assays and Product Formation A baculoviral system was used to produce sufficient enzyme to analyze IcCYP9T1 functionally. The V5-epitope was used to follow recombinant protein production, as an antibody for IcCYP9T1 was unavailable. Western blot analysis indicated high expression of an approximately 65 kDa protein at 4 and 5 days post-infection (not shown). CO-difference absorption spectra of microsomes from Sf9 cells producing IcCYP9T1 had the characteristic peak at 450 nm, while microsomes from Sf9 cells without recombinant protein did not (not shown).

Assays with microsomes from Sf9 cells that co-express recombinant IcCYP9T1 and housefly P450 reductase were analyzed by GC-MS for myrcene hydroxylation products. Selective ion monitoring (SIM) analysis at *m/z*=85 (for ipsdienol) of microsomes incubated with unlabeled myrcene yielded a product with a retention time and mass spectrum identical to an ipsdienol standard (Fig. 4a). Products from reactions incubated with deuterium-labeled myrcene had the same retention time (not shown), but with a mass spectrum of appropriate *m/z* diagnostic fragments that was 1 amu larger. A time course of 5, 10, and 15-min reactions with unlabeled myrcene showed a linear increase in ipsdienol production relative to the *n*-octanol standard (not shown). Ipsdienol was not detectable by SIM analysis of products from reactions run in the absence of substrate (not shown), boiled microsomes (Fig. 4b), or microsomes infected with housefly P450 reductase baculovirus construct only (not shown). No other hydroxylated versions of myrcene (amitinol, *E*-myrcenol (2-methyl-6-methylene-2,7-octadien-1-ol), linalool (2,6-dimethylocta-2,7-dien-6-ol), or geraniol (2,6-dimethyl-2,6-octadien-8-ol)) were detected in any sample.

GC-MS analysis with a CycloSil-B column, of extracts of recombinant IcCYP9T1/P450 reductase, incubated with either unlabeled or deuterium-labeled myrcene, produced two major peaks with retention times corresponding to (*R*)-(−)-ipsdienol and (*S*)-(+)-ipsdienol. Reactions using unlabeled myrcene as substrate yielded enantiomeric percentages that averaged 85%-(*R*)-(−)-ipsdienol (Fig. 4c).

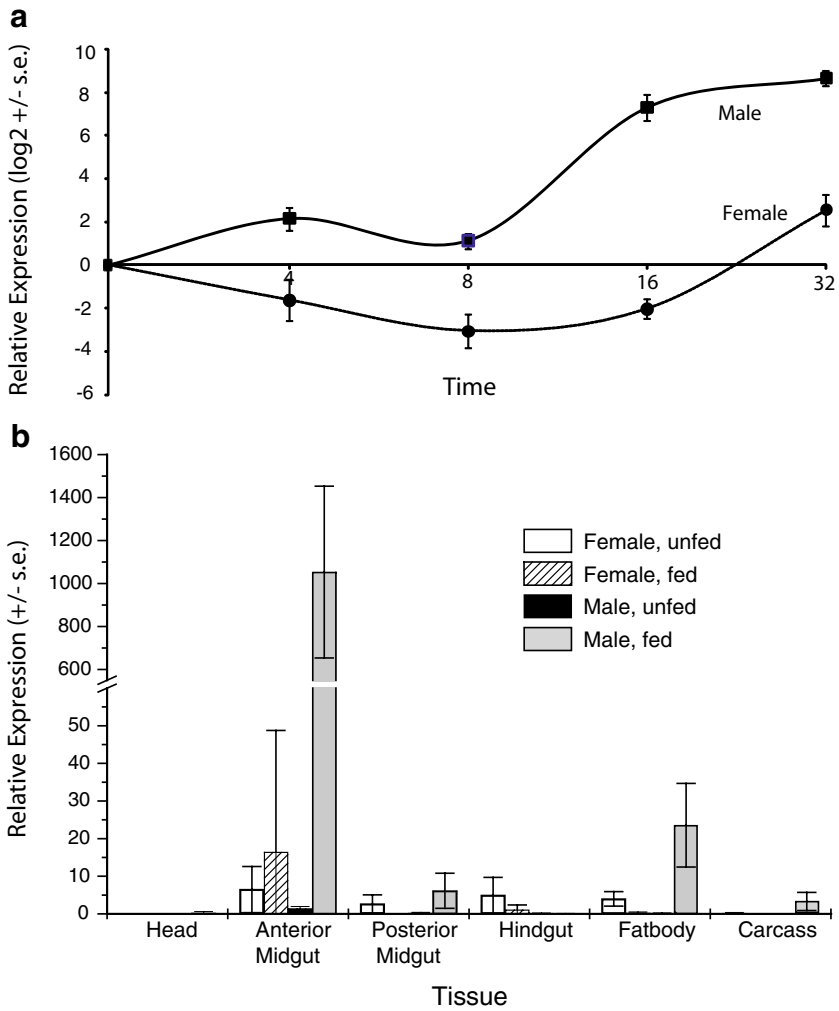

Fig. 3 Real time quantitative RT-PCR analysis of IcCYP9T1 mRNA levels. **a** Time course of midgut expression (log_2) comparing fed males and females. **b** Relative *IcCYP9T1* mRNA levels (+/− standard error) in various tissues of starved or fed (26 h) males and females

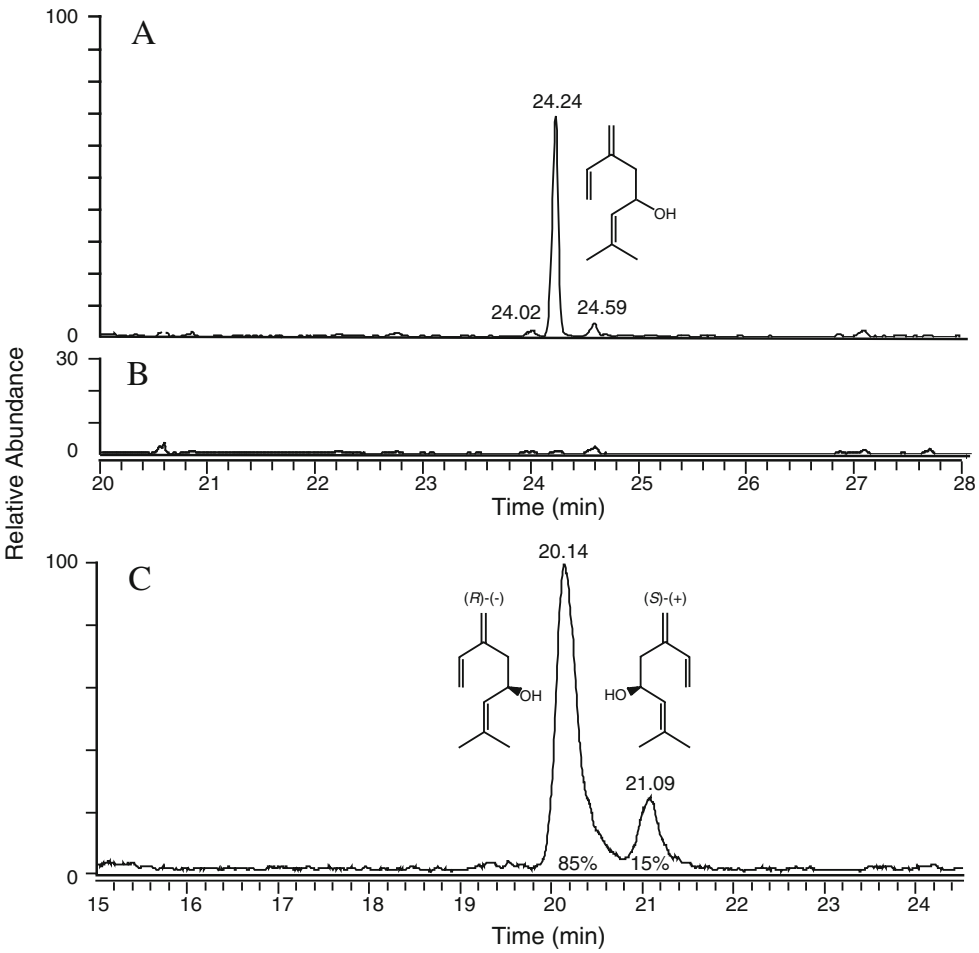

Fig. 4 Gas chromatography–mass spectrometry (GC-MS) analysis of ipsdienol formed from myrcene by recombinant IcCYP9T1. Selected ion monitoring (*m/z*=85 for ipsdienol) using a DB-5 column for reactions containing **a** unlabeled myrcene (identical to the ipsdienol standard, not shown) or **b** boiled microsomes (negative control). **c** Enantiomeric ratio of reaction products, determined by chiral GC-MS

Discussion

We report the isolation and functional expression of a monoterpene-oxidizing cytochrome P450 cDNA, IcCYP9T1, which produces the pheromone component, (*R*)-(−)-ipsdienol. The predicted translation product has a hydrophobic N-terminal target sequence and many motifs, including the classic heme-binding domain, common to P450s (Fig. 2). A CYP9T1 partial cDNA was first isolated and characterized from the California five-spined ips, *I. paraconfusus*, although its biochemical function was not determined (Huber et al. 2007). *I. paraconfusus* and *I. confusus* are sibling species, and once were considered a single species. It is, therefore, not surprising that the IcCYP9T1 and IpCYP9T1 orthologs share such high (98%) sequence identity (Fig. 2). The seven non-identical amino acids are nevertheless conserved, and appear located in positions that do not contribute to the active site (not shown), suggesting that the activities of the two enzymes should be similar.

Bark beetle pheromone-biosynthetic genes are typically up-regulated by feeding (Keeling et al. 2004), transcribed predominantly in the anterior midgut (Tillman et al. 2004; Gilg et al. 2005; Bearfield et al. 2006), and have basal expression levels that are higher in the pheromone-producing sex (Keeling et al. 2004, 2006). IcCYP9T1 mRNA levels increased substantially in male, but not in female, anterior midguts in response to feeding (Fig. 3a,b). *IcCYP9T1* mRNA was predominantly localized to the anterior midgut of pheromone-producing male *I. confusus* (Fig. 3b), and there was a fivefold basal level difference between male and female expression. These data correlate well with expression patterns of other *I. confusus* (Bearfield et al., unpublished), *I. paraconfusus* (Ivarsson et al. 1998; Tittiger et al. 1999; Huber et al. 2007) and *I. pini* pheromone-biosynthetic genes (Keeling et al. 2006), and strongly support a role for *IcCYP9T1* in male-specific aggregation pheromone biosynthesis.

Interesting differences in transcript accumulation patterns exist between *CYP9T2, IcCYP9T1*, and *IpCYP9T1*. Feeding induced an approximately 28-fold *CYP9T2* mRNA expression in male *I. pini* midguts (Sandstrom et al. 2006), over 400-fold expression in male *I. confusus* midguts (Fig. 3a,b), and an astonishing 85,000-fold expression in male *I. paraconfusus* whole bodies (Huber et al. 2007).

There are also differences in the timing of the up-regulation, with male *I. paraconfusus* achieving high levels within only 8 h of feeding (Huber et al. 2007), while the highest expression in *I. confusus* (Fig. 3a) and *I. pini* (Sandstrom et al. 2006) occurred by 32 h. Finally, while the difference between male and female basal levels of expression was not reported for *I. paraconfusus*, male *I. confusus* had an approximately five-fold higher expression level than females (not shown), which was much less than the ~500-fold difference in basal CYP9T2 mRNA levels in observed in male and female *I. pini* (Keeling et al. 2006). It should be emphasized that the study by Huber et al. (2007) investigated whole bodies, and the tissue distribution of IpCYP9T1 is unknown. Furthermore, IpCYP9T1 levels in that study were normalized to *CYP4G27*, rather than *cytoplasmic actin*. We analyzed the stability of expression of four candidate normalizing genes, *CYP4G27*, *cytoplasmic actin*, *β-tubulin*, and *ubiquitin C* in *I. confusus* by using qBase (Hellemans et al. 2007) and found that *CYP4G27* and *cytoplasmic actin* were similarly stable (A. Griffith, unpublished data). We chose to continue to use *cytoplasmic actin* as a normalizing gene in order to maintain continuity with previous studies. Thus, while quantitative comparisons between our studies and that of Huber et al. (2007) may have reduced value, the general trends among the three species appear qualitatively similar.

Similarities in sequence and expression patterns with the myrcene hydroxylase CYP9T2 (Sandstrom et al. 2006) suggested myrcene as a logical IcCYP9T1 substrate. A baculovirus system was used to produce sufficient enzyme for functional assays. Sf9 microsomes that contain recombinant IcCYP9T1 readily hydroxylated myrcene to ipsdienol (Fig. 4). The amount of ipsdienol produced increased linearly with incubation time, and myrcene hydroxylation activity was abolished when microsomes were heat-denatured prior to the reaction (Fig. 4b), confirming that ipsdienol is an enzymatic product. Furthermore, microsomes from cells that were infected with recombinant housefly P450 reductase baculovirus (without recombinant IcCYP9T1) did not hydroxylate myrcene (not shown). Thus, ipsdienol production was due to IcCYP9T1 and not to an endogenous activity of Sf9 cells. Other possible myrcene hydroxylation products were not detected, suggesting high product specificity.

Recombinant IcCYP9T1 produced ~85%-(−)-ipsdienol, similar to the ~81%-(−)-ipsdienol produced by recombinant CYP9T2 (Sandstrom et al. 2006). This enantiomeric excess is nearly antipodal to the ~90%-(+)-ipsdienol found in the *I. confusus* pheromone blend. While the enantiomeric ratios of ipsdienol from recombinant enzyme assays may be affected by experimental conditions (i.e., the reactions were done with Sf9 microsomes, not purified recombinant enzyme), the observed excess of (*R*)-(−)-ipsdienol is assumed to be biologically relevant. Our data strengthen the observation that myrcene hydroxylases contribute little to the final enantiomeric blend of pheromonal ipsdienol (Sandstrom et al. 2006), and further support the suggestion that enzymes downstream from myrcene hydroxylation are required to achieve the crucial enantiomeric compositions of pheromone used by different species. The terminal steps in ipsenol and ipsdienol pheromone biosynthesis in scolytids likely involve oxidases and/or reductases that catalyze the interconversion between ipsdienol, ipsdienone, ipsenone, and ipsenol (Fish et al. 1979; Ivarsson 1997; Vanderwel, personal communication). There may be situations, such as those that can occur in hybrid zones, in which downstream enzymes with different product profiles may compete, or not be active, and the enantiomeric composition would then be determined by the P450 (Domingue and Teale 2008).

Functional expression of IcCYP9T1 provides insight into P450 evolution and control of *Ips* spp. pheromone production. The enantiomeric ratios of ipsdienol produced by IcCYP9T1 and CYP9T2 are similar and apparently unrelated to the final pheromone blend. Even though a single a.a. change can alter P450 activity (Feyereisen 2005), the 94% a.a. identity between IcCYP9T1 and CYP9T2 appears to be sufficient to confer similar activity. The conservation of activities can be taken to indicate a common ancestral function, most likely host-resin detoxification. However, both genes appear to have acquired, or else inherited from a common ancestor, regulatory motifs that coordinate their transcription with other pheromone-biosynthetic genes. Indeed, co-ordinate regulation would almost be necessary given the large amounts of toxic myrcene produced endogenously during pheromone biosynthesis. In this respect, these P450s still function as detoxification enzymes in pheromone-biosynthetic midguts. Thus, they may serve as an example of how enzymes acquire new biological roles by variations in their corresponding gene expression, despite retaining their original activities. This is consistent with the application of pre-existing metabolic activities to conserve resources and develop regulated pheromone component production. Future investigation will determine the substrate specificity of IcCYP9T1 and CYP9T2. The study of these proteins and additional homologues in different *Ips spp*. or populations will lead to a better understanding of the origin and regulation of bark beetle pheromone biosynthesis, thus allowing for the development of new pest management tools.

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References

ALTSCHUL, S. F., GISH, W., MILLER, W., MYERS, E. W., and LIPMAN, D. J. 1990. Basic local alignment search tool. *J. Mol. Biol.* 215:403–410.

BEARFIELD, J. C., KEELING, C. I., YOUNG, S., BLOMQUIST, G. J., and TITTIGER, C. 2006. Isolation, endocrine regulation, and mRNA distribution of the 3-hydroxy-3-methylglutaryl coenzyme A synthase (HMG-S) gene from the pine engraver, *Ips pini*. *Insect Mol. Biol.* 15:187–195.

BROWNE, L. E. 1972. An emergence cage and refrigerated collector for wood-boring insects and their associates. *J. Econ. Entomol.* 65:1499–1501.

BYERS, J. A. 1989. Chemical ecology of bark beetles. *Cel. Mol. Life Sci.* 45:271–283.

DOMINGUE, M., and TEALE, S. 2008. The genetic architecture of pheromone production between populations distant from the hybrid zone of the pine engraver, *Ips pini*. *Chemoecology.* 17:255–262.

DOMINGUE, M., STARMER, W., and TEALE, S. 2006. Genetic control of the enantiomeric composition of ipsdienol in the pine engraver, *Ips pini*. *J. Chem. Ecol.* 32:1005–1026.

FEYEREISEN, R. 2005. Insect Cytochromes P450. pp 1–77. in L. I. Gilbert, K. Iatrou, S. S. Gill (eds.). Comprehensive Molecular Insect Science: Elsevier BV.

FISH, R. H., BROWNE, L. E., WOOD, D., and HENRY, D. L. 1979. Conversions of deuterium-labelled ipsdienol with sexual and enantioselectivity in *Ips paraconfusus* Lanier. *Tetrahedron Lett.* 17:1465–1468.

FISH, R., BROWNE, L. E., and BERGOT, B. J. 1984. Pheromone biosynthetic pathways: conversion of ipsdienone to (-)-ipsdienol, a mechanism for enantioselective reduction in the male bark beetle, *Ips paraconfusus*. *J. Chem. Ecol.* 10:1057–1064.

FURNISS, R. L. and CAROLIN, V. M. 1977. Western Forest Insects. USDA Forest Service Misc. Pub. 1339:654.

GASTEIGER, E., GATTIKER, A., HOOGLAND, C., IVANYI, I., APPEL, R. D., BAIROCH, A. 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 2003 31 (13):3784–8.

GILG, A. B., BEARFIELD, J. C., TITTIGER, C., WELCH, W. H., and BLOMQUIST, G. J. 2005. Isolation and functional expression of the first animal geranyl diphosphate synthase and its role in bark beetle pheromone biosynthesis. *Proc. Nat. Acad. Sci. U. S. A.* 102:9760–9765.

GILG, A. B., TITTIGER, C., and BLOMQUIST, G. J. Unpublished data. Geranyl diphosphate synthase functions as both an isoprenyl diphosphate synthase and a monoterpene synthase (myrcene synthase) in the bark beetle, *Ips pini* (Say). *Naturwissenschaften*.

HALL, G. M., TITTIGER, C., ANDREWS, G. L., MASTICK, G. S., KUENZLI, M., LUO, X., SEYBOLD, S. J., and BLOMQUIST, G. J. 2002. Midgut tissue of male pine engraver, *Ips pini*, synthesizes monoterpenoid pheromone component ipsdienol *de novo*. *Naturwissenschaften.* 89:79–83.

HELLEMANS, J., MORTIER, G., DE PAEPE, A., SPELEMAN, F., and VANDESOMPELE, J. 2007. qBase relative quantification framework and software for management and automated analysis of real-time quantitative PCR data. *Genome Biol.* 8:R19.

HUBER, D. P., ERICKSON, M. L., LEUTENEGGER, C. M., BOHLMANN, J., and SEYBOLD, S. J. 2007. Isolation and extreme sex-specific expression of cytochrome P450 genes in the bark beetle, *Ips paraconfusus*, following feeding on the phloem of host ponderosa pine, *Pinus ponderosa*. *Insect Mol. Biol.* 16:335–49.

IVARSSON, P., BLOMQUIST, G. J., SEYBOLD, S. J., 1997. In vitro production of the pheromone intermediates ipsdienone and ipsenone by the bark beetles *Ips pini*(Say) and *I. paraconfusus* Naturwissenschaften 84:454–7. Lanier (Coleoptera: Scolytidae).

IVARSSON, P., TITTIGER, C., BLOMQUIST, C., BORGESON, C. E., SEYBOLD, S. J., and BLOMQUIST, G. J. 1998. Pheromone precursor synthesis is localized in the metathorax of *Ips paraconfusus* Lanier (Coleoptera: Scolytidae). *Naturwissenschaften.* 85:507–511.

KEELING, C. I., BLOMQUIST, G. J., and TITTIGER, C. 2004. Coordinated gene expression for pheromone biosynthesis in the pine engraver beetle, *Ips pini* (Coleoptera: Scolytidae). *Naturwissenschaften.* 91:324–328.

KEELING, C. I., BEARFIELD, J. C., YOUNG, S., BLOMQUIST, G. J., and TITTIGER, C. 2006. Effects of juvenile hormone on gene expression in the pheromone-producing midgut of the pine engraver beetle, *Ips pini*. *Insect Molec. Biol.* 15:207–216.

KEGLEY, S. J., LIVINGSTON, R. L., and GIBSON, K. E. 2002. Pine engraver, *Ips pini* (Say) in the western United States. Bark beetles of North America image and fact sheet library.

LANIER, G. N., and CAMERON, E. A. 1969. Secondary sexual characters in the North American species of genus *Ips* (Coleoptera: Scolytidae). *Can. Entomol.* 101:862–870.

LANIER, G. N., CLAESSON, A., STEWART, T., PISTON, J. J., and SILVERSTEIN, R. M. 1980. *Ips pini*: the basis for interpopulational differences in pheromone biology. *J. Chem. Ecol.* 6:677–687.

LIVAK, K. J., and SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods.* 25:402–8.

MARTIN, D., BOHLMANN, J., GERSHENZON, J., FRANCKE, W., and SEYBOLD, S. J. 2003. A novel sex-specific and inducible monoterpene synthase activity associated with a pine bark beetle, the pine engraver, *Ips pini*. *Naturwissenschaften.* 90:173–9.

MILLER, D., GIBSON, K., RAFFA, K. F., SEYBOLD, S., TEALE, S., and WOOD, D. 1989. Inter- and intrapopulation variation of the pheromone, ipsdienol produced by male pine engravers, *Ips pini* (Say) (Coleoptera: Scolytidae). *J. Chem. Ecol.* 23:2013–2031.

MILLER, D. R., BORDEN, J. H., and SLESSOR, J. H. 1997. Geographic variation in response of pine engraver, Ips pini, and associated species to pheromone, lanierone. *J. Chem. Ecol.* 23:2013–2031.

SAMBROOK, J., FRITSCH, E. F., and MANIATIS, T. 1989. Molecular Cloning, a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor.

SANDSTROM, P., WELCH, W. H., BLOMQUIST, G. J., and TITTIGER, C. 2006. Functional expression of a bark beetle cytochrome P450 that hydroxylates myrcene to ipsdienol. *Insect Biochem. Mol. Biol.* 36:835–845.

SEYBOLD, S. J., QUILICI, D. R., TILLMAN, J. A., VANDERWEL, D., WOOD, D. L., and BLOMQUIST, G. J. 1995. De novo biosynthesis of the aggregation pheromone components ipsenol and ipsdienol by the pine bark beetles *Ips paraconfusus* Lanier and *Ips pini* (Say) (Coleoptera: Scolytidae). *Proc. Nat. Acad. Sci. U. S. A.* 92:8393–8397.

TILLMAN, J. A., LU, F., STAEHLE, L., DONALDSON, Z., DWINELL, S. C., TITTIGER, C., HALL, G. M., STORER, A. J., BLOMQUIST, G. J.,

and SEYBOLD, S. J. 2004. Juvenile hormone regulates *de novo* isoprenoid aggregation pheromone biosynthesis in pine bark beetles, *Ips* spp. (Coleoptera: Scolytidae), through transcriptional control of HMG-CoA reductase. *J. Chem. Ecol.* 30:2459–2494.

TITTIGER, C., BLOMQUIST, G. J., IVARSSON, P., BORGESON, C. E., and SEYBOLD, S. J. 1999. Juvenile hormone regulation of HMG-R gene expression in the bark beetle *Ips paraconfusus* (Coleoptera: Scolytidae): implications for male aggregation pheromone biosynthesis. *Cel. Mol. Life Sci.* 55:121–7.

YOUNG, J. C., SILVERSTEIN, R. M., and BIRCH, M. C. 1973. Aggregation pheromone of the beetle *Ips confusus*: Isolation and identification. *J. Insect Physiol.* 19:2273–2277.

with intraspecific pheromone reception, but rather with the perception of nonpheromonal odorants.

Proteins with characteristics of OBPs also have been identified in many orders of insects, such as in Lepidoptera (Vogt et al. 1991b; Maleszka and Stange 1997; Scaloni et al. 1999; Wang et al. 2003; Maida et al. 2005; Xiu and Dong 2007), Diptera (Ozaki et al. 1995; Hekmat-Scafe et al. 1997; Biessmann et al. 2002; Ishida et al. 2002a, 2004; Xu et al. 2003), Heteroptera (Dickens et al. 1995), Coleoptera (Graham et al. 2003; Nagnan-Le Meillour et al. 2004), Orthoptera (Ban et al. 2003), Isoptera (Ishida et al. 2002b), and Hymenoptera (Lu et al. 2007). Twenty-one OBP-like genes have been characterized from the whole genome sequences of the honey bee, *Apis mellifera* L. (Forêt and Maleszka 2006). Proteins corresponding to specific OBP family member genes from *A. mellifera* include ASP1 (Danty et al. 1999), ASP2 (Briand et al. 2001), ASP4: AAL60417 (GenBank accession number), ASP5: AAL60422, and ASP6: AAL60421. These have been characterized from the antennae and legs of workers and drones according to their precise molar weights determined by mass spectrometry and N-terminal sequencing (Danty et al. 1998). The antennal specific water-soluble protein, ASP2 (Mr 13,695.2±1.6), was purified and characterized as the first Hymenoptera putative GOBP, based on its specific expression in olfactory areas and higher expression in the worker than in the drone (Danty et al. 1997). Briand et al. (2001) reported that it interacted with a wide range of volatile odorant molecules, but not with the major components of the queen pheromone.

The Asian honey bee, *Apis cerana cerana* Fabricius (Hymenoptera: Apidae), is an economically important indigenous species with about two million colonies being bred in China. *A. cerana cerana* has many unique characters, such as the resistance to ectoparasitic mites by a specific grooming behavior and tolerance for low environmental temperatures. The latter quality makes *A. cerana cerana* an important pollinator of flowering plants at high altitudes (Peng et al. 1987; Chen 2001). Biochemically, OBPs are thought to act as solubilizers and carriers of the lipophilic odorants in the sensillar lymph, as peripheral filters in odor discrimination by selectively binding certain kinds of odorants, as stimulus molecules to the receptor to facilitate signal transduction, as rapid deactivators of odorants after stimulation, and as cleaners to remove unwanted or toxic compounds from the perireceptor space (Vogt and Riddiford 1981; Steinbrecht 1998; Pelosi et al. 2006). Thus, we hypothesize that OBPs may play an important role in the olfactory system of *A. cerana cerana* related to the search for nectar and pollen and in detection of odorants from ectoparasitic mites.

In this study, we cloned a new OBP-encoding gene (*Acer-ASP2*) from *A. cerana cerana* and then expressed the corresponding protein in *Escherichia coli*. With polyclonal anti-(Acer-ASP2)-serum from purified recombinant Acer-ASP2 antigen, we investigated immunolocalization in the antennae of *A. cerana cerana* workers, and with real-time polymerase chain reaction (PCR), we characterized the spatiotemporal expression pattern of *Acer-ASP2*.

Methods and Materials

Insects Larval, pupal, and adult workers from *A. cerana cerana* colonies were bred at the Experimental Apiary at Zhejiang University, Hangzhou, China. Bees of known ages were obtained by marking the newly emerged bees from a frame of capped broods kept in an incubator; bees were returned to the hive and collected when required. The developmental stages of workers were classified according to the criteria of Rachinsky et al. (1990) and Michelette and Soares (1993).

Amplification of cDNA Encoding Acer-ASP2 From 100 antennae of adult worker bees of *A. cerana cerana*, total RNA was isolated with Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was synthesized by using SuperScript™ II Reverse Transcriptase system (Invitrogen). The DNA encoding *Acer-ASP2* was amplified by PCR from cDNA with primer pairs (Table 1) designed from ASP2 sequence from *A. mellifera* (GenBank no. AF393493). The amplification conditions were 4 min at 94°C, followed by 35 cycles of 45 s at 94°C, 45 s at 50°C, 1 min at 72°C, and final extension for 10 min at 72°C. Amplified DNA purified with a gel extraction kit (Qiagen, Valencia, CA, USA) was inserted into pGEM-T vector (Promega, Madison, WI, USA) and was transformed into *E. coli* TG1 competent cells. Positive colonies, screened for the presence of the insert by restriction analysis by using *Bam*H I and *Hin*d III, were sequenced (Center of Analysis &

Table 1 Oligonucleotide primers used for isolation and expression analysis of odorant-binding proteins of *A. cerana cerana*

Purpose/primer name	Sequence (5′–3′)
cDNA isolation (reverse transcriptase PCR)	
Sense	AAGGATCCATGAACACCCTCGTC
	Underlined BamH I restriction site
Antisense	CGCAAGCTTTTACGAGAACAGTT
	Underlined Hind III restriction site
Expression analysis (real-time PCR)	
Acer-ASP2	
Sense	CTCGTCACCGTTACTTGT
Antisense	TTAAACTCGGAATCTTCG
β-Actin	
Sense	TCCTGCTATGTATGTCGC
Antisense	AGTTGCCATTTCCTGTTC

Measurement, Zhejiang University) and shown to encode the mature protein Acer-ASP2.

Production and Purification of Recombinant Acer-ASP2 The 450-bp fragment excised with *Bam*H I and *Hin*d III from the pGEM-Acer-ASP2 plasmid, was purified with the gel extraction kit (Qiagen) and cloned into pET-30a (+) vector (Novagen, Darmstadt, Germany) digested with the same restriction enzyme, and the recombinant expressed plasmid pET-Acer-ASP2 was transformed into *E. coli* BL21 (DE3) competent cells. Single colonies were grown overnight in 10 ml Luria–Bertani broth (including 30 μg/ml kanamycin). The culture was diluted 1:100 with fresh medium and grown at 37°C until absorbance at OD_{600} reached 0.4, at which point isopropyl-β-D-thiogalactopyranoside (Merck, Darmstadt, Germany) was added to the culture to a final concentration of 1.5 mM to induce expression of the target products. After 5 h at 28°C, the bacterial cells, harvested by centrifugation and resuspended in lysis buffer [50 mM Tris/HCl (pH 8.0), 100 mM NaCl, TritonX-100 (0.5%), 2 mM ethylenediaminetetraacetic acid], were lysed by sonication and centrifuged again. The inclusion body of recombinant protein Acer-ASP2 was severely precipitated in 1.5 M urea in ddH_2O and finally freeze-dried and resuspended (1 mg/ml) in phosphate buffered saline (PBS; pH 7.4) for injection into rabbit for producing antisera.

Preparation of Antisera Antisera were obtained by injecting an adult male rabbit subcutaneously and intramuscularly with purified inclusion body of recombinant protein of Acer-ASP2 (1 mg/time). The interval between the first and second injection was 21 days, whereas two additional injections were performed at 14-day intervals. The protein was emulsified with an equal volume of Freund's complete adjuvant for the first injection and incomplete adjuvant for further injections. For 10 days after the final injection, the rabbit was bled and the serum was collected for further application.

Western Blot Analysis After electrophoretic separation under denaturing conditions (12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)), purified Acer-ASP2 was electroblotted on a nitrocellulose membrane by using a Bio-Rad Transblot (Bio-Rad Laboratories, Hercules, CA, USA). After treatment in TBS (containing 3% BSA and 0.05% Tween 20) at 4°C overnight, the membrane was incubated with the crude antiserum against Acer-ASP2 (dilution 1:500) and then with goat antirabbit IgG horse radish peroxidase conjugate (dilution 1:1,000). Immunoreacting bands were treated with the color development solution (2 mg diaminobenzidine in 10 ml TBS, 10 μl 30% H_2O_2).

Scanning Electron Microscopy For scanning electron microscopy (SEM), the antennae of worker bees were immersed in osmic acid (1%) for 30 min and in a concentration series of ethanol (50%, 70%, 80%, 90%, 95%, and 100%) for 15 min each. After air drying, samples were mounted on holders and examined by using a SEM of XL30-ESEM (Philips Research, Eindhoven, The Netherlands) after gold coating with K500X sputter coater (Emitech Ltd., Ashford, Kent, UK).

Immunocytochemical Localization Antennae of worker bees were chemically fixed in a mixture of formaldehyde (2%) and glutaraldehyde (1%) in 0.1 M PBS (pH 7.4) at 4°C for 2–3 h, then dehydrated in an ethanol series and polymerized embedded in K4 M (Sigma, St. Louis, MO, USA) and irradiated with UV at −20°C for 72 h. Ultrathin sections (50–100 nm) were cut with a glass knife on an Ultracut E ultramicrotome (Reichert-Jung, Austria). For immunocytochemistry, the nickel grids adhering to ultrathin sections were floated on droplets of the following solutions on parafilm with sequential steps: ddH_2O, BL (50 mM PBS containing 1% bovine serum albumin and 0.02% PEG2000, 10 mM NaCl, and 1% NaN_3), primary antiserum diluted with BL (dilution 1:50), twice each on ddH_2O, BL, secondary antibody that goat anti-rabbit IgG coupled to 10-nm colloidal gold (AuroProbe EM, GAR G10, Amersham Biosciences, Piscataway, NJ, USA) diluted with BL (dilution 1:20), and three–five times on ddH_2O. The results of immunolocalization of Acer-ASP2 were observed and photographed through the transmission electron microscope of JEM-1230 (JEOL Ltd., Tokyo, Japan).

Expression Profiling of Acer-ASP2 with Real-Time PCR For spatial expression profiling, antennae (A), heads (H), thoraces (Th), abdomens (Ab), wings (W), and legs (L) were dissected from 50 adult worker bees. For expression profiling across ages of worker bees, 7-day-old larvae and pupae, and the antennae of 1, 4, 6, 9, 12, 15, 18, 21, 24, 27, and 30-day-old adult worker bees were prepared. Total RNA of all above material from individual honeybee colonies was extracted separately and cDNA was synthesized as described above. Specific primer pairs were designed based on the primers used for cloning Acer-ASP2 (Table 1) and *A. cerana* β-actin (GenBank accession no. AB072495). For real-time quantitative RT-PCR, primers for *A. cerana* β-actin were designed such that the paired primers (Table 1) were expected to amplify a 301-bp fragment. An iCycler iQ™ real-time PCR detection system (Bio-Rad) was used to detect the spatiotemporal transcriptional profiling of *Acer-ASP2* mRNA of *A. cerana cerana*. The total 20-μl reaction system consisted of 1 μl cDNA (about 20 ng), 10 μl iQ™ SYBR® Green Supermix (Bio-Rad), and 10 μM of each primer. The thermal cycling

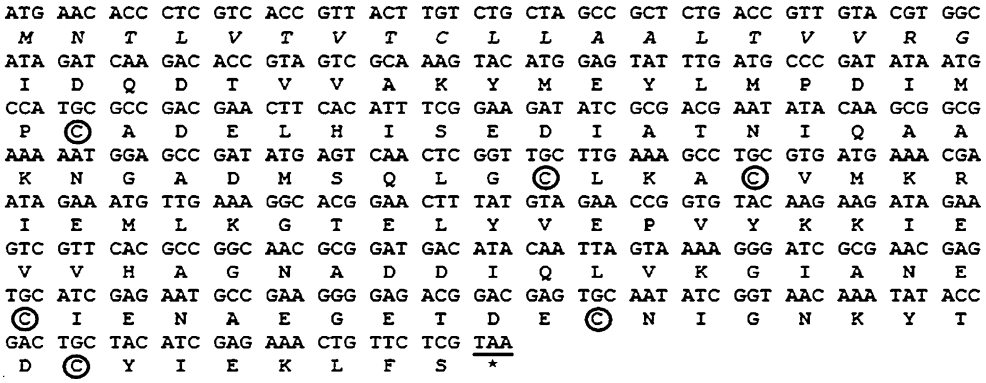

Fig. 1 Full cDNA sequence of the open reading frame and deduced amino acid sequence of Acer-ASP2 from an Asian honey bee, *A. cerana cerana*. The *asterisk* marks the stop codon, six conserved Cys residues are *circled*, and the 19 amino acid residues in *italicized font* represent the N-terminal signal peptide sequence

conditions for real-time PCR were 5 min at 94°C, followed by 40 cycles of 30 s at 94°C, 30 s at 50°C, and 30 s at 72°C. PCR reactions were performed in triplicate, and data were processed by using the relative quantification $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

Results

cDNA Sequence of Acer-ASP2 To characterize the *Acer-ASP2* cDNA, we designed specific primers and obtained a 393-bp open reading frame (ORF; Fig. 1). After cloning and sequencing, we obtained the cDNA sequence and submitted it to GenBank (accession number DQ449667). The ORF encodes 142 amino acids and contains a hydrophobic signal peptide with 19 amino acids at the N terminus (predicted by the software SignalP 3.0; Bendtsen et al. 2004). By using the proteomics server ExPASy (Gasteiger et al. 2003), the theoretical isoelectric point and molar weight of mature Acer-ASP2 were computed as 4.36 and 15,656.16, respectively, in agreement with other OBPs. The protein has the typical six-cysteine signature of OBPs and has low similarity (14.9–24.4% of identical residues) with OBPs from other insect species (Fig. 2). However, it does have high similarity (98.6%) with *Amel*-ASP2

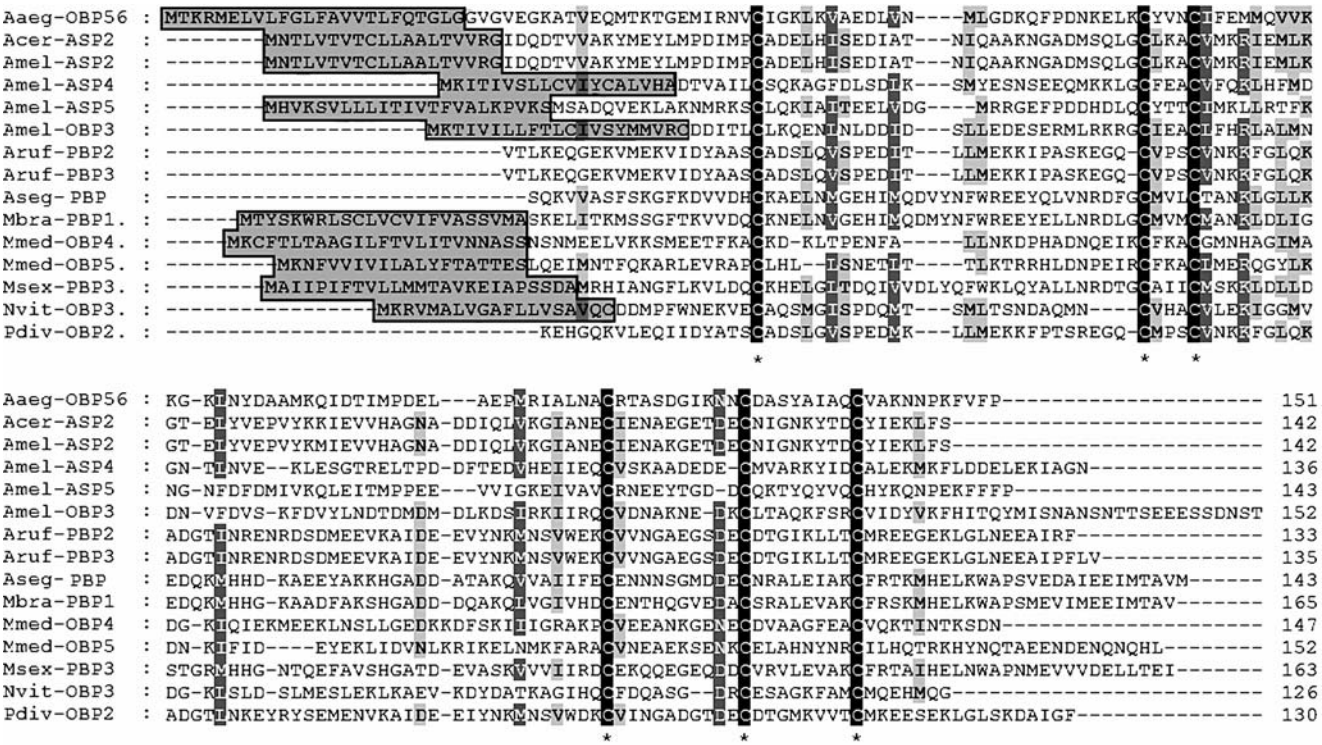

Fig. 2 Alignment of deduced amino acid sequence of Acer-ASP2 with homologous proteins from other insect species (GenBank BLASTP). Sequences used in alignment are indicated by the following species abbreviations: *Acer Apis cerana cerana*, *Aaeg Aedes aegypti*, *Amel Apis mellifera*, *Asch Anomala schonfeldti*, *Aruf Anomala rufocuprea*, *Pdiv Phyllopertha diversa*, *Nvit Nasonia vitripennis*, and *Mmed Microplitis mediator*. Residues common to most sequences are *shaded*. Six conserved Cys residues are labeled by *asterisks*. Signal peptide sequences are highlighted by enclosing them in *shaded rectangles*

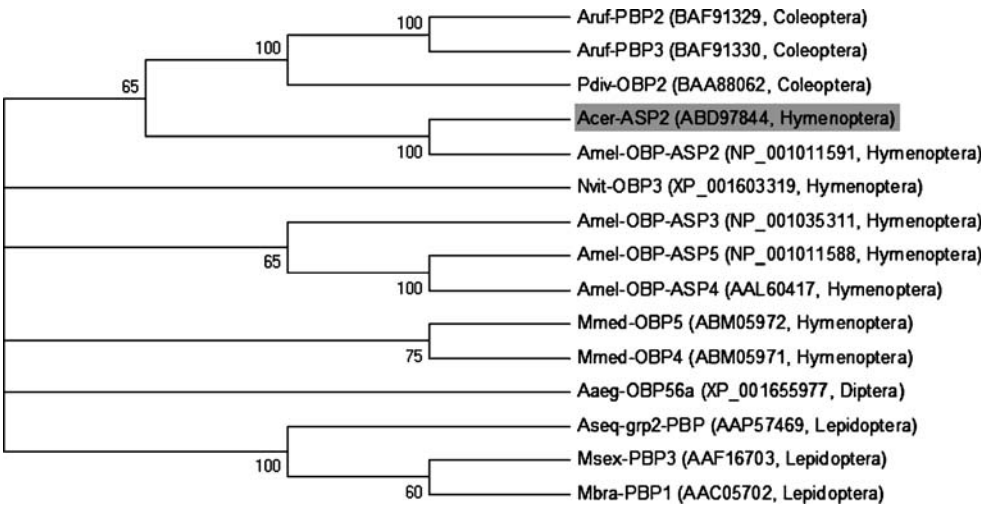

Fig. 3 Neighbor-joining (NJ) tree (condensed tree, cutoff value is 50%) of sequences similar to Acer-ASP2. All OBPs from the alignment analysis (Fig. 2) were included in the NJ tree. Bootstrap support values (%) based on 1,000 replicates are indicated

(Fig. 2). These results were emphasized in the evolutionary tree from the neighbor-joining analysis (Fig. 3).

Production and Purification of Acer-ASP2 and Western Blot Analysis with Antisera To obtain the amounts of recombinant protein Acer-ASP2 suitable for preparation of antisera, we digested the pGEM-Acer-ASP2 plasmid with *Bam*H I and *Hin*d III and the resultant purified DNA was inserted into the expression vector pET-30a (+) and transformed into *E. coli* BL21(DE3) competent cells. Electrophoretic analysis of the crude cell extracts (pellet and supernatant) under denaturing conditions (12.5% SDS-PAGE) revealed that the recombinant protein Acer-ASP2 migrated as an intense band of 23 kDa (Fig. 4). The protein was recovered almost entirely in the form of an insoluble inclusion body and in high yields (generally, 1 l of culture yielded 50 mg of protein at final purification). Recombinant Acer-ASP2 was purified by precipitation in urea, freeze-dried, and injected into a rabbit as described above. The polyclonal anti-(Acer-ASP2)-serum obtained was applied to detect Acer-ASP2 by Western blot analysis (Fig. 4, WB).

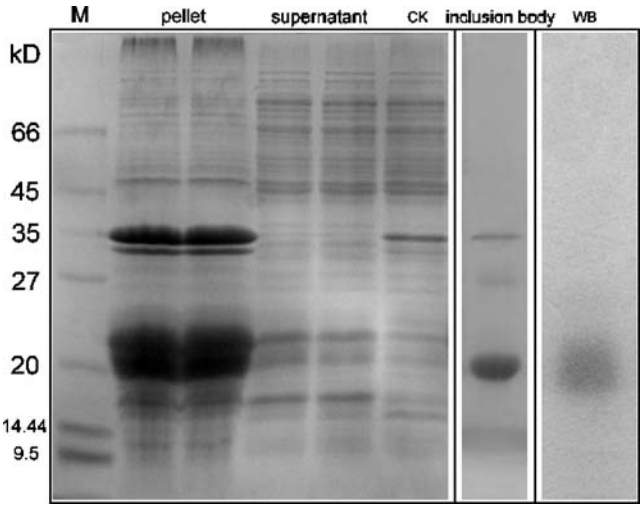

Fig. 4 Electrophoretic analysis (12.5% SDS-PAGE) of expression products from *E. coli* BL21(DE3) cells. The inclusion body protein of Acer-ASP2 was purified by urea with differing concentrations, showing an intense band of 23 kDa, present almost entirely in the pellet. Western blot analysis showed the specificity of anti-(Acer-ASP2)-serum with antigen Acer-ASP2. The low molecular weight protein markers are shown in the *lane furthest to the left*

Immunocytochemical Localization Imaging of *A. cerana cerana* worker antennae by scanning electron microscopy revealed at least six different types chemosensilla: sensilla placodea (sp), sensilla basiconica (sb), sensilla campaniform (scf), and sensilla trichoidea A,B,CD (Fig. 5, which shows only sp and stA). Sensilla placodea, the most numerous type on worker bee antennae, are oval discs. As observed in other honey bees (Ågren 1977, 1978), the plate is raised and resembles a hemispherical dome separated from the surrounding surface by a crevice. These clefts may either result from air drying during sample processing (i.e., an artifact) or may correspond to a lower point at which the plate rim is fastened to the cuticle and the outer rim of the

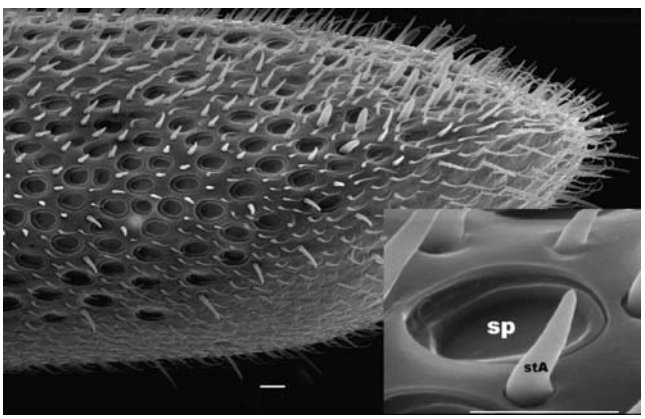

Fig. 5 Scanning electron micrograph of the sensilla on the tip of the flagellar segment of the antennae of *A. cerana cerana*. The *magnified figure* illustrates the two main olfactory sensilla: s. placodea (*sp*) and s. trichoidea A (*st A*). *Scale bar* is 10 μm

sensillum is raised (Fig. 5, sp). Sensilla trichodea A, which are more numerous than the remaining types listed above, appear slender, glabrous, and bent distally with the tip pointed slightly up. They are characterized by a thick cuticular wall with unbranched outer dendritic segments in the lumen (Fig. 5, stA).

The polyclonal antiserum against Acer-ASP2 was used for immunocytochemical analysis of the antennae. In sections of tarsal segments of antennae, significant gold particles only labeled the sp and the stA. With the sp, gold particles accumulated on the dendrites in the sensillar lymph, but not in the narrow pore areas on the top of the wide brim of the oval discs of the sp (Fig. 6, A1,A2). With the stA, more gold particles accumulated on the dendrites in the trichogen cell; fewer particles accumulated in the outer and inner sensillar lymph in the cell (Fig. 6, B1,B2).

Expression Profiling of Acer-ASP2 Based on the normalized relative quantification $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) for real-time PCR with SYBR I, spatial expression profiling of *Acer-ASP2* revealed that the transcript was specifically expressed on antennal tissue, but never in other anatomical regions tested (based on the final melt curve of real-time PCR; Fig. 7a). On the other hand, the developmental and temporal expression analysis indicated no expression in larvae or pupae, and then two ages in the adult worker bee antenna during which expression was elevated: (1) highest expression between 9 and 15 days (although it declined at the 12 days measurement) and (2) relatively high expression between 27 and 30 days (Fig. 7b). Transcript abundance was calculated based on the difference in threshold cycle (C_t) values between *Acer-ASP2* and β-actin transcripts.

Discussion

In this study, we cloned and identified a novel gene encoding Acer-ASP2, an OBP from the Asian honey bee, *A. cerana cerana*. The protein has typical hexapod OBP characteristics, e.g., conserved residues of six cysteines, and a similar isoelectric point and molecular weight. When compared with Amel-ASP2 of *A. mellifera*, the amino acid

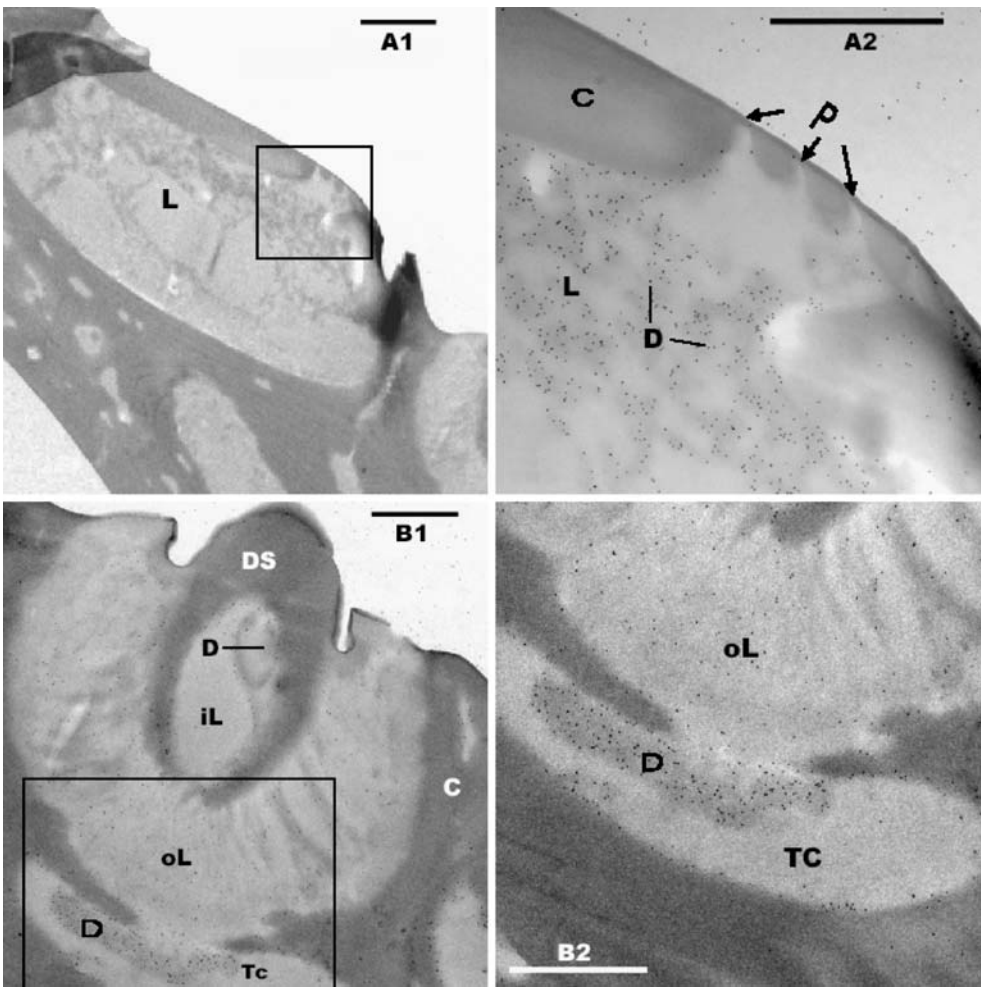

Fig. 6 Immunocytochemical localization of OBP in the antennal sensilla of *A. cerana cerana*. Acer-ASP2 was specifically expressed on the s. placodea (*A1* and *A2*, *A2* is an enlargement of the square section in *A1*) and the s. trichodea A (*B1* and *B2*, B2 is an enlargement of the square section in *B1*). In this image of the s. placodea, the gold particles were labeled on the dendrites (*D*) and the sensillar lymph (*L*), but not in the narrow pore areas (*P*) on the top of the olfactory sensilla (*A2*). In this image of the s. trichodea A, more gold particles were labeled on the dendrites in trichogen cell (*TC*) and fewer particles were labeled in the outer (*oL*) and inner (*iL*) sensillar lymph, the cuticular hair wall (*C*), or the dendritic sheath (*DS*). *Scale bar* is 1 μm

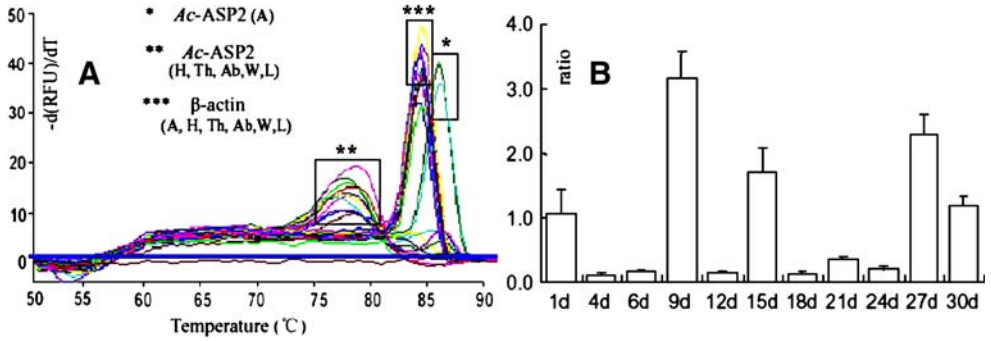

Fig. 7 Relative spatial and developmental quantification of Acer-ASP2 transcripts from various honey bee organs by real-time PCR. In spatial expression profiles (**a**), the melting curve within real-time PCR showed that Acer-ASP2 was specifically expressed on antennae (*A*; the high peak at 85–90°C labeled with *one asterisk*) but was never detected (except as primer dimers) in other organs: heads (*H*), thoraces (*Th*), abdomens (*Ab*), wings (*W*), and legs (*L*; the lowest peak at 75–80°C labeled with *two asterisks*). Another peak representing amplified products of β-actin present in all organs was labeled with *three asterisks*. In temporal expression profiles (**b**), Acer-ASP2 was not expressed in larvae and pupae (data not shown in the figure). However, in some adults, the highest levels of relative expression in the antennae occurred at 9 and 27 days (ratio>2.0), the next highest levels occurred at 1, 15, and 30 days (2.0>ratio>1.0), and the lowest levels occurred at 4, 6, 12, 18, 21, and 24 days (ratio<1.0)

sequence of Acer-ASP2 is highly similar (98.6%), but less similar (14.9–24.4%) to 13 other OBPs or PBPs (Fig. 2) from more distantly related species of insects.

Immunocytochemistry experiments have shown that OBPs are always localized in the diverse olfactory sensilla in many insects. For example, in Lepidoptera, the GOBPs of *Antheraea polyphemus* and *Bombyx mori* were localized only in s. trichodea and s. basiconica and principally in the sensillar lymph around the sensory dendrites (Steinbrecht et al. 1995). Similar anatomical patterns were also described for OBPs from two species of Orthoptera, *Schistocerca gregaria* and *Locusta migratoria* (Jin et al. 2005). In male *Helicoverpa armigera* (Lepidoptera), GOBP2*Harm* was mainly expressed in the s. basiconica, whereas in females, it was expressed abundantly in the s. trichodea (Wang et al. 2003). In the noctuid moths, *Agrotis segetum*, *Autographa gamma*, *H. armigera*, *Heliothis virescens*, and *Spodoptera littoralis*, PBPs were localized predominantly in s. trichodea and GOBP2 in s. basiconica (Zhang et al. 2001). In that study, interspecific immunolocalization utilized antisera raised against the PBP and GOBP2 of *A. polyphemus*, but the result reflected a good correlation with the stimulus specificity of the receptor cells in these types of sensilla.

Summarizing the immunochemical studies, we can conclude that PBPs of moths always have been localized predominantly in the s. trichodea, which respond to pheromones, whereas the GOBPs have been localized predominantly in the s. basiconica, which are generally involved in detecting plant odors. However, Acer-ASP2 was not found in the s. basiconica of *A. cerana cerana* (considered to be involved in taste and mechanoreception in *A. mellfera*; Esslen and Kaissling 1976), but in the s. placodea and s. trichodea A. This suggests that the latter sensillar types may be involved in detecting plant odors in bees. Although the allocation of function to the various types of sensilla in moths and bees is not completely identical, the anatomical placement of OBPs is consistent with their role in detecting general odors in both higher taxonomic groups. Perhaps this is a result of differences in classification of the antennal morphology of moths and bees or in the function of the sensilla in the two species. OBPs are synthesized in the trichogen cells along typical pathways for protein biosynthesis (Steinbrecht et al. 1992). In our study, the heavy labeling of Acer-ASP2 in the trichogen cells of the s. trichodea A supports this concept.

In most insect species, the prevailing model is that OBPs are expressed specifically in the antennae. Our real-time PCR data support this because the *Acer-ASP2* mRNA was present in abundance in antennae, but never in other anatomical tissues (e.g., heads, thoraces, abdomens, wings, or legs; Fig. 7a). The anatomical patterns of expression of Acer-ASP2 were similar to those of Amel-ASP2, which has been detected exclusively in the antennae of *A. mellifera* (Danty et al. 1997). Other OBPs of *A. mellifera* (e.g., Amel-ASP1, 4, 5, 6) have been reported as antennal specific in workers and drones, but with some localization in legs of drones (ASP1; Danty et al. 1998), equally expressed in the antennae, wings, and legs of various castes and age groups (ASP4 and ASP5) or detected in antennae and legs of both workers and drones (ASP6; Calvello et al. 2005). Furthermore, OBPs have been expressed equally in antennae, wings, and legs of all castes and ages of the paper wasp, *Polistes dominulus* (Calvello et al. 2003), and in legs and wings of another wasp, *Vespa crabro*.

From the developmental and temporal expression profiles of *Acer-ASP2*, we learned that it is not expressed in larvae and pupae, and in adults, there are two periods of elevated expression. The first period occurs at 9 and 27 days (mean expression ratio was 2.7±0.4) and the second occurred at 1, 15, and 30 days (mean ratio was 1.3±0.3).

Throughout the remaining times that we assayed for expression, the mean ratio was 0.2±0.03 (Fig. 7b). Because the expression ratios during the first two periods are tenfold greater than that of the lowest period, Acer-ASP2 may play an important functional role in adults during the two periods. At the 9-day timepoint (the highest expression of *Acer-ASP2*), worker bees are making the transition in maturation and are starting to secrete plentiful beeswax to construct their comb (Yang 1998). After 15 days, maturing bees begin to fly out and forage for nectar and pollen. Their highest level of foraging activity occurs around the 27-day timepoint, which coincides with the second highest level of *Acer-ASP2* expression that we recorded. We do not understand why transcript amounts were lower in the intervening periods of 18, 21, and 24 days. However, taken together, our results suggest that Acer-ASP2 may play an important role in the processes of producing beeswax and acquiring the odorant molecules related to foraging for nectar and pollen, or other general odorants.

As a native Chinese bee species, the habitat of *A. cerana cerana* is threatened by the invasion of exotic bees as well as deteriorating environmental conditions (Tan 2006). Future research necessary to protect this species might involve the molecular mechanism of the unique characteristics of the olfactory system. Recently, the genomic sequencing project of *A. mellifera* was completed (The Honeybee Genome Sequencing Consortium 2006), and examination of genes related to signal transduction suggested that *A. mellifera* has fewer genes for gustatory reception and more genes for odorant reception, which is consistent with its ecology and social organization. *A. cerana cerana* appears to have greater olfactory sensitivity when compared with *A. mellifera*, so elucidating the biochemical mechanism of the olfactory process in *A. cerana cerana* should be a high priority in the future.

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References

ÅGREN, L. 1977. Flagellar sensilla of some Colletidae (Hymenoptera: Apoidae). *Int. J. Insect Morphol. Embryol.* 6:137–146.

ÅGREN, L. 1978. Flagellar sensilla of two species of *Andrena* (Hymenoptera: Andrenidae). *Int. J. Insect Morphol. Embryol.* 7:73–79.

BAN, L., SCALONI, A., D'AMBROSIO, C., ZHANG, L., YAHN, Y., and PELOSI, P. 2003. Biochemical characterization and bacterial expression of an odorant-binding protein from *Locusta migratoria*. *Cell. Mol. Life Sci.* 60:390–400.

BENDTSEN, J. D., NIELSEN, H., VON HEIJNE, G., and BRUNAK, S. 2004. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* 340:783–795.

BIESSMANN, H., WALTER, M. F., DIMITRATOS, S., and WOODS, D. 2002. Isolation of cDNA clones encoding putative odourant binding proteins from the antennae of the malaria-transmitting mosquito, *Anopheles gambiae*. *Insect Mol. Biol.* 11:123–132.

BRIAND, L., NESPOULOUS, C., HUET, J. C., TAKAHASHI, M., and PERNOLLET, J. C. 2001. Ligand binding and physico-chemical properties of ASP2, a recombinant odorant-binding protein from honeybee (*Apis mellifera* L.). *Eur. J. Biochem.* 268:752–760.

CALVELLO, M., GUERRA, N., BRANDAZZA, A., D'AMBROSIO, C., SCALONI, A., DANI, F. R., TURILLAZZI, S., and PELOSI, P. 2003. Soluble proteins of chemical communication in the social wasp *Polistes dominulus*. *Cell. Mol. Life Sci.* 60:1933–1943.

CALVELLO, M., BRANDAZZA, A., NAVARRINI, A., DANI, F. R., TURILLAZZI, S., FELICIOLI, A., and PELOSI, P. 2005. Expression of odorant-binding proteins and chemosensory proteins in some Hymenoptera. *Insect Biochem. Mol. Biol.* 35:297–307.

CHEN, S. L. 2001. The Apiculture Science in China. pp. 123–131. Agricultural Publishing House of China, Beijing, China(in Chinese).

DANTY, E., MICHARD-VANHEE, C., HUET, J. C., GENECQUE, E., PERNOLLET, J. C., and MASSON, C. 1997. Biochemical characterization, molecular cloning and localization of a putative odorant-binding protein in the honey bee *Apis mellifera* L. (Hymenoptera: Apidea). *FEBS Lett.* 414:595–598.

DANTY, E., ARNOLD, G., HUET, J. C., HUET, D., MASSON, C., and PERNOLLET, J. C. 1998. Separation, characterization and sexual heterogeneity of multiple putative odorant-binding proteins in the honeybee *Apis mellifera* L. (Hymenoptera: Apidae). *Chem. Senses* 23:83–91.

DANTY, E., BRIAND, L., MICHARD-VANHEE, C., PEREZ, V., ARNOLD, G., GAUDEMER, O., HUET, D., HUET, J. C., OUALI, C., and MASSON, C. 1999. 7468–7475. Cloning and expression of a queen pheromone-binding protein in the honeybee: an olfactory-specific, developmentally regulated protein. *J. Neurosci.* 19:7468–7475.

DICKENS, D. J., CALLAHAN, W. P., WERGIN, W. P., and ERBE, E. F. 1995. Olfaction in a hemimetabolous insect: antennal-specific proteins in adult *Lygus lineolaris* (Heteroptera: Miridae). *J. Insect Physiol.* 41:857–867.

ESSLEN, J., and KAISSLING, K. -E. 1976. Number and distribution of the sensilla on the antennal flagullum of the honeybee (*Apis mellifera* L.). *Zoomorphologie* 83:227–251.

FORÊT, S., and MALESZKA, R. 2006. Function and evolution of a gene family encoding odorant binding-like proteins in a social insect, the honey bee (*Apis mellifera*). *Genome Res.* 16:1404–1413.

GASTEIGER, E., GATTIKER, A., HOOGLAND, C., IVANYI, I., APPEL, R. D., and BAIROCH, A. 2003. ExPASy: The proteomics server for in-depth protein knowledge and analysis. *Nucleic Acids Res.* 31:3784–3788.

GRAHAM, L. A., BREWER, D., LAJOIE, G., and DAVIES, P. L. 2003. Characterization of a subfamily of beetle odorant-binding proteins found in hemolymph. *Mol. Cell. Proteomics* 2:541–549.

HEKMAT-SCAFE, D. S., STEINBRECHT, R. A., and CARLSON, J. R. 1997. Coexpression of two odorant-binding protein homologs in *Drosophila*: implications for olfactory coding. *J. Neurosci.* 17:1616–1624.

ISHIDA, Y., CORNEL, A. J., and LEAL, W. S. 2002a. Identification and cloning of a female antenna-specific odorant-binding protein in the mosquito *Culex quinquefasciatus*. *J. Chem. Ecol.* 28:867–871.

ISHIDA, Y., CHIANG, V. P., HAVERTY, M. I., and LEAL, W. S. 2002b. Odorant-binding proteins from a primitive termite. *J. Chem. Ecol.* 28:1887–1893.

ISHIDA, Y., CHEN, A. M., TSURUDA, J. M., CORNEL, A. J., DEBBOUN, M., and LEAL, W. S. 2004. Intriguing olfactory proteins from the yellow fever mosquito, *Aedes aegypti*. *Naturwissenschaften* 91:426–431.

JIN, X., BRANDAZZA, A., NAVARRINI, A., BAN, L., ZHANG, S., STEINBRECHT, R. A., ZHANG, L., and PELOSI, P. 2005. Expression and immunolocalisation of odorant-binding and chemosensory proteins in locusts. *Cell. Mol. Life Sci.* 62:1156–1166.

LEAL, W. S., NIKONOVA, L., and PENG, G. 1999. Disulfide structure of the pheromone binding protein from the silkworm moth, *Bombyx mori*. *FEBS Lett.* 464:85–90.

LIVAK, K. J., and SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25:402–408.

LU, D., LI, X., LIU, X., and ZHANG, Q. 2007. Identification and molecular cloning of putative odorant-binding proteins and chemosensory protein from the bethylid wasp, *Scleroderma guani* Xiao et Wu. *J. Chem. Ecol.* 33:1359–1375.

MAIDA, R., MAMELI, M., MULLER, B., KRIEGER, J., and STEINBRECHT, R. A. 2005. The expression pattern of four odorant-binding proteins in male and female silk moths, *Bombyx mori*. *J. Neurocytol.* 34:149–163.

MALESZKA, R., and STANGE, G. 1997. Molecular cloning, by a novel approach, of a cDNA encoding a putative olfactory protein in the labial palps of the moth *Cactoblastis cactorum*. *Gene* 202:39–43.

MICHELETTE, E. R. F., and SOARES, A. E. E. 1993. Characterization of preimaginal developmental stages in Africanized honey bee workers (*Apis mellifera* L.). *Apidologie* 24:431–440.

NAGNAN-LE MEILLOUR, P., FRANCOIS, M. C., and JACQUIN-JOLY, E. 2004. Identification and molecular cloning of putative odorant-binding proteins from the American palm weevil, *Rhynchophorus palmarum* L. *J. Chem. Ecol.* 30:1213–1223.

OZAKI, M., MORISAKI, K., IDEI, W., OZAKI, K., and TOKUNAGA, F. 1995. A putative lipophilic stimulant carrier protein commonly found in the taste and olfactory systems. A unique member of the pheromone-binding protein superfamily. *Eur. J. Biochem.* 230:298–308.

PELOSI, P. 1996. Perireceptor events in olfaction. *J. Neurobiol.* 30: 3–19.

PELOSI, P., and MAIDA, R. 1995. Odorant-binding proteins in insects. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* 111:503–514.

PELOSI, P., ZHOU, J. J., BAN, L. P., and CALVELLO, M. 2006. Soluble proteins in insect chemical communication. *Cell Mol. Life Sci.* 63:1658–1676.

PENG, Y. S., FANG, Y. Z., XU, S. Y., and GE, L. S. 1987. The resistance mechanism of the Asian honey bee *Apis cerana* Fabr., to an ectoparasitic mite, *Varroa jacobsoni* Oudemans. *J. Inverteb. Pathol.* 49:54–60.

RACHINSKY, A., STRAMBI, C., STRAMBI, A., and HARTFELDER, K. 1990. Caste and metamorphosis: hemolymph titers of juvenile hormone and ecdysteroids in last instar honeybee larvae. *Gen. Comp. Endocrinol.* 79:31–38.

SCALONI, A., MONTI, M., ANGELI, S., and PELOSI, P. 1999. Structural analysis and disulfide-bridge pairing of two odorant-binding proteins from *Bombyx mori*. *Biochem. Biophys. Res. Commun.* 266:386–391.

STEINBRECHT, R. A. 1998. Odorant-binding proteins: expression and function. *Ann. N. Y. Acad. Sci.* 855:323–332.

STEINBRECHT, R. A., OZAKI, M., and ZIEGELBERGER, G. 1992. Immunocytochemical localization of pheromone-binding protein in moth antennae. *Cell Tissue Res.* 270:287–302.

STEINBRECHT, R. A., LAUE, M., and ZIEGELBERGER, G. 1995. Immunolocalization of pheromone-binding protein and general odorant-binding protein in olfactory sensilla of the silk moths *Antheraea* and *Bombyx*. *Cell Tissue Res.* 282:203–217.

TAN, X. M. 2006. Wild resources of *Apis cerana cerana* are reducing rapidly. *J. Bee (in Chinese)* 4:16.

THE HONEYBEE GENOME SEQUENCING CONSORTIUM 2006. Insights into social insects from the genome of the honeybee *Apis mellifera*. *Nature* 443:931–949.

VOGT, R. G., and RIDDIFORD, L. M. 1981. Pheromone binding and inactivation by moth antennae. *Nature* 293:161–163.

VOGT, R. G., and LERNER, M. R. 1989. Two groups of odorant binding proteins in insects suggest specific and general olfactory pathways. *Neurosci. Abstr.* 15:1290–1296.

VOGT, R. G., PRESTWICH, G. D., and LERNER, M. R. 1991a. Odorant-binding-protein subfamilies associate with distinct classes of olfactory receptor neurons in insects. *J. Neurobiol.* 22:74–84.

VOGT, R. G., RYBCZYNSKI, R., and LERNER, M. R. 1991b. Molecular cloning and sequencing of general odorant-binding proteins GOBP1 and GOBP2 from the tobacco hawk moth *Manduca sexta*: comparisons with other insect OBPs and their signal peptides. *J. Neurosci.* 11:2972–2984.

WANG, G. R., WU, K. M., and GUO, Y. Y. 2003. Cloning, expression and immunocytochemical localization of a general odorant-binding protein gene from *Helicoverpa armigera* (Hubner). *Insect Biochem. Mol. Biol.* 33:115–124.

XIU, W. M., and DONG, S. L. 2007. Molecular characterization of two pheromone binding proteins and quantitative analysis of their expression in the beet armyworm, *Spodoptera exigua* Hübner. *J. Chem. Ecol.* 33:947–961.

XU, P. X., ZWIEBEL, L. J., and SMITH, D. P. 2003. Identification of a distinct family of genes encoding atypical odorant-binding proteins in the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol. Biol.* 12:549–560.

YANG, C. H. 1998. The development and lives of male and worker bees of *Apis cerana cerana*. *J. Bee (in Chinese)* 8:16.

ZHANG, S., MAIDA, R., and STEINBRECHT, R. A. 2001. Immunolocalization of odorant-binding proteins in noctuid moths (Insecta, Lepidoptera). *Chem. Senses* 26:885–896.

al. 2006), and many containerized baits are not practical for landscape scale applications. Poison baits also may carry non-target risks to pets and wildlife. In natural areas, particularly those sensitive to perturbation or of high ecological value, impacts on native invertebrates can be considerable (Balança and de Visscher 1997; Zhakharov and Thompson 1998). Thus, effective and environmentally benign control tactics are needed for management and control of these invasive ants.

Wright (1964) proposed that if sufficient synthetic sex pheromone were distributed in the air, the normal pheromone communication systems of certain species would be disrupted, and the sexes might be incapable of locating each other. The feasibility of the disruption approach by using pheromones was demonstrated for the cabbage looper (Gaston et al. 1967), and "mating disruption" is now an effective direct control option against certain pests (Suckling and Karg 2000; El-Sayed 2008).

The development of more environmentally benign treatments based on semiochemicals could be used for managing invasive ants in sensitive natural ecosystems. Despite the discovery and characterization of a variety of ant pheromones, relatively little work has been undertaken using them for management (Robinson and Cherrett 1978; Shorey et al. 1992).

The Argentine ant uses (*Z*)-9-hexadecenal (Cavill et al. 1979) as its trail pheromone to orient and communicate the location of food, and it is probably critical to successful mass recruitment to ephemeral food resources. Van Vorhis Key et al. (1981) and Van Vorhis Key and Baker (1982a, b, c) studied this system and recognized that it might be possible, without elaborating how, to modify trail-following behavior with synthetic compounds. Shorey et al. (1992) tested other volatile compounds as barrier treatments for disruption of Argentine ant trails, but these were not very effective.

The recent demonstration of partial disruption of Argentine ant trails by using polyethylene tubing dispensers that release (*Z*)-9-hexadecenal in orchards (Tatsuki et al. 2005) raises the possibility of development of a new control tactic against this species in different environments, including sensitive ecosystems, by using formulations that could be applied at landscape level. Trail disruption could have a potential as a stand-alone pest management tactic or in combination with others.

In this study, we first examined close-range disruption of trail-following behaviors on different substrates when a single point source was placed near a trail. Second, we carried out small plot field tests to examine foraging at bait cards in areas by using a widely dispersed point source strategy. In both cases, the pheromone was placed within the boundary layer of the foraging ants, which was not the case in the study by Tatsuki et al. (2005).

Methods and Materials

Chemicals The Argentine ant trail pheromone (*Z*)-9-hexadecenal (94% purity; Bedoukian Research, Danbury, CT, USA) was loaded in micronized carnuba wax (Eggar & Co. Ltd. (Reading, UK), as plant wax is an effective carrier for pheromones (Karg et al. 1994). In single point source tests at close range, the pheromone source was prepared either as (a) 0.1 g pheromone in hexane (10% by vol.) steeped in 1 g of carnuba wax in a closed 1-ml glass screw cap vial at room temperature for 2 h, and then the solvent was evaporated off, or (b) by coating particles of white quartz sand (−50 + 70 mesh, Sigma Aldrich, Saint Louis, MO, USA) with melted wax (20% wax by weight) in a rotary evaporator, and then adding pheromone to give a mixture consisting of 1 g quartz sand/200 mg wax/1 mg pheromone.

Disrupting Trail Following from a Point Source Ant density and trail walking behaviors, before and after treatment, were used to characterize disruption from a single point source on a range of vertical and horizontal substrates in and around a wooden office building located at ca 1,200 m altitude in Hawaii Volcanoes National Park, Hawaii. A Logitech webcam (640×480 pixels, Notebook Pro, Logitech, Freemont, CA, USA) recorded avi files at rate of 15 frames/s onto a laptop computer (actual screen size 10×12 cm at 480×640 pixels). A plastic ruler was used for frame calibration before treatment. All experimental data were collected between 10 A.M. and 3 P.M.

Video Recording and Statistical Analysis The position of ants in the frame, number of ants per frame, and walking track angle were measured in the first trial, but only the position of ants was analyzed for the subsequent three experiments. The position and movement of individual ants was analyzed with MaxTraq v1.92 trial edition (Innovision Systems, Lapeer, MI, USA). The *x*–*y* positions of the ants were either recorded continuously (15 frames/s) or from individual frames at 5-s intervals, as this was sufficient time for the ants to leave the video frame under control conditions, thus rendering the samples independent. Walking angles of individual ants were analyzed from sequential frames and were calculated as: $\theta\,^{\circ} = \text{Arctan}\,\Delta X/\Delta Y \times 180/\pi$, where $\theta\,^{\circ}$ is the walking angle, and ΔX and ΔY are the distance traveled between two consecutive frames on the *X*–*Y* axes, respectively. Values generated when an ant apparently did not move in two consecutive frames were discarded, as these rare cases would be erroneous. Regression statistics for trail integrity (r^2) were calculated

from the position of digitized ants, before and after treatment. A *t* test was used to determine the significance of trail integrity in each of the tests, following Shapiro and Wilk tests for normality (Zar 1984). For experiment 1, the number of ants present in the video frame on the wall before and after the vial was introduced was analyzed by regression of count over time over 40 min. Sampling of the avi file at 5-s intervals was undertaken at the beginning and end of the time series to improve precision. The walking track angles of individual ants walking on a painted internal wall at high traffic density were compiled into histograms, and the track angle distributions were compared by two-sample *F* test for variance for before and after exposure to the pheromone.

Test 1.1 Ants (between 20 and 40 per frame) on a vertical interior wall were recorded as in a continuous stream, diagonally across the frame for 2 min at 300 and 120 s before the experiment, and confirmed that the foraging trail was linear. Activity was recorded continuously for 60 s before the glass vial with pheromone was opened within the camera view (2 cm to the right of the lower left corner) and after a 20-s behavior was recorded for another 60 s. Recordings were made continuously over the next 30 min, at which time the vial was removed and activity was recorded for an additional 7 min. The same experimental procedure was carried out on a painted outdoor wall (*Test 1:2*) at low to moderate density (range one to 13 ants per frame), but in this case, the ant positions were digitized for 100 s following treatment.

Test 1.3 and 1.4 Ant walking tracks, on an interior wooden floor (five to 30 ants per frame) or on an outdoor concrete step covered by a roof eave (four to 13 ants per frame), were recorded as above, before and after a 10-mg sample of carnuba wax containing 1 mg trail pheromone was placed in the view, ca. 4 cm from the trail. Ants were recorded, and positions were digitized for up to 340 s post-treatment.

Experiment 2.1: Small Plot Disruption (1 m^2) Semi-field plots (>3 m between plots) were established under the eaves of the office building (to avoid rainfall). There were four treatments (a sand alone control or 2.5, 7.5, and 25 mg of pheromone), and in each case, the ant sand was mixed with local Kilauea cinder soil to give a final amount of 50 g that was applied to each 1 m^2 plot by using a hand-held rotary spreader (Handy Green II, Scott's, Mayville, OH, USA).

A plastic-coated 3×5 cm card was baited with a small dab of 1:1 macerated tuna (chunky light tuna in water, Bumblebee Foods, San Diego, CA, USA) and Karo light corn syrup (ACH Food Companies, Memphis TN, USA) and placed in the center of a white sheet of paper taped to a firm backing in the center of each plot for 1 h before treatment. The number of ants at the bait card and the presence of the trails (a 1-min visual assessment) were recorded pre-treatment and then at 1, 2, 3, 24, 48, 72, 120, 144, and 196 h after treatment. Baits were covered each night with inverted plastic bins to protect them against vermins. The experiment was replicated three times, and fresh baits were used for each assessment in each replicate. Video files (15 frames/s) were also recorded in the sand control and the high rate (25 mg/m^2) plots, 24 h post-treatment, so that walking track angles could be calculated as with the single point experiment above.

Experiment 2.2: Trail Disruption Impacts on Ant Foraging Twenty-four plots (4 m^2), at least 10 m apart, were established in the Broomsedge Burn (19°25′59.6″ N, 155° 17′35.2″ W) of Hawaii Volcanoes National Park (1,200 m elevation), an area with few trees but dominated by clumps (1–3/m^2) of about 1-m-high broomsedge bluestem (*Andropogon virginicus*). Thus, the study site was exposed to prevailing trade winds, making it a challenging natural ecosystem to test rigorously the concept of trail disruption.

Sand samples were mixed with Kilauea cinder soil to give 200 g lots of soil containing 0, 10, 30, and 100 mg of pheromone, and at the start, each of the six replicates were applied to the plots by using a hand-held rotary spreader. Bait cards were placed directly on the ground at the center of each plot and protected from the direct rays of the sun by inverted plastic plates (25 cm diam.) suspended 15 cm above ground level. New baits were used for each observation period, except between the pre-treatment and first post-treatment ones.

Measurements of ant density at the bait card and visual assessment of the presence/absence of visible trails ants in 1-m^2 core of each plot were carried out just before treatment and then 1, 2, 3, 24, and 48 h after treatment. Wind speeds at ground level (2 cm), as well as at 1 and 2 m height, and shaded ground temperature were recorded at the time of application and toward the end of the observation period each day (2–3 P.M.).

In both field trials, analyses of variance were conducted on log-transformed counts of ants for each time interval, with Tukey tests to separate treatment effects.

Pheromone Release Rate Ant sand was aged in a laboratory fume hood (ca. 20°C) with an airflow (0.5 m/s) at Lincoln, New Zealand so that pheromone release rates could be estimated. The pheromones in three subsamples were extracted by adding 1,800 μl ethanol with 200 μl of 1 mg/ml decanal as the internal standard, vortexed, sonicated at 25°C for 30 min, centrifuged at 3,000 rpm for 10 min, and then filtered through 0.45-μm PTFE filter. Samples were analyzed by gas chromatography with a GC-

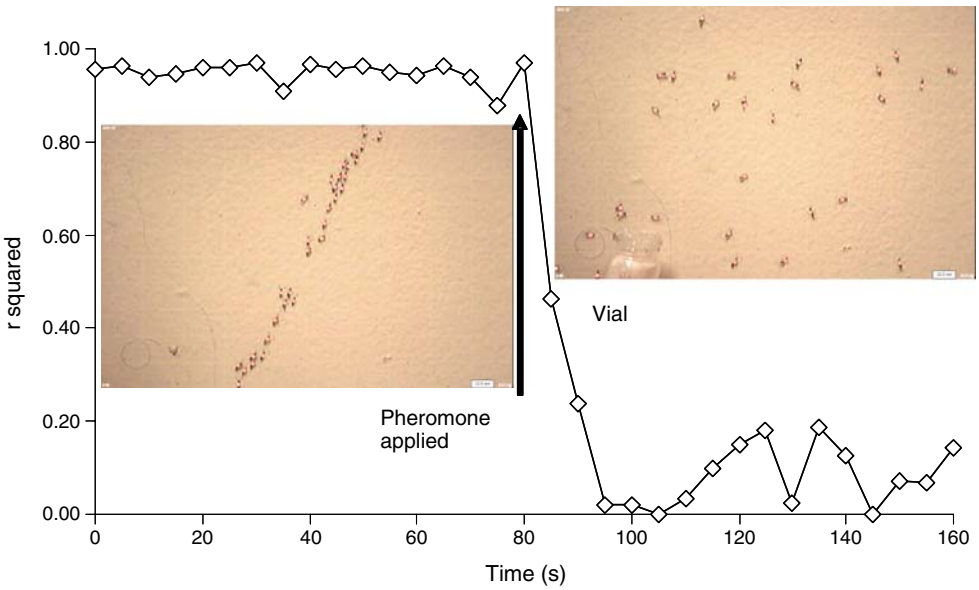

Fig. 1 Disruption of trail integrity (r^2) of high-density foraging trails of Argentine ant after the introduction of a vial at 80 s, containing a slow release formulation of carnuba wax with 100 mg of the trail pheromone, (*Z*)-9-hexedecenal. Ants are shown with *numbered dots*, before and after treatment

FID BPX-70 polar column, splitless, injector 220°C, flow 1 ml/min He, with a temperature program of 80°C for 1 min then ramped up to 220°C at 5°C/min and held for 1 min (total 30 min). The entire procedure was repeated twice.

Results

Disrupting Trail Following from a Point Source Ant trails at high traffic density on the wall (between 20 and 40 ants in the field of view) were <1 cm wide and linear. Upon introduction of the synthetic trail pheromone, the integrity of the trail was disrupted completely within seconds (Fig. 1; Test 1.1, Table 1; supplementary material, video A), as evidenced by an immediate drop in the value of the r^2 statistic. Trail-following behavior was disrupted for 30 min that the vial was present and for at least 7 min after its removal. Inspection the next day showed some evidence of disruption, although this was not quantified, possibly due to absorption of pheromone in the wall paint.

The number of ants increased significantly over time at the site (Fig. 2), probably due to ants arriving for both the food source and the nest but unable to leave by using the normal trail because of the pheromone treatment.

Ants initially showed a unimodal distribution of walking angles (with a SE 7.18 from 560 observations), but after presentation of the trail pheromone, there was no clear peak (an SE of 2.94 from 340 observations; Fig. 3; $F_{18,18}=5.94$, $P<0.001$).

Experiment 2.1: Small Plot Disruption (1 m^2) There was no difference in bait card visitation between bait cards before treatment and after 1 h in the sand control ($F_{3,8}=0.34$, $P=0.798$), but there was a significant decline in visitation in all pheromone-treated plots (Fig. 4). There was an overall difference in the number of foraging workers at bait cards between pheromone and control over 24 h (1 h, $F_{3,8}=4.71$, $P=0.035$; 2 h, $F_{3,8}=10.05$, $P=0.004$; 3 h, $F_{3,8}=12.62$, $P=0.002$; 4 h, $F_{3,8}=13.20$, $P=0.002$; 24 h, $F_{3,8}=11.05$, $P=0.003$). The disruption effect was marginally significant

Table 1 Impact of treatment with synthetic argentine ant trail pheromone ((*Z*)-9-hexadecenal) on trail integrity (r^2), recorded at 5-s intervals before and after treatment with a single point source of pheromone, on four different substrates

Test/substrate	Pheromone quantity, formulation	Before treatment		After treatment		n1, n2[a]	*t*	*P*
		Mean trail integrity, r^2 (no. ants)[b]	SEM	Mean trail integrity, r^2 (no. ants)[b]	SEM			
Interior painted wall	100 mg, vial	0.947 (517)	0.060	0.164 (530)	0.058	16, 16	13.55	0.001
Exterior painted wall	100 mg, vial	0.945 (59)	0.019	0.135 (70)	0.044	9, 7	17.06	0.001
Interior wooden floor	1 mg, wax	0.867 (170)	0.04	0.298 (150)	0.091	12, 11	5.76	0.001
Exterior concrete floor	1 mg, wax	0.967 (85)	0.025	0.229 (147)	0.061	11, 13	12.01	0.001

[a] Number of samples of r^2 used in the *t* test before (n1) and after (n2) treatment
[b] Total number of ants digitized for position

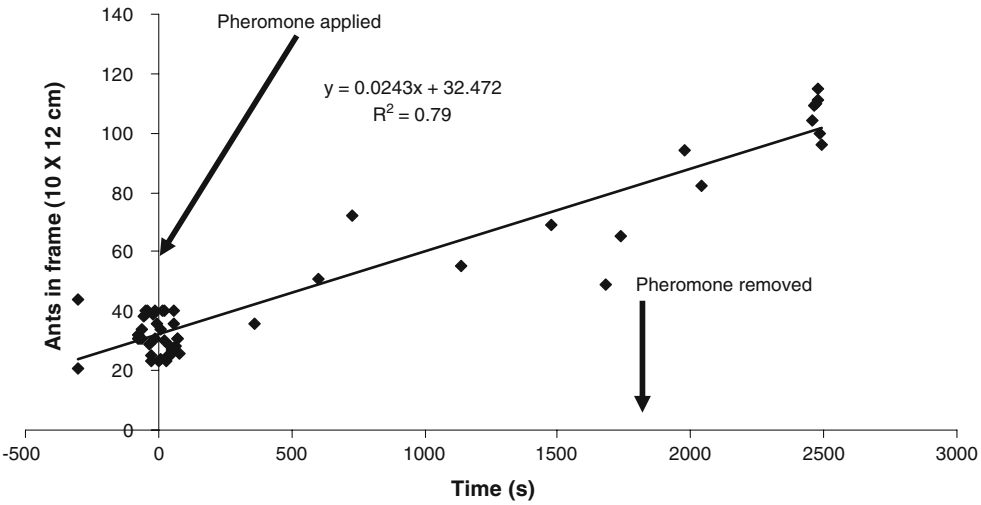

Fig. 2 Increase in the number of Argentine ants in the video frame after the introduction of a vial containing a slow release formulation of carnuba wax with 100 mg of the trail pheromone (*Z*)-9-hexedecenal

after 48 h ($F_{3,8}$=0.041) but not at any later times (Fig. 4). Tukey tests indicated significant differences (P<0.05) between the control and all pheromone treatments at 1, 2, 3, and 4 h, while at 24 h, only the 2.5- and 7.5-mg/m^2 application rates differed from the control. By 48 h, this effect had started to decline in these treatments. The absence of evident trails lasted 72 h in all three treated plots (Supplementary material, Fig. A).

Ants in the three sand controls all showed unimodal distributions of walking track angles, which was not the case in the 25 mg/m^2 pheromone treatment (Fig. 5). The difference in walking track angles between the treatment and controls was significant (P<0.05, t=4.06, df=3).

Experiment 2.2: Trail Disruption Impacts on Ant Foraging There was no difference in the number of foraging ants in the different plots before treatment ($F_{3,\ 20}$=0.53, P=0.66). However, there was a decline in the number of visiting bait cards for 2 h (1 h, $F_{3,\ 20}$=8.61, P<0.001; 2 h, $F_{3,\ 20}$=4.63, P=0.013, Fig. 6) when comparing treated vs. control plots, but after that, there were no significant differences (3 h, $F_{3,\ 20}$=2.92, P=0.059; 24 h, $F_{3,\ 20}$=0.66, P=0.59). The significant differences observed were due to the two higher concentrations of pheromone (Tukeys tests, P<0.05). There was evidence of trail-following behaviors in control plots for the duration of each trial but none for 24 h after treatment in the pheromone ones (Supplementary material, Fig. B). There was a correlation (r^2=0.36, P< 0.05) between the methods assessing disruption of counts at bait cards and disruption of trails in this experiment.

Pheromone Release Rate The experimental wax formulation had a high first-order release rate with ca. 30% loss of the applied material in 2 days at 20°C under laboratory conditions, giving an estimated half-life of 30 h for the ant sand preparation (Supplementary material, Fig. C). Using this laboratory data, the estimated release rates for the 25 mg/m^2 treatment in the field, where temperatures averaged about 22°C, were 260 and 200 μg/h/m^2 1 and 4 h after treatment (Figs. 4 and 6).

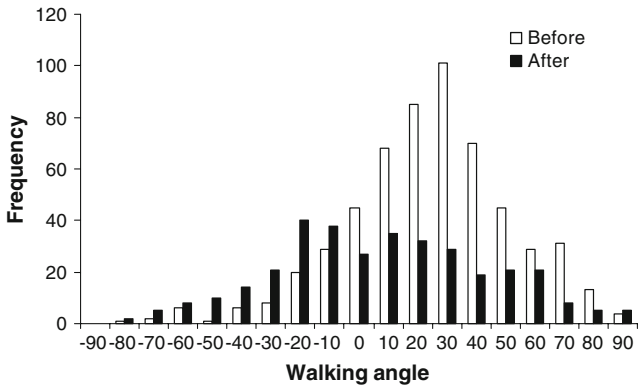

Fig. 3 Change in walking track angles of Argentine ant on a vertical kitchen wall before and after the introduction of a vial containing a slow release formulation of carnuba wax containing 100 mg of (*Z*)-9-hexedecenal

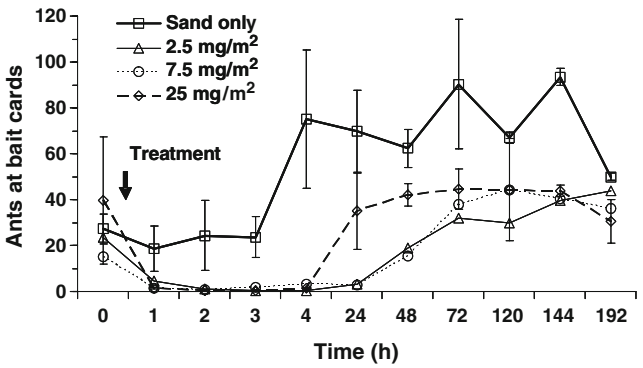

Fig. 4 Mean (±SEM) number of Argentine ants present at bait cards before and after treatment with a trail pheromone-laden wax and sand formulation at three rates in 1-m^2 plots at Hawaii Volcanoes National Park

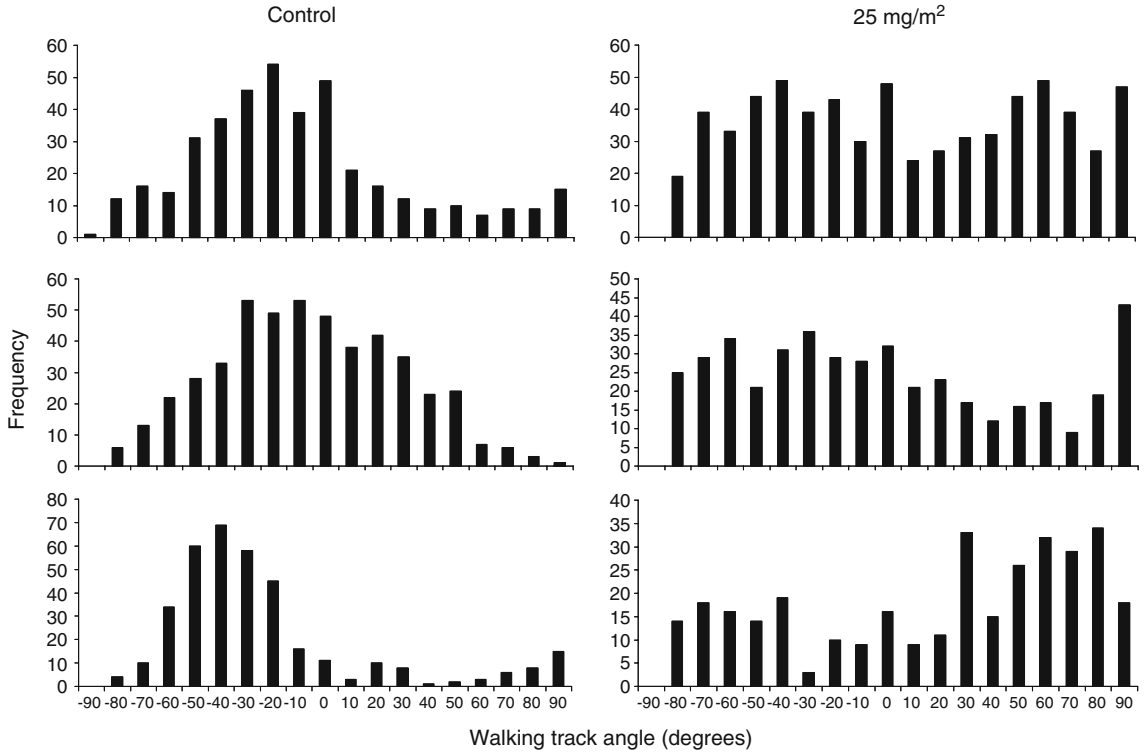

Fig. 5 Walking track angles for Argentine ants, 24 h after exposure to untreated control sand and sand laden with wax containing trail pheromone (active ingredient at 25 mg/m^2), from webcam recordings in the center of each of three replicate plots (1 m^2)

Discussion

The results of this study demonstrate the proof of concept of trail disruption against the Argentine ant indoors, as well as in small semi-field and field plots. Close-range trail orientation behaviors were significantly disrupted when the ants were exposed to an excess of their trail pheromone, resulting in a virtually instantaneous increase in turning behavior and a complete lack of orientation to the natural foraging trail. Furthermore, the deployment of a dispersible formulation, such as the ant sand used here, would have a natural advantage over a few large emission rate point sources for trail disruption (Suckling and Angerilli 1996). When applied, the material would fall within the boundary layer of the pest species, thus ensuring maximum atmospheric concentrations at the most suitable site (Suckling et al. 1999).

However, the effects observed were of quite short duration, especially in field plots where a variety of abiotic conditions (such as temperature, UV, and wind; Supplementary material, Fig. D) could markedly affect both release rates and persistence of the pheromone. Clearly, more research is required to determine the feasibility of this technique at a practical level. The fact that the highest concentration tested varied in efficacy in the field trials underlines the need to develop new formulations to provide longer and more consistent pheromone release under a range of different climatic conditions.

One also needs to find the minimum dose that would effectively disrupt trail-following behavior for an extended period of time. (*Z*)-9-Hexadecenal, being a lepidopteran sex pheromone (Nesbitt et al. 1975; Piccardi et al. 1977; Mayer and McLaughlin 1991), is more readily available than many other ant trail pheromones, but if the concentration required is too high, then trail disruption may not be economically

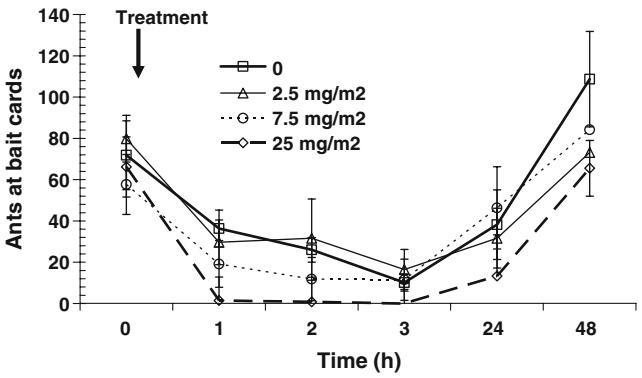

Fig. 6 Mean (±SEM) number of Argentine ants present at bait cards before and after treatment with three rates (milligrams per square meter) in 4-m^2 plots of a trail pheromone-laden wax and sand formulation at the Broomsedge Burn area of Hawaii Volcanoes National Park

viable as a stand-alone management tool, even for sensitive ecosystems. Cavill et al. (1979) reported many other compounds from the Argentine ant, and these merit investigation, as some of these minor compounds might improve both trail following and its disruption. Even if this were not the case, the integration of trail disruption with other control tactics warrants investigation.

Additional work on the trail-following behavior of the ants also is required, including further evaluation of the proposed trail integrity statistic, r^2. Van Vorhis Key and Baker (1986) reported that workers are more likely to follow trails if they meet an engorged con-specific, and they have other complex behaviors that may render trail disruption unsuccessful. For example, ants crossing an uninterrupted portion of the trail deposit more pheromone than those crossing "odor" gaps. Thus, if workers respond by increasing the concentration they release, this could reduce the duration of trail disruption.

Our results suggest that it would be worth trying a similar approach for the disruption of trail following in the Red Imported Fire Ant since they are an even more significant ecological problem in urban and sensitive environments world wide, causing billions of dollars of loss every year (Pimentel et al. 2000). Successful application of trail pheromones to tackle such pest ants may have significant environmental and social benefits.

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References

BALANÇA, G., and DE VISSCHER, M.-N. 1997. Impacts on nontarget insects of a new insecticide compound used against the desert locust [*Schistocerca gregaria* (Forskal 1775)]. *Arch. Environ. Contam. Toxicol*. 32:58–62.

CAVILL, G. W. K., ROBERTSON, P. L., and DAVIES, N. W. 1979. An Argentine ant *Iridomyrmex humilis* aggregation factor. *Experientia (Basel)* 35:989–990.

COLE, F. R., MEDEIROS, A. C., LOOPE, L. L., and ZUEHLKE, W. W. 1992. Effects of the Argentine ant on arthropod fauna of Hawaiian high-elevation shrubland. *Ecology* 73:1313–1322.

EL-SAYED, A. M. 2008. The pherobase: database of insect pheromones and semiochemicals. http://www.pherobase.com. Accessed May 1 2008.

GASTON, L. K., SHORY, H. H., and SAARIO, S. A. 1967. Insect population control by the use of sex pheromone to inhibit orientation between the sexes. *Nature* 213:1155.

GREENBERG, L., KLOTZ, J. H., and RUST, M. K. 2006. Liquid borate bait for control of the Argentine ant, *Linepithema humile*, in organic citrus (Hymenoptera: Formicidae). *Fla. Entom*. 89:469–474.

HOLWAY, D. A. 1999. Competitive mechanisms underlying the displacement of native ants by the invasive Argentine ant. *Ecology* 80:238–251.

HOLWAY, D. A., LACH, L., SUAREZ, A. V., TSUTSUI, N. D., and CASE, T. J. 2002. The causes and consequences of ant invasions. *Annu. Rev. Ecol. Syst*. 33:181–233.

HOOPER-BUI, L. M., and RUST, M. K. 2000. Oral toxicity of abamectin, boric acid, fipronil, and hydramethylnon to laboratory colonies of Argentine ants (Hymenoptera: Formicidae). *J. Econ. Entomol*. 93:858–864.

HUMAN, K. G., and GORDON, D. M. 1997. Effects of Argentine ants on invertebrate diversity in Northern California. *Conserv. Biol*. 11:1242–1248.

ITIOKA, T., and INOUE, T. 1996. The role of predators and attendant ants in the regulation and persistence of a population of the citrus mealybug *Pseudococcus citriculus* in a Satsuma orange orchard. *Appl. Entomol. Zool*. 31:195–202.

KARG, G., SUCKLING, D. M., and BRADLEY, S. J. 1994. Absorption and release of pheromone of *Epiphyas postvittana* (Lepidoptera: Tortricidae) by apple leaves. *J. Chem. Ecol*. 20:1825–1841.

KLOTZ, J., GREENBERG, L., and VENN, E. C. 2000. Evaluation of two hydramethylnon granular baits for control of Argentine ant (Hymenoptera: Formicidae). *Sociobiology* 36:201–207.

KRUSHELNYCKY, P. D., and REIMER, N. J. 1998. Efficacy of Maxforce bait for control of the Argentine ant (Hymenoptera: Formicidae) in Haleakala National Park, Maui, Hawaii. *Environ. Entomol*. 27:1473–1481.

LACH, L. 2005. Interference and exploitation competition of three nectar-thieving invasive ant species. *Insect. Soc.* 52:257–262.

LIEBHERR, J. K., and KRUSHELNYCKY, P. D. 2007. Unfortunate encounters? Novel interactions of native *Mecyclothorax*, alien *Trechus obtusus* (Coleoptera: Carabidae), and Argentine ant (*Linepithema humile*, Hymenoptera: Formicidae) across a Hawaiian landscape. *J. Insect Conserv.* 11:61–73.

MAYER, M. S., and MCLAUGHLIN, J. R. 1991. Handbook of insect pheromones and sex attractantsCRC, Boca Raton, FL1083pp.

MORENO, D. S., HANEY, P. B., and LUCK, R. F. 1987. Chlorpyrifos and diazinon as barriers to Argentine ant (Hymenoptera: Formicidae) foraging on citrus trees. *J. Econ. Entomol*. 80:208–214.

NESBITT, B. F., BEEVOR, P. S., HALL, D. R., LESTER, R., and DYCK, V. A. 1975. Identification of the female sex pheromones of the moth, *Chilo suppressalis*. *J. Insect Physiol*. 21:1883–1886.

PICCARDI, P., CAPIZZI, A., CASSANI, G., SPINELLI, P., ARSURA, E., and MASSARDO, P. 1977. A sex pheromone component of the old world bollworm *Heliothis armigera*. *J. Insect Physiol*. 23:1443–1445.

PIMENTEL, D., LACH, L., ZUNIGA, R., and MORRISON, D. 2000. Environmental and economic costs of nonindigenous species in the United States. *Bioscience* 50:53–65.

ROBINSON, S. W., and CHERRETT, J. M. 1978. The possible use of methyl 4-methylpyrrole-2-carboxylate, an ant trail pheromone, as a component of an improved bait for leaf-cutting ant (Hymenoptera: Formicidae) control. *Bull. Ent. Res*. 68:159–170.

SHOREY, B. H., GASTON, L. K., GERBER, R. G., PHILLIPS, P. A., and WOOD, D. L. 1992. Disruption of foraging by Argentine ants, *Iridomyrmex humilis* (Mayr) (Hymenoptera: Formicidae), in citrus trees through the use of semiochemicals and related chemicals. *J. Chem. Ecol*. 18:2131–2142.

SUAREZ, A. V., and CASE, T. J. 2002. Bottom-up effects on persistence of a specialist predator: ant invasions and horned lizards. *Ecol. Appl*. 12:291–298.

SUAREZ, A. V., BOLGER, D. T., and CASE, T. J. 1998. Effects of fragmentation and invasion on native ant communities in coastal southern California. *Ecology* 79:2041–2056.

SUCKLING, D. M., and ANGERILLI, N. P. D. 1996. Pheromone point source distribution affects spike frequency and communication disruption of *Epiphyas postvittana* (Lepidoptera: Tortricidae). *Environ. Entomol*. 25:101–108.

SUCKLING, D. M., and KARG, G. 2000. Pheromones and semiochemicals, pp. 63–99, in J. Rechcigl, and N Rechcigl (eds.). Biological and Biotechnical Control of Insect PestsCRC, Boca Raton, FL.

SUCKLING, D. M., GREEN, S. R., GIBB, A. R., and KARG, G. 1999. Predicting atmospheric concentration of pheromone in treated apple orchards. *J. Chem. Ecol*. 25:117–139.

TATSUKI, S., TERYAMA, M., TANAKA, Y., and FUKUMOTO, T. 2005. Behavior-disrupting agent and behavior disrupting method of Argentine ant. Patent pub. no. US2005/0209344A1.

VAN VORHIS KEY, S. E., and BAKER, T. C. 1982a. Specificity of laboratory trail following by the Argentine ant, *Iridomyrmex humilis* (Mayr), to (*Z*)-9-hexadecenal, analogs, and gaster extract. *J. Chem. Ecol*. 8:1057–1063.

VAN VORHIS KEY, S. E., and BAKER, T. C. 1982b. Trail-following responses of the Argentine ant, *Iridomyrmex humilis* (Mayr), to a synthetic trail pheromone component and analogs. *J. Chem. Ecol*. 8:3–14.

VAN VORHIS KEY, S. E., and BAKER, T. C. 1982c. Trail pheromone-conditioned anemotaxis by the Argentine ant, *Iridomyrmex humilis*. *Entomol. Exp. Appl*. 32:232–237.

VAN VORHIS KEY, S. E., and BAKER, T. C. 1986. Observations on the trail deposition and recruitment behaviors of the Argentine ant, *Iridomyrmex humilis* (Hymenoptera: Formicidae). *Ann. Entomol. Soc. Am*. 79:283–288.

VAN VORHIS KEY, S. E., GASTON, L. K., and BAKER, T. C. 1981. Effects of gaster extract trail concentration on the trail following behavior of the Argentine ant, *Iridomyrmex humilis* (Mayr). *J. Insect Physiol*. 27:363–370.

VEGA, S. J., and RUST, M. K. 2001. The Argentine ant—a significant invasive species in agricultural, urban and natural environments. *Sociobiology* 37:3–25.

WRIGHT, R. H. 1964. After pesticides—what? *Nature* 204:121–125.

ZAR, J. H. 1984. Biostatistical analysisPrentice-Hall International, London.

ZHAKHAROV, A. A., and THOMPSON, L. C. 1998. Effects of repeated use of fenoxycarb and hydramethylnon baits on nontarget ants. *J. Entomol. Sci*. 33:212–220.